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Narendra Tuteja,
Sarvajeet Singh Gill,
and Renu Tuteja

Improving Crop Productivity in Sustainable Agriculture

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Improving Crop Productivity in Sustainable Agriculture

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Professor G.S. Khush (August 22, 1935)

Professor G.S. Khush was born in a small village in Punjab, India, and did B.Sc. in 1955 from Government Agricultural College (now Punjab Agricultural University), Ludhiana, and Ph.D. in 1960 from the University of California, Davis. After serving as an Assistant Geneticist at University of California, Davis, for 7 years, he joined International Rice Research Institute (IRRI), Los Banos, Philippines (1967), as a Plant Breeder. He was promoted as Head of Plant Breeding Department in 1972 and became Principal Plant Breeder and Head of Division of Plant Breeding, Genetics and Biochemistry (1986). Professor Khush is a world-renowned plant breeder who has made enormous contribution to the development of more than 300 high-yielding rice varieties that played significant role toward achieving "Green Revolution," thereby boosting rice production. Professor Khush provided excellent leadership for the global rice improvement program benefiting millions of resource-poor rice growers in the world. A semi-dwarf rice variety IR36 developed by him was one of the most widely grown rice varieties in the world during 1980s. IR64 developed during 1980s is the most widely planted rice variety in the world. In India, Professor Khush has been actively involved in the development of Plant Breeding and Agriculture Biotechnology. He has authored 3 books, edited 6 books, 40 review articles, 45 book chapters, and 160 research papers. His scientific work featured in the most prestigious international journals. Professor Khush received many awards and honors from various scientific bodies, such as Borlaug Award (1977), Japan Prize (1987), World Food Prize (1996), Rank Prize (1998), Wolf Prize (2000), and Padma Shri from the President of India. He received D.Sc. (hc) degrees from 10 universities, including Punjab Agricultural University, Jawahar Lal Nehru Agriculture University, De Montfort University, Cambridge University, and Ohio State University. He is elected to the Fellowship of Indian Academy of Sciences, Bangalore; National Academy of Sciences (India), Allahabad; National Academy of Agricultural Sciences, New Delhi; Indian National Science Academy (INSA), New Delhi; the Academy of Sciences for the Developing World; Chinese Academy of Sciences; Russian Academy of Agricultural Sciences; US National Academy of Sciences; and The Royal Society (London). At present, Professor Khush is serving as Adjunct Professor in University of California, Davis.

This book is dedicated to Prof. G.S. Khush, the undisputed Hero of Rice Revolution.

Contents

Foreword

Agriculture is now at a crossroad of conservation and sustainability along with the challenge of increasing productivity. Global agricultural land is limited; the same is true for the water availability and other natural resources. However, population is increasing, particularly in the developing countries. The vertical growth of crop productivity is the only way to meet the daunting task and ensure food security for ever-increasing population. I am glad to see this book addressing those issues and providing scientific know-how to solve some of the problems. I firmly believe that genetic potential of the crop productivity can be utilized and further improved through science and technology interventions.

Several chapters including (1) Climate Change and Food Security by Dr R.B. Singh, (2) Improving Crop Productivity under Changing Environment by Drs Dhillon, Gosal, and Kang, (3) Are Virus Always Villains? The Roles Plant Viruses May Play in Improving Plant Responses to Stress by Drs Wylie and Jones, (4) Risk Assessment of Abiotic Stress-Tolerant GM Crops by Drs Howles and Smith, (5) Rice: Genetic Engineering Approaches for Abiotic Stress Tolerance – Retrospects and Prospects by Dr Singh *et al*., (6) Groundnut: Genetic Approaches to Enhance Adaptation of Groundnut (*Arachis hypogaea* L.) to Drought by Rao *et al*., (7) Pulse Crops: Biotechnological Strategies to Enhance Abiotic Stress Tolerance by Drs Ganeshan, Gaur, and Chibar, and so on make focused discussions on the subject. All chapters are well written and create a scientific interest in the learners/readers and researchers.

Congratulations and my best compliments to editors of this book Drs N. Tuteja, Sarvajeet S. Gill, and R. Tuteja who performed an outstanding work in getting valuable contributions from some world experts on the relevant subject. I am sure the readers in the field of agriculture and particularly in abiotic stress management, biotechnology, and new genetics in plant breeding would find this book very useful. The publisher also deserves congratulations for publishing this useful book.

ICAR, New Delhi
April 11, 2012

Prof. Swapan K. Datta, FNAAS, FNASc
Deputy Director General (Crop Science)
ICAR, New Delhi

Preface

The world's population is projected to hit ~9.2 billion in 2050. On the other hand, agricultural production is decreasing because of negative implications of global climate change. Therefore, it has become essential to increase the global agricultural production to feed the increasing population. Globally, a major loss in crop production is imposed by a suite of stresses, resulting in 30–60% yield reduction every year. Abiotic stress factors such as heat, cold, drought, salinity, wounding, heavy metal toxicity, excess light, floods, high-speed wind, nutrient loss, anaerobic conditions, radiation, and so on represent key elements affecting agricultural productivity worldwide. In an agriculturally important country, agriculture is the main driver of agrarian prosperity and comprehensive food and nutritional security. The loss of productivity is triggered by a series of morphological, physiological, biochemical, and molecular stress-induced changes. Therefore, minimizing these losses is a major area of concern for the whole world.

Genetic engineering of abiotic stress-related genes is an important objective for increasing agricultural productivity. Plant adaptation to environmental stresses is dependent on the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Consequently, these genes that protect and maintain the function and structure of cellular components can enhance tolerance to stress. Genetic engineering of important genes and QTLs have now become valuable tools in crop improvement for rapid precision breeding for specific purposes. Additionally, drip irrigation and fertigation, leaf color chart (LCC) for need-based application of nitrogen, sensor-based yield monitors, nitrogen sensors/green seekers, special-purpose vehicles with sensor-based input applicators, integrated nutrient management (INM) systems, integrated pest management (IPM) systems, integrated disease management (IDM) systems, site-specific management systems using remote sensing, GPS, and GIS, and Web-based decision support systems for controlling diseases and insect pests have been developed and are being commercialized for precision farming.

In this book "Improving Crop Productivity in Sustainable Agriculture," we present a collection of 19 chapters written by 55 experts in the field of crop improvement and abiotic stress tolerance. This volume is an up-to-date overview of current progress in improving crop quality and quantity using modern methods. Included

literature in the form of various chapters provides a state-of-the-art account of the information available on crop improvement and abiotic stress tolerance for sustainable agriculture. In this book, we present the approaches for improving crop productivity in sustainable agriculture with a particular emphasis on genetic engineering; this text focuses on crop improvement under adverse conditions, paying special attention to such staple crops as rice, maize, and pulses. It includes an excellent mix of specific examples, such as the creation of nutritionally fortified rice and a discussion of the political and economic implications of genetically engineered food. The result is a must-have hands-on guide, ideally suited for the biotech and agro industries. This book best complements our previous title "Improving Crop Resistance to Abiotic Stress" (ISBN 978-3-527-32840-6, Volumes 1 and 2, Wiley-Blackwell, 2012).

For the convenience of readers, the whole book is divided into three major parts, namely, Part I: Climate Change and Abiotic Stress Factors; Part II: Methods to Improve Crop Productivity; and Part III: Species-Specific Case Studies. Further, Part III has been divided into three sections, namely, Section IIIA: Graminoids; Section IIIB: Leguminosae; and Section IIIC: Rosaceae. Part I covers four chapters. Chapter 1 deals with climate change and food security, where emphasis has been paid to food security and climate resilient agriculture. Chapter 2 uncovers the ways for improving crop productivity under changing environment. Chapter 3 deals with the approaches such as genetic engineering for acid soil tolerance in crop plants, whereas Chapter 4 focuses on the evaluation of tropospheric O_3 effects on global agriculture. Part II covers five chapters. Chapter 5 deals with "-omics" approaches for abiotic stress tolerance where emphasis has been paid to understand the importance of mitogen-activated protein kinases in abiotic stress tolerance in crop plants. Chapter 6 unravels the importance of plant growth promoting rhizobacteria for the amelioration of abiotic and biotic stresses for increasing crop productivity. Chapter 7 interestingly uncovers the importance of viruses in reducing damage from both biotic and abiotic stressors in crop plants. This chapter focuses on the new technologies that revealed that viruses are far more abundant and diverse than previously known and unexpected roles as symbionts and as sources of genetic raw material for evolution are informing a new appreciation of the roles plant viruses play in nature. Chapter 8 is on risk assessment of abiotic stress-tolerant GM crops. This chapter outlines the likely issues for consideration in risk assessment for the commercial release of a GM plant with a novel abiotic stress tolerance trait. Chapter 9 is on biofertilizers as potential candidate for crop improvement under stressed conditions. Part III deals with different crop plants under three sections. Section IIIA covers four chapters that deal with rice, pearl millet, and bamboo. In this section, Chapter 10 deals with the genetic engineering approaches for abiotic stress tolerance in rice – retrospects and prospects. Chapter 11 uncovers the genetic engineering approaches to enhance grain iron content in rice. The creation of nutritionally fortified rice can have a dramatic impact on human health because it is a major staple crop in the world. Chapter 12 deals with the genetic improvement for tolerance to abiotic stresses in pearl millet. Chapter 13 deals with the application of plant tissue culture techniques for genetic improvement of bamboo

(*Dendrocalamus strictus* Nees). Section IIIB includes four chapters on groundnut, chickpea, grain legumes, and pulse crops. Chapter 14 deals with genetic approaches to enhance adaptation of groundnut (*Arachis hypogaea* L.) to drought stress. Chapter 15 discusses the strategies for crop improvement under changing environment conditions in chickpea. Chapter 16 deals with grain legumes, where biotechnological interventions in crop improvement for adverse environments have been discussed. Chapter 17 uncovers the biotechnological strategies to enhance abiotic stress tolerance in pulse crops. Section IIIC includes two chapters on *Fragaria* and rose. Chapter 18 deals with improving crop productivity and abiotic stress tolerance in cultivated *Fragaria* using "-omics" and systems biology approach. Chapter 19 discusses the strategies for improving crop productivity in rose. The editors and contributing authors hope that this book will add to our existing knowledge of improving crop productivity in sustainable agriculture that, in turn, may eventually open up new avenues for improving the stress tolerance in crop plants.

We are highly thankful to Dr. Ritu Gill, Centre for Biotechnology, MD University, Rohtak, for her valuable help in formatting and incorporating editorial changes in the manuscripts. We would like to thank Prof. Swapan K. Datta, Deputy Director General (Crop Science), ICAR, New Delhi, for writing the foreword and Wiley-Blackwell, Germany, particularly Gregor Cicchetti, Senior Publishing Editor, Life Sciences, and Anne Chassin du Guerny for their professional support and efforts in the layout. This book is dedicated to Professor G.S. Khush, the undisputed Hero of Rice Revolution.

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Part I
Climate Change and Abiotic Stress Factors

Improving Crop Productivity in Sustainable Agriculture, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, and Renu Tuteja.

1
Climate Change and Food Security

R.B. Singh

Abstract

The Green Revolution ushered in the 1960s brought unprecedented transformation in agricultural production, productivity, food security, and poverty reduction. But, it has now waned. The numbers of hungry, undernourished, and poor remain stubbornly high. Moreover, the natural agricultural production resources, particularly water and land, have shrunk and degraded. The problem has further exacerbated by the global climate change and extreme weather fluctuations widely depressing agricultural yields, increasing production instability, and degrading natural resources. If the change is not managed adequately, the agricultural yields will drop by up to 20% by the year 2050 and the national GDP will erode annually at least by 1%. A series of adaptation and mitigation pathways involving business unusual have been suggested toward developing climate smart agriculture by increasing agricultural resilience to climate change through integrating technology, policy, investment, and institutions with special reference to the resource poor, women, and other more vulnerable people.

1.1
Background and Introduction

Toward the year 2050, the world population is projected to stabilize at around 9.2 billion. In order to adequately feed this population, the global agriculture must double its food production, and farm productivity would need to increase by 1.8% each year – indeed a tall order. On the other hand, the natural resources – the agricultural production base, especially land, water, and biodiversity – are fast shrinking and degrading. For instance, by 2025, 30% of crop production will be at risk due to the declining water availability. Thus, in order to meet the ever-intensifying demand for food and primary production, more and more is to be produced from less and less of the finite natural and nonrenewable resources.

Improving Crop Productivity in Sustainable Agriculture, First Edition.
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The challenges of attaining sustainably accelerated and inclusive growth and comprehensive food security have been exacerbated by the global climate change and extreme weather fluctuations. The global warming due to rising concentration of greenhouse gases (GHGs) causing higher temperature, disturbed rainfall pattern causing frequent drought and flood, sea level rise, and so on is already adversely impacting productivity and stability of production, resulting in increased vulnerability, especially of the hungry and resource-poor farmers, and is a growing threat to agricultural yields and food security. World Bank projects that the climate change will depress crop yields by 20% or more by the year 2050. Livestock and fish production will likewise be impacted. Pathogen virulence, disease incidences, pest infestations, epidemic breakouts, and biotic stresses in general are predicted to intensify.

The impact of the climate change will vary across ecogeographic and demographic domains. As projected, the bulk of the population increase will materialize in developing countries. Most of these are agriculture based, and several of them are food deficit. Moreover, these countries have high concentration of smallholder resource-poor farmers and their agriculture is predominantly rainfed, which is inherently low yielding and vulnerable to weather fluctuations. In such countries, sustained and accelerated agricultural growth is fundamental not only to achieving food security but also to generating economic growth and opportunity for overall livelihood security.

India will be the most populous country in the world by 2050, with a projected population of over 1.5 billion, and will need to double its food production by then to ensure its food security. The country was able to overcome its food crisis and insecurity through ushering in the Green Revolution in the mid-1960s. Between 1965 and 1995, the food and agriculture production and productivity had more than doubled and the intensity of hunger and poverty had halved [1]. This revolution was largely due to the synergy of technology, policies, services, farmers' enthusiasm, and strong political will. However, the Green Revolution has now waned. During the past decade or so, while the overall national GDP had registered a high growth rate of 7–9%, the agricultural growth had gone sluggish (although recovered lately).

Unethical as it is, the country is still home to almost one-fourth of the world's hungry and poor, and over 40% of our children are undernourished. The income gap between farmers and nonfarmers has widened rather unacceptably, farmers' income being about one-fifth of that of the nonfarmers (Figure 1.1). This is primarily due to the steadily declining share of agriculture in GDP while the intensity of dependence on agriculture for livelihoods has remained high. The agriculture's share in GDP fell from about 35% in 1980 to 15% in 2010, and is expected to fall to 5% in 2040 (Figure 1.2), whereas in 2010 over 50% of the population was dependent primarily on agriculture.

In an agriculturally important country like ours, agriculture is the main driver of agrarian prosperity and comprehensive food and nutritional security. The past Five-Year Plans have been aiming at an agricultural growth rate of 4% and above to achieve a balanced overall GDP growth rate of about 8–9%. But, we have not been able to achieve the targeted growth in the agricultural sector, being 3.3% during

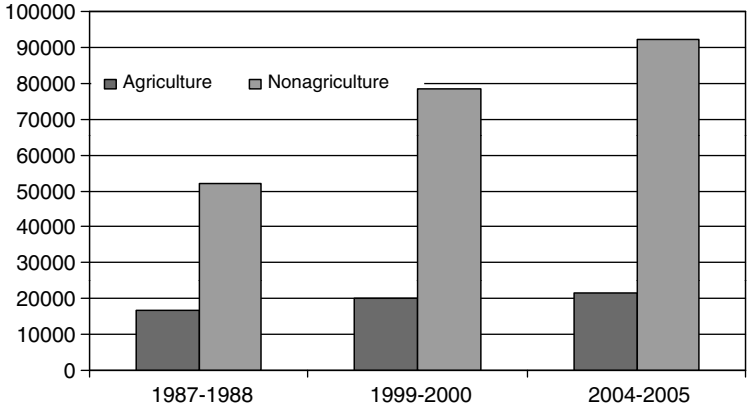

Figure 1.1 Per worker GDP in agriculture and nonagriculture sectors, Rs. at 1999–2000 prices. (*Source*: Ref. [2].)

1980–2010 and only 2.3% during the decade ending 2010. The coefficient of variation (CV) of the agricultural growth has been high and, if not managed, will further increase with the increasing volatilities caused due to climate change. The XII Plan also targets an overall agricultural output growth of 4.0–4.5% coupled with inclusiveness and gender sensitivity. A business unusual rooted in the principles of ecology, environment, economics, and equity is called for ensuring sustained and enhanced livelihood security of our people in face of the fast changing climate.

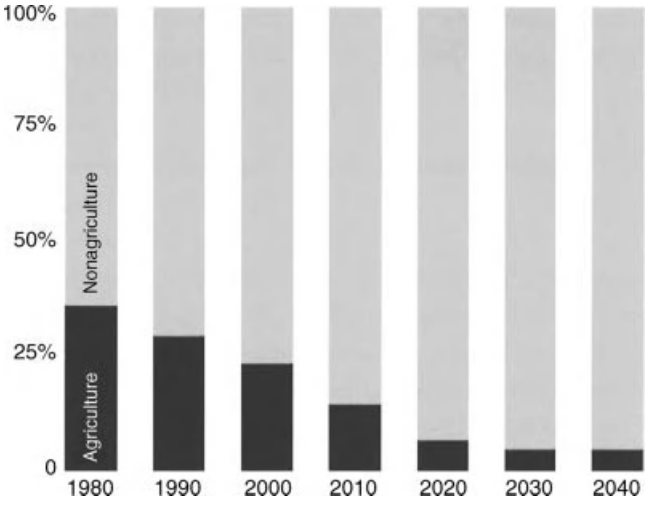

Figure 1.2 Agriculture's share in GDP steadily declining. (*Source*: Refs [3, 4].)

1.2 State of Food Security

The number of undernourished people in the world had been increasing for a decade or so and the number of hungry for the first time had crossed the 1 billion mark in 2008–2009 [2], but the number came down to 925 million in 2009–2010. Nearly all hungry people were from developing countries. The gains made in the 1980s and early 1990s in reducing chronic hunger have been lost and the hunger reduction targets of the Millennium Development Goal 1 (MDG1) as well as of the World Food Summit (WFS) remain elusive. The soaring food prices of 2007–2008 had drawn the poor farther from food, resulting in the unusual increase in the number and even proportion of undernourished. Despite the fall in international food and fuel prices starting in the late 2008, the prices in domestic markets remained 15–25% higher in real terms than the trend level – continuing the distress for the poor. High food inflations and the associated household food security have been recurrent features in India.

The continued neglect of agriculture during the past decades had denied hundreds of millions of people access to adequate food and has kept them below the poverty line. Globally, as also in India, the hunger and poverty incidences are mirror images (Table 1.1) and cause and consequence of each other. The rapid increase in the number of hungry and poor in the recent years reveals that food, fuel, and economic crises arise from the fragility of present food systems and livelihood security programs. Necessary structural adjustment and macroeconomic stabilization policies should be designed to minimize the impact of the shocks, particularly through enhanced investment in agriculture (including nonfarm rural activities and employment), expanding safety nets and social assistance programs, and, of course, improving governance.

In India, as mentioned earlier, the Green Revolution had transformed the country from the status of a food-deficit country to a food self-sufficient nation (at macro level). Per caput dietary energy supply (DES) increased from 2370 kcal/day in 1990–1992 to 2440 kcal/day in 2001–2003, and prevalence of undernourishment in total population decreased correspondingly from 25 to 20%. Between 1993/1994 and 1999/2000, 58 million individuals came out of the poverty trap, the number of poor dropping from 317 million to 259 million. Other livelihood indicators such as literacy rate and longevity also increased significantly. Life expectancy at birth in

Table 1.1 Poverty ($1.25 a day or less) and hunger levels in the developing world.

Region	% Poverty	% Hunger (undernourished)
Asia-Pacific	27	17
Latin America and Caribbean	8	10
Sub-Saharan Africa	51	32
Total developing countries	29	20

Source: Ref. [5].

Table 1.2 Number and percentage of undernourished people in India since the base year 1990–1992.

Year	Total population (million)	Undernourishment	
		Number (million)	Percent
1990–1992	863	215	25
1995–1997	949	202	21
2001–2003	1050	212	20
2005–2007	1116	221	20
2009–2010	1168	245 (est.)	21

Source: Refs [3, 5].

2005/2006 was over 63 and 66 years, respectively, for males and females against 58 and 59 years in 1986–1991 [3].

Despite India's national level food self-sufficiency and security, the number of food insecure people in India has remained stubbornly high, in recent years hovering around 245 million, one-fourth of the world's food insecure people. In fact, during 2007–2010, the number of hungry in the country, as in the world as a whole, had increased due to soaring food prices. In percentage term, however, food insecurity in India had reduced from 25% in 1990–1992 to 20% in 2001–2003, but in recent years has increased to 21% (Table 1.2). The record food grain production of over 230, 240, and 250 million tons during the years 2009–2010, 2010–2011, and 2011–2012, respectively, should have improved the situation, which should be known from the latest household survey reports. In any case, one-fifth to one-fourth of our people are still hungry and poor.

The above situation is ascribed primarily to the high and increasing population pressure (nearly 16 million being added annually to the already 1.20 billion population) and to the distributional and economic access problems, aggravating the household and individual level food insecurities. Obviously, India has made little progress toward WFS and MDG targets, whereas China and Brazil are fairly close to achieving the targets. India must critically analyze the situation and take necessary measures to get on the track to meet the targets, as done by Brazil and China.

The Global Hunger Index (GHI), which incorporates three interlinked hunger-related indicators – the proportion of undernourished in the population, the prevalence of underweight in children, and the mortality rate of children, worldwide improved from 19.8% in 1990 to 15.1% in 2010. The higher GHI was for South Asia at 22.9. In 2010, among the 84 countries (for which data could be available) having GHI above 5.0 (ranging from 5.2% in Syrian Arab Republic to 41.0% in Democratic Republic of the Congo), India with GHI at 24.1 ranked 67. This alarming GHI in India was driven by high levels of child underweight (Table 1.3). Unfortunately, over 40% of the undernourished children of the world have their homes in India (Figure 1.3). Thus, in order to improve the GHI score, India must

Table 1.3 Contributions of the three components of GHI and the underlying data for calculating the 1990 and 2010 GHI in India and in its major neighboring countries.

Country	Proportion of undernourished in the population (%)		Prevalence of underweight in children under 5 years (%)		Under 5 mortality rate (%)		GHI	
							With data from 1988 to 1992	With data from 2003 to 2008
	1990–1992	2004–2006	1990–1992	2003–2008	1990	2008	1990	2010
India	24.0	22.0	59.5	43.5	11.6	6.9	31.7	24.1
Bangladesh	36.0	26.0	56.5	41.3	14.9	5.4	35.8	24.2
China	15.0	10.0	15.3	6.0	4.6	2.1	11.6	6.0
Pakistan	22.0	23.0	39.0	25.3	13.0	8.9	24.7	19.1
World	—	—	—	—	—	—	19.8	15.1

Source: Ref. [6].

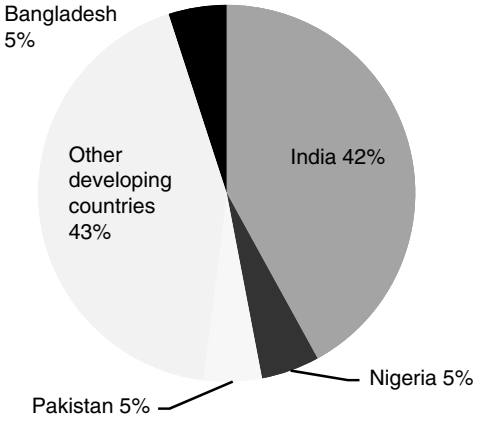

Figure 1.3 Share of underweight children under 5 years of age. (*Source*: Ref. [7].)

accelerate progress in reducing child underweight by improving childhood nutrition. Seasonal oscillations in food grain production often caused due to extreme weather conditions exacerbate the situation.

1.3
Climate Change Impact and Vulnerability

Climate change, especially aberrations in rainfall, temperature, soil, and water availability and quality, is emerging as a major threat to the food security and agricultural sustainability.

India with its huge population being dependent on agriculture and experiencing excessive pressure on its natural resources, coupled with its poor coping mechanisms, is highly vulnerable to the climate change, especially the poor and resource-poor farmers. The warming trend in India over the past 100 years has indicated an increase of 0.60 °C. Negative impacts of these changes on yield of wheat and paddy in parts of India due to increased temperature, increased water stress, and reduction in number of rainy days are already felt. Significant negative impacts have been projected with medium-term climate change, for example, yield reduction by 11–18%, depending on the magnitude and distribution of warming, eroding roughly 1.5% of the GDP per year.

The ICAR's National Network Project on "Impact, Adaptation, and Vulnerability of Indian Agriculture to Climate Change," started in 2004 involving 23 institutes/universities covering all major sectors of agriculture, namely, crops, horticulture, plantations, livestock, inland and marine fisheries, poultry, and natural resources such as water and soil, has the following objectives [8]:

- Quantify the sensitivities of current food production systems to different scenarios of climate change by integrating the response of different sectors.

- Quantify the least risk or "no regrets" options in view of uncertainty of global environmental change that would also be useful in sustainable agricultural development.
- Determine the available management and genetic adaptation strategies for climatic change and climatic variability.
- Determine the mitigation options for reducing global climatic changes in agroecosystems.
- Provide policy support for the international negotiations on global climatic changes.

The project has revealed both negative and positive trends in annual rainfall and minimum and maximum temperature. For instance, the project report, based on the past six decades' data, revealed an increasing trend in maximum 1-day precipitation in the west coast of Maharashtra, south Madhya Pradesh, east Bihar, Assam, north and west Karnataka, eastern Uttar Pradesh, western Jharkhand, and Ganga Nagar area of Rajasthan. A declining trend was observed in parts of southern Karnataka, western Maharashtra, northern Chhattisgarh, northern Madhya Pradesh, and western Uttar Pradesh.

As regards temperature, the number of cold days significantly declined in northwestern Madhya Pradesh, southern Chhattisgarh, and western Gujarat, and in parts of peninsular India. Number of cold nights declined in major parts of north India, south and west Gujarat, west Maharashtra, coastal Andhra Pradesh, southern Karnataka, northwestern Tamil Nadu, and northern Kerala, whereas it increased in north Chhattisgarh and northern Jammu and Kashmir states.

The occurrence of warm days significantly increased in parts of southern Rajasthan, western Madhya Pradesh, western Gujarat, northern Jammu and Kashmir, and Manipur, while it declined in parts of West Bengal, Jharkhand, southern Bihar, eastern Himachal Pradesh, Uttarakhand, northwestern Uttar Pradesh, and northern Haryana. In peninsular India, the number increased except in north and eastern Andhra Pradesh, southern Tamil Nadu, northern Karnataka, and in south and north of Maharashtra.

The report further showed that the emission of greenhouse gases has been increasing from agricultural soils. Emission of methane from Indian rice fields has remained almost constant, but emission of nitrous oxide is increasing. The CO_2 emission of marine fishing boats has increased.

The impact of climate change on wheat, rice, and maize yields will be significantly negative, varying from region to region. For instance, the climate change is projected to reduce the timely sown irrigated wheat production by about 6% in 2020 scenario. But, when late and very late sown wheat are also considered, the loss could be 18% in 2020, 23% in 2050, and 25% in 2080 scenarios, if no adaptation measures are followed (Figure 1.4). Likewise, in northwest India toward 2050 irrigated rice will have a yield loss of 15–17%. However, with adoption of suitable adaptation measures, the losses can be averted and even additional yields could be harvested. This underpins the importance of developing new techniques such as stress-tolerant

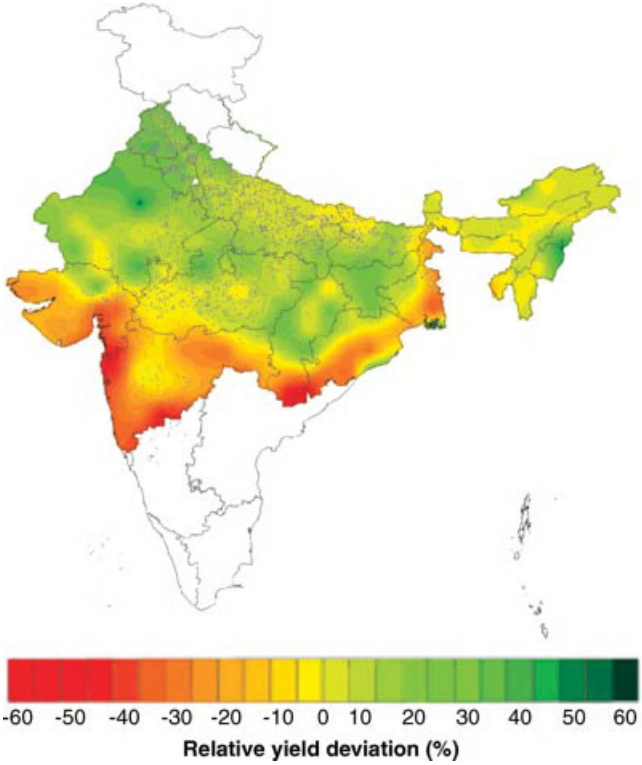

Figure 1.4 Net vulnerability of wheat in 2020 scenario. (*Source*: Ref. [8], with kind permission by Naresh Kumar, ICAR.)

varieties, efficient nutrient and water use technologies, and management practices suited to different agroecological regimes. Similar trends were projected for potato.

The simulation results had shown that the future climate change will have a positive impact on productivity of rainfed soybean and to a lesser extent on groundnut. The maximum positive impact was on chickpea productivity, ranging from 23 to 57%. The differential behavior of soybean and groundnut was due to increased rainfall and that of chickpea due to increased crop season temperature.

Regarding horticultural crops, the coconut model indicated positive impact on coconut yield in west coast and parts of Tamil Nadu and Karnataka and negative effects in east coast. The overall national level impact is simulated to be positive. The climate change is projected to shift the apple belt upward and cause shifts in cropping patterns.

As regards livestock, global warming is expected to lead to a loss of 1.6 million tons in milk production by 2020 and 15 million tons by 2050 under the usual scenario. Based on temperature–humidity index (THI, Figure 1.5), this loss in 2020 is valued at about Rs. 2661 crores at current prices. The loss will be highest in Uttar Pradesh. The crossbred cows are expected to suffer more from climate change.

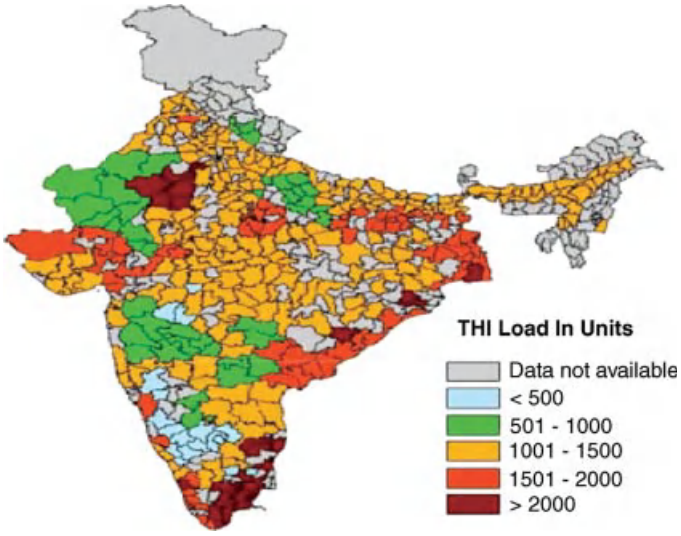

Figure 1.5 Annual THI load on livestock. (*Source*: Ref. [8], with kind permission by Naresh Kumar, ICAR.)

This underpins the need for conserving and improving local breeds that are tolerant to the climatic fluctuations and stresses.

As regards fisheries, even a 1 °C temperature increase would have profound impact. The marine and freshwater fishes will respond differently. Moreover, within each group, the interspecies differences in their responses to climate change will be profound. For instance with warming of sea surface, the oil sardine is able to find temperature to its preference in the northern latitudes and eastern longitudes, thus expanding its distributional boundaries. Likewise, the spawning season of several fishes will be changed. As regards inland fisheries, a major shift in spawning and breeding behavior of Indian carps has been seen. A number of fish species that were predominantly available in lower and middle Ganga about 50 years ago are now found in the upper cold water stretch up to Tehri.

As regards impact of climate change on natural resources, while in general there will be an increase in mean annual streamflow, it will decrease in summer months. With the increase in latitude, the annual runoff and soil loss will decrease. However, in most part of the country, mostly during monsoon season, the runoff and soil loss will increase considerably, being 6–12 times during 2071–2100 that of 1961–1990 in Coimbatore, Tamil Nadu. In other places, the increase will range from 56 to 309 % (Figure 1.6).

Water supplies will be impacted the most by the climate change. The flow of rivers originating in the Himalayas will be disrupted. For instance, Brahmaputra could lose 20% of its flow by 2050, adversely impacting the livelihoods of an estimated 28–41 million people. This underpins the need for sharp improvement in water use efficiency.

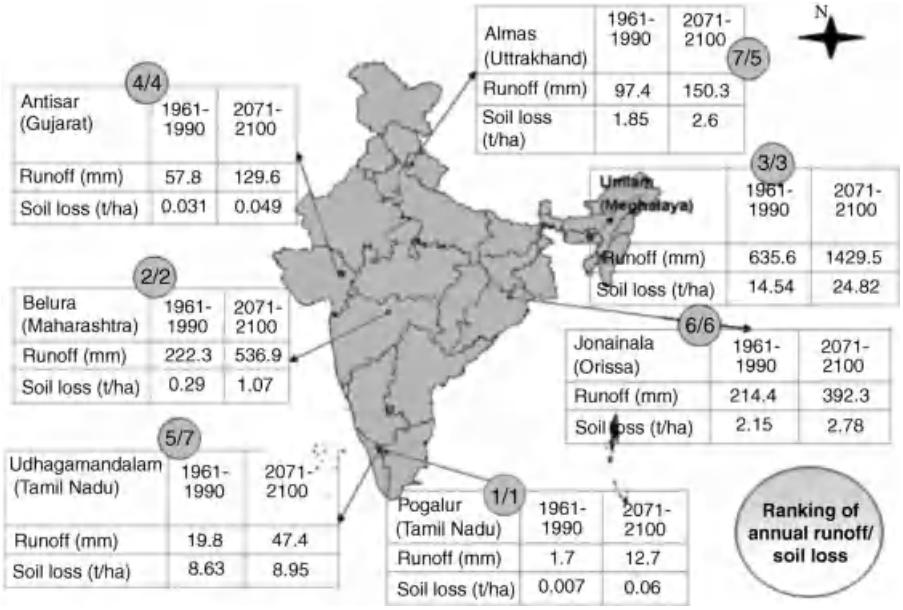

Figure 1.6 Annual runoff and soil loss during 1961–1990 and 2071–2100 from different agroecological regions of the country. (*Source*: Ref. [8], with kind permission by Naresh Kumar, ICAR.)

1.4
Natural Resources Management

Climate change impacts agriculture mostly through its impact on the natural resources, especially land, water, and biodiversity. In order to attain and maintain desired level of TFP growth, it is essential to launch an integrated management of these resources. As regards soil fertility management, thousands of well-equipped and well-functioning soil testing laboratories should be established and strategically located throughout the country (in which the private sector, agriclinics, and entrepreneurs can be effectively partnered) and each farmer should be issued a soil health card. Farmers should be oriented and convinced to get their soils tested on regular basis and manage their soil fertility through integrated nutrient application. Conservation agriculture involving technologies such as zero/reduced tillage is both time and cost saving in popular intensive cropping systems; for example, rice–wheat or maize–wheat rotation or potato plus maize (intercropped) and other diversified systems should be adopted extensively.

The sharp fall in water quantity and quality is the foremost threat to food security. Despite a viable national water policy being in place, water continues to be the most misused commodity. A countrywide campaign is required to conserve water and to

optimize its use as per resources. Other policy measures for water conservation and efficient use should include the following:

- Restoring water bodies around the country including village ponds, implementing the Million Well Recharge Program, promoting mandatory rainwater harvesting in rural as well as urban India, and managing water bodies and reservoirs by stakeholders and water users with the participation of Gram Panchayats and other local democratic bodies and self-help groups (SHGs) including women representatives.
- Withdrawing and not repeating all populist orders such as free electric supply to farmers by various State Governments that encourage excessive pumping of ground water and its wastage.
- Regulating and rationalizing the city water supply, and recycling the city wastewater to save the rivers from silting and pollution, thus increasing freshwater supply for domestic, agricultural, and industrial uses, and educating public at large continuously, making schools as base on importance of water and its rational use, and imposing penalty on the offenders. (Nearly Rs. 10 000 crores have gone down the drains under the Clean Ganges Campaign, but Ganga remains polluted as ever and is "dying" at places.)
- Extending the technologies for dryland farming to the small and marginal farmers and the National Rainfed Area Authority can have as its mandate the launching of second green revolution in dry farming areas beginning with pulses and oilseeds.
- On the basis of carbon credits, there can be water credits with each water user, be that a farmer, industrialist, or household, and he or she can earn credits or be punished accordingly.

The background paper for 12th Five-Year Plan has rightly identified the following NRM issues that require focused attention:

- Securing ecology of watershed and catchments.
- Cumulative environmental impact assessments (CEIAs) for vulnerable regions.
- Carrying capacity studies in selected river basins.
- Maintaining acceptable water quality and quantity through pollution control of water resources.
- Restoration of wetlands and lakes.
- Management of wastewater discharge from industrial and commercial establishments into major water bodies.

Several of the above issues were identified also in the past two Plans, but commensurate progress has not been made. Targeted activities should be defined under each district plan and judiciously implemented.

Bulk of the allocations to agriculture in the past Plans has gone to irrigation, which had significantly contributed to the Green Revolution process, but due to low water use efficiency at the farm level and losses in conveyance from source to the field, the overall payoff has been unsatisfactory. Misuse of water has resulted in widespread waterlogging and salinization. Several of the irrigation projects were delayed, but lately the accelerated completion of irrigation projects, micro-

irrigation, and participatory water management have been emphasized. India has been a leader in agroecological zoning and watershed-based integrated land and water use. Recent reviews of watershed programs have revealed that the programs had generally ignored the social, humanware, and equity concerns and had suggested to adopt watershed plus plus approach. A water productivity atlas should be prepared for the whole nation.

Rainfed regions, despite accounting for 40% of the population, 40% of food grain production, and 60% of the livestock population, are 13–15 times underinvested compared to irrigated command development. Effective rainwater harvesting and recycling and risk-proofing technologies and devices should encourage farmers to adopt improved farming practices and to bridge the huge yield gaps. Nearly 28 million ha of rainfed area has good potential for runoff harvesting. This harvested water can be used for at least one supplementary irrigation with high payoff yielding additional 9–10 million tons of food grains (Table 1.4).

Table 1.4 Additional production estimate of proper management of rains in India.

Crop group	Traditional production (million tons)	Additional production (million tons) with limited irrigation	
		Normal rains	Subnormal rains
Rice	7.612	3.549	3.776
Coarse cereals	8.300	4.410	3.415
Oilseeds	4.213	1.658	1.590
Pulses	3.717	1.152	1.078
Total	23.842	10.769	9.859

Source: Ref. [9].

Biodiversity, besides providing diversified food and nutrition resources, has direct implications on natural resources conservation, regional and global ecologies, and sustainability of agricultural production systems. Fortunately, India has been endowed with one of the richest biodiversity reserves in the Himalayas, river valley basins, costal areas, including offshore islands, and rain forests. But the pace and manner of development is presenting an imminent threat to the multiplicity of biodiversity. As a part of strategy to save and conserve, intertwining of biodiversity conservation should be an integral part of the large river valley projects, railroad construction, industrial and urban expansion, mining projects, and special economic zones (SEZs). Any further loss of biodiversity will have an adverse effect on the conservation and upkeep of land and water resources, which have a direct bearing on food and agriculture production, productivity, and food security. India having ratified the International Convention on Biodiversity (CBD) and being a party to the convention must be committed to conserve its biodiversity and also play a leading role globally. A national biosecurity umbrella encompassing food safety, environmental safety,

human and livestock health, and SPS and other risk management and regulatory provisions particularly under TRIPS should be created.

Forests and the benefits they provide in the form of food, income, and watershed protection have an important and often critical role in enabling people around the world to secure a stable and adequate food supply. Forest food resources are important to the most food insecure people because to them the forest food is most accessible and nutritious. Tropical forests present a range of uncultivated foods such as fruits, grains, seeds, leaves, roots, and tubers of food value, fish and a range of wild animals, as well as feed and fodder for animals. Need, however, is to check the deforestation and their degradation through increasing the awareness, educating the masses, and strict implementation of laws and policies. Rights of forest tribes, as detailed in the forest and tribal bill, should be protected.

Enhanced sustainability is a must for obviating instability in food availability and only a safe environment can sustain high productivity. Sustainable agriculture in safer environment is essential to save the environmental parameters in atmosphere, lithosphere, and hydrosphere while conducting agricultural operations. This is to be achieved through adoption of an ecotechnology approach encompassing integrated crop management (ICM) inclusive of integrated nutrient management (INM), integrated water management (IWM), and integrated pest management (IPM) – all grouped under "Green Agriculture." In addition to being eco-friendly, the technology should be cost effective and suited to the resource-poor farmers, encompassing the three E concerns for food security: economics, ecology, and equity. The paradigm shifts toward integrated farming system and ecotechnologies should be widely operationalized.

State and Central Governments, SAUs, KVKs, ICAR institutes, ATMAs, NGOs, private sector, farmers, women groups, small farmers' estates, cooperatives, Panchayati Raj Institutions, and other grassroot rural and community-based institutions must work in a participatory and interactive mode through adopting watershed-based approach that provides multiple entry points and synergistically converges efforts of the various partners. Panchayati Raj Institutions should be suitably strengthened to bring the necessary convergence at grassroot level.

Regional diagnostic centers equipped with modern facilities should be established for seed testing, soil and plant analysis, and diagnosis of insect pests and diseases of crops, animals, and fishes. These centers should be capable of addressing the farmers' problems of the region in all agricultural, horticultural, animal husbandry, poultry, fishery, postharvest management, and marketing subsectors. Also, they should have the backward links with district development programs and forward links with SAUs and ICAR institutes in the region. There has to be a strong team of specialists including socioeconomists placed at the centers. Selected KVKs and ATMAs with due strengthening could perform this role. Some of the KVKs should strategically be earmarked primarily for catering to the needs of women farmers. It may be remembered that other things being similar, women suffer more than men when exposed to climate change stresses. Therefore, women-specific adaptation and mitigation programs and plans should be designed.

1.5
Adaptation and Mitigation

Agricultural diversity is a manifestation of climatic adaptation. Farmers and farming communities have shaped and channeled the adaptation depending on technology availability, economic viability, and their socioeconomic capacity. A two-dimensional approach is needed to increase adaptive capacity of agriculture to climate change. In the longer term, adaptation to increased mean temperature and rainfall intensity will be important. In the short term, adaptation to unpredictable weather extremes such as drought, flood, heat, and cold will assume priority. Short-term land and water use plans must therefore be developed for ensuring climate resilience.

Adaptation should include autonomous as well as planned measures. Autonomous adaptation – the ongoing implementations of existing knowledge and technology in response to changes in climate – would include variety and crop substitution, altering irrigation, water management practices, fertilizer and other inputs timing, quantity and mix to cut cost without compromising yield, water harvesting, watershed management and increased water use efficiency, community-based management of water, adjusting overall farming system, and making intelligent use of weather forecasting.

The long-term adaptation is a must now. It will need additional knowledge, information, technologies, investments, infrastructures, and institutions integrated with the decision support system. Insurances, safety nets, cash transfers, and other risk management options to reduce vulnerability to shocks are also important parts of the solution. Greater emphasis must be placed on land and water use planning, monitoring, and community action. The Government must have a dynamic policy, plan, and strategy on climate change management to address the various issues.

As regards mitigation, agriculture being both a victim of and a contributor to GHGs and other environmental pollutions, an integrated resource and pollutant management strategy should be adopted. As regards GHGs, a three-pronged approach based on reducing emissions, enhancing removals, and avoiding (or displacing) emissions should prove effective. Grassroot organizations, primary agricultural cooperatives, and other cooperative systems should be empowered to increase carbon sequestration and their participation in the carbon market and income generation from environmental services without jeopardizing short-term production at smallholder farms. Governments, international agencies, private sector, farmers' organizations, CSOs, investors, and donors should collaborate in developing a win–win situation.

Research on genetic improvement, promotion of resource conservation technologies, and diversification would help the smallholder farmers in empowering them to adapt to and cope up with the situations. Research on organic recycling, alternative sources of energy, and enhanced and efficient biomass production and utilization will have high payoff. Successful models of identifying and applying appropriate technologies and supporting collective action of farmers with natural

resource management in the most fragile environment and in tribal areas should be up- and outscaled. Systems such as mobile telephony for carrying the viable technologies, weather forecasts and weather-related information, and information on markets, prices, and pest and disease management should be promoted.

As the smallholder farmers are most vulnerable, an affordable and effective insurance system has to be introduced and implemented. The main risks of such farmers are related to (i) crop, (ii) weather, (iii) life, (iv) health, and (v) price. Whereas an effective price support system and marketing infrastructure may cover price-related risks and National Agriculture Insurance Schemes may cover the crop-related risks, the ITGI's products relating to weather insurance, general insurance, health insurance, and accidental death and injury insurance are particularly useful for climate change management. Rural cooperatives and other private organizations could become nodal points and agents for these insurance products and cover their members. A transparently and professionally managed Agriculture Risk Fund, as recommended by the National Commission on Farmers, should be established to insulate extremely poor and distressed farmers in the event of extreme weather and natural disaster conditions [10].

Since climate change poses complex challenges such as multiple abiotic stresses on crops and livestock, shortage of water, land degradation, and loss of biodiversity, a focused and long-term research is required to find solutions to the problems specific to our country. In this context, it is gratifying to note that a National Initiative on Climate Resilient Agriculture (NICRA) with a substantive budgetary outlay has been launched by the ICAR under the supervision of NRM Division with implementation by CRIDA. The scheme attempts to develop and promote climate resilient technologies in agriculture that will address vulnerable areas of the country. The outputs of the scheme will help the districts and regions prone to extreme weather conditions such as drought, floods, frost, heat waves, and so on to cope with such extremes, with special consideration to small and marginal farmers in rainfed, coastal, and hill areas.

The Initiative, however, should develop common metrics and tools for demonstrating the impacts on a wider scale, and also for benefiting from external financing (e.g., Clean Development Mechanism, Adaptation Fund of the Kyoto Protocol, Green Climate Fund, and private sector sources) to support community-based efforts to implement CSA practices and projects. In this context, effective GHG accounting methods should prove helpful.

1.6
Climate Resilient Agriculture – The Way Forward

The developing countries bear 75–80% of the costs of damages caused by the changing climate and the disturbed ecological, environmental, economic, social, and biological settings. Recognizing that agriculture is both a victim of and a contributor to GHGs and other environmental pollutions, a two-pronged approach to reduce the emission on one hand and to develop adaptive measures to increase

agricultural resilience on the other hand will be needed. Fortunately, alternative agricultural practices designed for specific agroecologies are proving effective in reducing GHG emissions from agriculture and at the same time in improving yields under extreme weather.

The road ahead to agricultural adaptation to climate change should therefore integrate technology, policy, and finance options toward lowering emission and promoting inclusive growth. Agroclimatic zone-specific approaches for management of water and other natural resources through adaptation of appropriate agricultural practices such as integrated watershed management, designer crops that may tolerate extreme biotic and abiotic stresses, conservation agriculture, system of rice intensification (SRI), development and use of climate analogues, and carbon finance for adaptation should be actively promoted.

It is well recognized that growth in agricultural sector of developing countries reduces hunger and poverty more effectively than do urban and industrial growth. This is particularly true under the fast changing climate. Bridging the yield gaps and enhancing sustainability of production systems should be the main pillars of achieving the desired accelerated and inclusive agricultural growth. The following actions are called for:

- Getting technology moving and ensuring access of farmers to the technology by re-establishing a trained, retooled, and dedicated cadre of extension workers, and strengthening of agricultural research and technology development.
- Increasing investment, efficiency, and systems support, rationalizing subsidy, and ensuring timely flow of cost-effective quality inputs and credit, insurance, and other institutional support systems.
- Augmenting the physical and economic connectivity of farm to market, postharvest operations including the role of food processing industries, cautious diversification without jeopardizing food security, and ultimately enhancing farmers' income and rural employment security.
- Promoting inclusiveness by enhancing access to land, water, credit, market, skills, and technology on the part of the poor and women, especially in the hot spots.

Food grain production and productivity, particularly of small and marginal farmers, should be accelerated to achieve the desired growth rate and equity. While the ongoing miniaturization of farm sizes should be halted and reversed through promoting off- and nonfarm rural employment, land reforms and land leasing, and other measures, improving small farm productivity must be the most important single development strategy to empower the resource-poor farmers to manage the stresses caused due to climate change. Village seed and food banks, preferably managed by women, should be established in each Panchayat.

Low-yield areas should be mapped and location-specific causes of the productivity gaps and land factor productivity should be identified and specific land and water use decisions should be promoted by restructured and retooled State Land Use Boards to realize the yield and income potential. Agricultural diversification should be promoted in consonance with market opportunities, farmers' income, and ecological sustainability.

The slowdown in livestock and horticulture subsectors where huge yield gaps and unexploited potential exist should be arrested and small farm estates for horticulture, cotton, poultry, and aquaculture should be created to promote group farming and postharvest management. Knowledge-based and market-led diversification and extension should be promoted by institutionalizing the Every Village a Knowledge Center or Gyan Choupal movement. Climate change extension agents should be deployed in village clusters and should be equipped with relevant information and communication tools and devices.

In the livestock subsector, productivity of livestock in India is low due to fodder, feed, healthcare, market, and price constraints. Integrated crop–livestock–fish farming systems, cooperatives (Amul being world famous experience), and SHGs, especially women SHGs for livestock and agriclinics operated by veterinary and farm science graduates and paravets, coupled with fodder and feed banks, will immensely increase the productivity and income of livestock owners. Livestock insurance should particularly be accessible to smallholders. In view of the setback to poultry industry due to bird flu outbreaks, quarantine and testing facilities at all ports of entry should be established. Poultry rearing should be recognized as an agricultural activity and appropriate support should be extended to backyard poultry farmers to establish smallholders' poultry estates.

As regards the fisheries subsector, the following steps are suggested for accelerating production:

- Introduce integrated coastal zone management and scientific fish rearing, harvesting, and processing, including introducing mother ships, and develop suitable dynamic policies and governance, particularly for the management of exclusive economic zone (EEZ) extending to nearly 2 million km^2 of sea surface, which amounts to two-thirds of the land surface available to India. Strengthen early warning systems and instant communications to escape damages from typhoons, cyclones, and other weather aberrations.
- Undertake and institutionalize well-planned Aquarian Reforms to provide landless labor families access to village ponds and other water bodies in the public domain for aquaculture, and clarify property and use rights.
- The National Fisheries Development Board (NFDB) should promote the Aquarian Reforms and ensure congruence of ecology, economics, gender equity, and employment generation and should help resolve the conflicts between aquaculturists and agriculturists as well as resolve problems of local population due to salt water entering into the aquifer and pollution caused by intensive systems of aquaculture, and settle conflicts related to seaweed farming and introduction of exotic carps and other alien invasive species.
- Establish Fish for All Training and Capacity Building Centers (decentralized) for comprehensive training of the capture/culture–consumption chain, quality, hygienic handling, and biosecurity (with due attention to needs of fisherwomen) to enable fisher families to take up additional income earning activities.
- Institutionalize Responsible Fisheries (ecosystem approaches), promote wider application of semi-intensive production systems, and strengthen research and

extension for integration of aquaculture and other subsectors of agriculture and public–private partnership particularly in hatcheries and stocking programs.

Under the United Nations Framework Convention on Climate Change (UNFCCC) in 2011, the Commission on Sustainable Agriculture and Climate Change encouraged policy action to help achieve food security while addressing climate change. It recommended seven priority actions [11]. Two of the most important priority actions in the context of climate smart agriculture (CSA) are (1) sustainably intensify agricultural production while reducing emissions and other environmental impacts and (2) create comprehensive information systems on human and ecological dimensions. Expanding CSA that results in increased food production, limits greenhouse gas emissions, and prepares for future climate change is a major goal and challenge especially in agriculture-based countries such as India where rural livelihoods depend primarily on shrinking and degrading ecosystems.

Research and technology development (supported by policy and institutions) will need to be geared to meet the veritable challenges. The much needed congruence of high productivity and sustainability in face of the intensifying volatilities due to climate change, biotic and abiotic stresses, and market instabilities, let alone the challenges of adequately feeding the swelling population from shrinking and degrading natural resources, can be underpinned only by developing smart technological solutions and innovations. New and modern sciences and cutting-edge technologies, especially intensive characterization of germplasm, molecular breeding, and genetic engineering for crop improvement and development of designer crops, coupled with associated resource management practices, including indigenous knowledge, practices, and technologies, will increasingly be called upon to provide the desired solutions.

There is a need for more integrated research and improved knowledge systems on the specific agricultural production methods that would work best for individual regions, farming systems, and landscapes. In addition, for agricultural practitioners in general, there is a need for public domain systems that provide information on "repeated observations" of successful CSA practices that are suitable for both large- and smallholder agriculture. Impacts of such practices should be tracked and reported through (1) design of standardized tracking metrics for measuring the impacts of CSA practices/strategies in terms of food production, GHG emissions, and climate resilience, (2) development or adoption of a public domain system for reporting and illustrating the results of these efforts, and (3) the potential establishment of a private sector-supported Climate Fund providing a mechanism for corporate to participate in the promotion and sustainability of climate smart agricultural practices.

The system may customize data collection and communication of successful project activities and adapt the Web-based software tool, such as CarbonCounts™ being used for monitoring, reporting, and verification (MRV) in Mexico for tracking of GHG mitigation and adaptation activities. Some modifications to this tool would likely be necessary; otherwise, a reporting tool specifically for Indian activities should be designed. Further, national investments in climate financing should

be streamlined to attract sustained national and international funds. With the increasing emphasis on corporate social responsibility (CSR) initiatives, a "Climate Fund" should be established to leverage the financial capacity of the private sector to direct additional philanthropic resources toward climate-friendly adaptation interventions that will target and support the advancement of India's poorest farmers and help uptake of CSA practices.

Finally, commensurate with priority policy actions, as also suggested by UNFCC/CSACC, the following policy options are needed: (1) integrate food security and sustainable/resilient agriculture into global and national policies, (2) increase global and national investments in resilient/sustainable agriculture and food systems, and (3) target programs and policies to assist vulnerable populations. Scientists should inform policies regarding the relative importance of adaptation and mitigation across various subsectors of agriculture (crop, livestock, fishery, and forestry) and provide guidelines to ensure synergies for improving food security and livelihoods. Effective methodologies should be developed for assessing risks and benefits and for evidence-based evaluations to enable scaling up to "safe operating space."

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2
Improving Crop Productivity under Changing Environment

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Abstract

Climate change is affecting agriculture directly or indirectly, worldwide. Temperature (high and low), level of greenhouse gases, rainfall, and high humidity directly affect the crops, pathogens, insects, and weeds. Several new diseases, weeds, and insect pests have started appearing with the changing climate. Some horticultural crops such as apple and pear will not be getting minimum chilling hours, which will reduce their production. New technologies such as plant genetic engineering and DNA markers have now become valuable tools in crop improvement for rapid precision breeding for specific purposes. Several climate resilient agricultural technologies such as zero tillage (no tillage), raised-bed planting, tensiometer, laser land leveling, happy seeder, and rotavator have been developed for conservation agriculture. Further, drip irrigation and fertigation, leaf color chart (LCC) for need-based application of nitrogen, sensor-based yield monitors, nitrogen sensors/green seekers, special-purpose vehicles with sensor-based input applicators, integrated nutrient management (INM) systems, integrated pest management (IPM) systems, integrated disease management (IDM) systems, site-specific management systems using remote sensing, GPS, and GIS, and Web-based decision support systems for controlling diseases and insect pests have been developed and are being commercialized for precision farming and to mitigate the climate change.

2.1
Introduction

Climate change refers to changes in the statistical distribution of weather across a period of time that ranges from decades to millions of years. It can be a change in the average weather or a change in the distribution of weather events around an average. Climate change may be limited to a specific region, or may occur across the whole Earth, and this type of climate change has been documented [1]. In the context of environmental policy, climate change usually refers to changes in modern climate. It may be experienced as anthropogenic climate change, more

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generally known as "global warming" or "anthropogenic global warming" (AGW). Recent environmental change attribution is the effort to observe the Earth's climate scientifically and to know the mechanisms responsible for recent changes. The effort has focused on changes observed during the period of instrumental temperature record, when records are most reliable, particularly for the past 50 years, when human action has grown fastest and observations of the troposphere have become accessible. The dominant mechanisms [2] responsible for recent climate change have been attributed to the following human activities:

- Deforestation that is responsible for global changes to land surface.
- Increasing atmospheric concentrations of greenhouse gases (GHGs).
- Increasing atmospheric concentrations of aerosols.

Recent reports from the Intergovernmental Panel on Climate Change (IPCC) have concluded that most of the observed increase in globally averaged temperatures since the mid-twentieth century is very likely caused by the observed increase in anthropogenic greenhouse gas concentrations [3]. It is extremely unlikely (<5%) that the global warming pattern during the past half-century can be explained without invoking external forces (i.e., it is contradictory to being the result of internal variability), and it is very unlikely that it can be attributed to only known natural external causes. The warming occurred in both the ocean and the atmosphere and took place at a time when natural external forcing factors would likely have shaped cooling [4]. Further, from new estimates of the combined anthropogenic forcing due to greenhouse gases, aerosols, and land surface changes, it is extremely likely that human actions have exerted a considerable net warming influence on climate since 1750. It is virtually certain that anthropogenic aerosols produce a net radioactive forcing (negative cooling) influence with a greater magnitude in the Northern Hemisphere than in the Southern Hemisphere. The panel defines "very likely," "extremely likely," and "virtually certain" as indicating probabilities greater than 90, 95, and 99%, respectively [2]. Environmental change is also attributed to the land use patterns. While 66% of anthropogenic CO_2 emissions during the past 250 years have resulted from burning fossil fuels, 33% have resulted from changes in land use such as deforestation. Deforestation both reduces the quantity of carbon dioxide absorbed by deforested regions and releases greenhouse gases, jointly with aerosols, through biomass burning that normally accompanies it. Further, the climate change has been attributed to land use; that is, terrestrial land is often altered by use. This effect is more noteworthy locally than globally [5].

Livestock production has also contributed to climate change. Scientists attribute more than 18% of anthropogenic greenhouse gas emissions to livestock and livestock-related activities such as deforestation and increasingly fuel-intensive farming practices. Attributions to the livestock include 9% of global carbon dioxide emissions, 35–40% of global methane emissions (primarily caused by enteric fermentation and manure), and 64% of global nitrous oxide emissions, primarily caused by fertilizer use [6]. Agriculture is strongly influenced by

changes in climate. During the past few decades, climate change caused by global warming has become the focus of worldwide attention. The most eminent change is the increase in the atmospheric temperature caused by increased levels of greenhouse gases, that is, carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and chlorofluorocarbons (CFCs) in the atmosphere. The CO_2, CH_4, and N_2O concentrations were 280 ± 6 ppm, 700 ± 60 ppb, and 270 ± 10 ppb between 1000 and 1750 AD. In 2005, these values had increased to 379 ppm, 1774 ppb, and 319 ppb, respectively. The global increases in CO_2 concentrations are primarily attributable to fossil fuel use and land use change, whereas those of methane and nitrous oxide are primarily attributable to agriculture. These increases in GHGs have resulted in the warming of the climate system by 0.74 °C between 1906 and 2005. Eleven of the 12 years (1995–2006) rank among the 12 warmest years in the instrumental record of global surface temperature kept since 1850 [7].

The United Nations Environment Programme (UNEP), along with the World Meteorological Organization (WMO), established the IPCC in 1988 to periodically access the state of global environment and to advise various UN agencies on climate change. According to a recent report of the IPCC [7], the rate of global warming has been much higher in the recent decades and the nighttime minimum temperatures have been increasing at twice the rate of daytime maximum temperatures. The quantity of rainfall and its distribution have also become more uncertain. In some places, climatic extremes such as droughts, floods, timing of rainfall, and snowmelt have also increased. The sea level has risen by 10–20 cm, with regional variations. Similarly, snow cover is also believed to be gradually decreasing. All these changes can have tremendous effects on agricultural production and hence food security of any region.

Human and industrial activities are primarily responsible for the rise in the concentration of greenhouse gases in the atmosphere. The increasing levels of CO_2 are mostly because of fossil fuel combustion. The CO_2 is often referred to as "polluting gas," which is true only if it is present in too large a quantity. But in reality it is also a "life savior." Nature has provided us with a miraculously balanced energy cycle, in which CO_2 plays an important role by acting as a sink for trapping the energy from the sun. There is a large fixation of CO_2 in agriculture, but estimates are generally not available because of continuous consumption of its products by the human beings and other secondary consumers. In India, fixation of CO_2 assumes importance because almost 190 million ha of land is being used for farming. The estimated dry matter production from agriculture in India is almost 800 million tons/year. This is equivalent to fixation of 320 Tg of C or 1000 Tg of CO_2 per annum. Only a part of this is retained across time, while the rest is released back to the atmosphere. The data in Table 2.1 show that the United States is the major contributor to CO_2 emission, accounting for about one-fourth of the total global emissions (23.06%), whereas India's contribution is about 4%. Per capita production of carbon is also highest in the United States followed by Canada [8].

Table 2.1 Global share (%) of some countries in CO_2 emission [8].

Country	Year	
	1990	2003
Unites States	23.04	23.06
China	10.41	14.07
Russia	9.67	6.38
Japan	5.54	4.79
India	2.63	4.07
Germany	4.24	3.35
Canada	2.19	2.39
United Kingdom	2.76	2.24
Italy	1.91	1.85
France	1.80	1.63
Rest of the world	38.61	36.17

The IPCC has recently compiled the magnitude of change in CO_2 for different parts of the world and according to it, by 2010, CO_2 level will increase to 397–416 ppm and by 2070 to 605–755 ppm. The industrial processes and products cause CFC emissions, whereas the increased agricultural activities and organic waste management are presumed to be contributing to the buildup of CH_4 and N_2O in the atmosphere. The greenhouse gases produced by various activities are considered to be the agents of climate change and their relative contribution is given in Figure 2.1.

Crop productivity is constrained by the interseasonal and intraseasonal variation of the weather parameters through their direct as well as indirect effects,

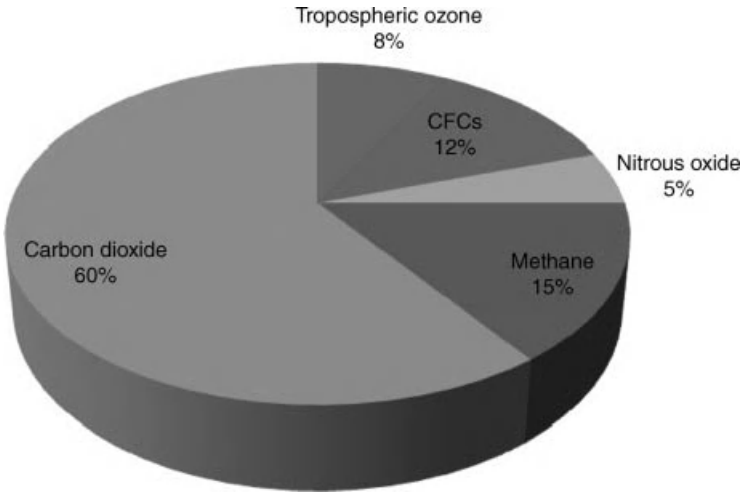

Figure 2.1 Relative contribution to the anthropogenic greenhouse effect. (*Source*: Ref. [7].)

such as weather-induced changes, on incidence of pests and diseases, and requirement or availability of water for irrigation. The given uncertainties in regional climates are even aggravated by global warming, which may have serious direct and indirect consequences on crop production and, hence, on food security. It is, therefore, important to have an assessment of the consequences of climatic variability on crops, especially cereals, and of possible adaptation strategies.

The CO_2 is vital for photosynthesis and hence for plant growth. An increase in the atmospheric CO_2 concentration affects agricultural production by changing climate, and photosynthesis and transpiration rates. The rise in atmospheric CO_2 concentration from pre-industrial level of about 280 ppm to about 377 ppm currently is well documented [9]. It is, therefore, important to assess the combined effects of elevated atmospheric concentration and climate change on the productivity of a region's dominant crops. The direct effects of increased concentrations of CO_2 are generally beneficial to vegetation, especially for C_3 plants (wheat, rice, barley, oats, peanut, cotton, sugar beet, tobacco, spinach, and soybean), as elevated levels lead to higher assimilation rates and to an increase in stomatal resistance, resulting in a decline in transpiration and improved water use efficiency of crops.

Simulation studies have been conducted for predicting the plausible effects of elevated levels of CO_2 on yields of crops. Under elevated CO_2 levels, yields of rice and wheat increased by 15 and 28%, respectively, for a doubling of CO_2 in northwest India. We have also found that CO_2 levels are able to counteract the adverse effects of temperature increase on growth and yield of crops to some extent. A temperature increase of 1.0 °C from normal and doubled CO_2 concentration of 600 ppm increased maximum leaf area index of wheat, rice, and maize by 7.8, 0.8, and 3.6% from normal, respectively, and grain yield of wheat, rice, and maize by 10.4, 0.5, and 2.3% from normal, respectively [10].

To feed the almost 7 billion people living on this planet, the production of high-quality food must increase, with reduced inputs, but this will be challenging under changing environment. Plant breeders need to focus on traits that increase yield and also improve quality. Thus, new technologies must be developed to accelerate breeding through improving genotyping and phenotyping methods and by increasing and utilizing the available genetic diversity in breeding germplasm. The major gain will come from delivering these technologies in developing countries, but the technologies will have to be economically accessible and readily disseminated. Breeding and agronomic improvements have achieved a linear increase in food production globally, at an average rate of 32 million metric tons per year. To meet the recent Declaration of the World Summit on Food Security target of 70% more food by 2050, an average annual increase in production of 44 million metric tons per year is required, which represents a 38% increase over past increases in production, and it must be sustained for 40 years. This magnitude of sustained increase in global food production is unprecedented and requires substantial changes in methods relative to agronomic and crop improvement practices [11].

2.1.1 Global Environmental Change Alters Crop Targets

Rising CO_2 acts as a fertilizer for C_3 crops and accounts for approximately 0.3% of the observed 1% rise in global wheat production [12], although this benefit is likely to shrink, because rising temperatures will increase photorespiration and nighttime respiration. Rising temperature benefit is the alleviation of low-temperature inhibition of growth, which is a limitation at higher latitudes and altitudes. Offsetting these benefits, however, are the obvious detrimental changes, such as an increased frequency of damaging high-temperature events, new pest and disease pressures, and distorted patterns of drought. Negative effects of other pollutants, particularly ozone, will also lessen benefits from rising CO_2 and temperature to plant growth. Particularly exigent for society will be changes in weather patterns that will require modifications in farming practices and infrastructure, for example, transport networks and water storage. Because one-third of the world's food is produced on irrigated land [13], the expected impacts on global food production are many. Along with agronomic and management-based approaches to improving food production, improvements in a crop's capability to maintain yields and quality with lower water supply will be critical. Put cleanly, we need to increase the tolerance of crops to drought, salinity, and high temperature.

Nitrogen use efficiency (NUE) has also emerged as a key target in the context of global environmental change. Human actions have already more than doubled the amount of atmospheric N_2 fixed annually, which has led to environmental impacts, such as increased water pollution, and the emission of greenhouse gases, such as nitrous oxide. Nitrogen inputs are gradually being managed by legislation that limits fertilizer use in agriculture. Furthermore, rising energy costs mean that fertilizers are now commonly the highest input cost for farmers. New crop varieties with improved NUE will need to be developed. Consequently, it is important that breeding programs should aim at developing strategies to select for high yield, quality, and NUE.

2.1.2 Crop Productivity

Crop productivity is measured as the ratio of agricultural outputs to agricultural inputs. While individual products are usually measured by weight, their varying densities make measuring overall agricultural output difficult. Therefore, output is usually measured as the market value of final output, which excludes intermediate products, such as corn feed used in the meat industry. This output value may be compared with many different types of inputs such as labor and land (yield). These are called partial measures of productivity. Agricultural productivity may also be measured by what is referred to as total factor productivity (TFP). This method of calculating agricultural productivity compares an index of agricultural inputs to an index of outputs, which was established to remedy the shortcomings of the partial

measures of productivity; notably, it is often hard to identify the factors that cause them to change. Changes in TFP are usually attributed to technological improvements [14]. Increase in agricultural productivity is often linked with questions about sustainability and sustainable development. Changes in agricultural practices necessarily bring changes in demands on resources. This means that as regions implement measures to increase the productivity of their farmland, they must also find ways to ensure that future generations will also have the resources that they will need to live and thrive.

2.1.3 Climatic Factors Affecting Crop Production

Man cannot control the climate, but crop management practices can be altered to maximize yield. Climate is the most dominating factor influencing the suitability of a crop to a particular region. The yield potential of a crop mainly depends on climate. More than 50% of yield variation of crops is attributable to solar radiation, temperature, rainfall, and relative humidity. Further, wind velocity also influences crop growth to some extent. Atmospheric factors that affect the crop plants are called climatic factors, which include

- precipitation;
- temperature;
- atmospheric humidity;
- solar radiation;
- wind velocity and atmospheric gases.

2.1.3.1 Precipitation

It results from evaporation of water from seawater and land surfaces. The process involved in the transfer of moisture from the sea to the land and back to the sea is known as the hydrologic cycle. Hydrologic cycle is the continuous circulation of water between hydrosphere, atmosphere, and lithosphere. Precipitation includes rainfall, snow, or hail; fog drip and dew also contribute to moisture. Fog consists of small water droplets, whereas dew is the condensation of the water vapor present in the air. Precipitation influences the vegetation of a place. Most of the crops receive their water supply from rainwater, which is the source of soil moisture so essential for the life of a plant. The yearly precipitation, both in total amount and in seasonal distribution, greatly affects the choice of cultivated crops of a place.

2.1.3.2 Temperature

It is regarded as a measure of intensity of heat energy. The range of maximum growth for most plants is between 15 and 40 °C. Every plant community has its own minimum, optimum, and maximum temperatures, known as their cardinal points. Temperature is determined by the distance from the equator (latitude) and by the altitude.

2.1.3.3 Atmospheric Humidity

Water that is present in the atmosphere in the form of invisible water vapor is termed as humidity of the air. Evapotranspiration of crop plants increases with the temperature but decreases with high relative humidity affecting the quantity of irrigation water. Moist air favors the growth of many fungi and bacteria that seriously affect crop production.

2.1.3.4 Solar Radiation

Solar energy provides two essential needs of plants:

a) Light required for photosynthesis and for many other functions of the plant, including seed germination, leaf expansion, growth of stem and shoot, flowering, fruiting, and even dormancy.
b) Thermal conditions required for the normal physiological functions of the plant. Light helps in synthesis of chlorophyll pigment. Light affects the plants in four ways: intensity, quality (wavelength), duration (photoperiod), and direction.

2.1.3.5 Wind Velocity

It affects growth mechanically (damage to crop) and physiologically (evaporation and transpiration). Hot, dry winds adversely affect photosynthesis and hence productivity, by causing closure of the stomata even when soil moisture is adequate. Moderate winds have beneficial effects on photosynthesis by continuously replacing the CO_2 absorbed by leaf surfaces.

Changing environment is regarded as a major threat to crop productivity, worldwide. To mitigate the effect of climate change, there is the need to develop matching crop varieties and production/protection technologies. New innovative approaches, such as conservation agriculture (CA), precision agriculture (PA), and biotechnology (BT), hold great promise for sustaining agricultural production. Conservation agriculture involves techniques of "no tillage" and crop residue management (recycling) and helps conserve natural resources such as water, soil, and nutrients. Several CA technologies have been developed, for example,

- zero tillage (no tillage);
- raised-bed planting;
- tensiometer;
- laser land leveling;
- happy seeder;
- rotavator.

The "precision agriculture" is the need of the hour to apply right amount of inputs, at right time, at right place, and in right manner with right hardware. Several techniques ensuring "precision agriculture" have been developed and are being commercialized. Most popular technologies include

- drip irrigation and fertigation;
- leaf color chart for need-based application of nitrogen;

- sensor-based yield monitors;
- nitrogen sensors/green seekers;
- special-purpose vehicles with sensor-based input applicators;
- integrated nutrient management (INM) systems;
- integrated pest management (IPM) systems;
- integrated disease management (IDM) systems;
- site-specific management systems using remote sensing, geographical positioning system (GPS), and geographical information system (GIS);
- Web-based decision support systems for controlling diseases and insect pests;
- germplasm enhancement for biotic and abiotic stress management.

"Seed is the carrier of technology"; therefore, plant breeding involving innovative approaches of biotechnology can play a dominant role in developing climate resilient varieties.

A series of high-yielding crop cultivars possessing resistance to diseases and insect pests and with improved quality have been developed following the conventional methods such as introduction, selection, hybridization, polyploidy, and mutation breeding. However, these methods are very time-consuming and laborious. Moreover, it has been rather difficult to develop improved varieties in vegetatively propagated and seedless crops. Plant genetic engineering and DNA marker technologies have now become a valuable adjunct in crop improvement for rapid precision breeding for specific purposes.

2.1.4
Plant Genetic Engineering

During the past 20 years, the combined use of recombinant DNA technology, gene transfer methods, and tissue culture techniques has led to efficient transformation and production of transgenics in a wide variety of crop plants [15]. In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops. Unlike conventional breeding, only cloned gene(s) of agronomic importance are being introduced without cotransfer of undesirable genes from the donor. The recipient genotype is least disturbed, which eliminates the need for repeated backcrosses. Above all, the transformation method provides access to a larger gene pool than available using conventional backcrossing, as the gene(s) can come from viruses, bacteria, fungi, insects, animals, human beings, unrelated plants, and even from chemical synthesis in the laboratory. Various gene transfer methods [16–20] have been developed for genetic transformation of plants. Rapid and remarkable achievements have been made in the production, characterization, and field evaluation of transgenic plants in several field, fruit, and forest plant species. However, the major interest has been in the introduction of cloned gene(s) of agronomic importance into commercial cultivars for their incremental improvement. Using different gene transfer methods and strategies, transgenics carrying useful agronomic traits have been developed and released in several crops.

2.1.4.1 Engineering for Herbicide Resistance

There have been two approaches to develop herbicide-resistant transgenic plants.

2.1.4.1.1 Transfer of a Gene Whose Enzyme Product Detoxifies a Herbicide (Detoxification) Using this approach, the introduced gene produces an enzyme that degrades the herbicide sprayed on the plant. For instance, introduction of bar gene cloned from bacteria *Streptomyces hygroscopicus* into plants makes them resistant to herbicides based on phosphinothricin. Bar gene produces an enzyme, phosphinothricin acetyltransferase (PAT), that degrades phosphinothricin into a nontoxic acetylated form. Plants engineered with bar gene were found to grow in phosphinothricin at levels 4–10 times higher than normal concentrations in field applications [21–23]. Likewise, bxn gene of *Klebsiella ozaenae*, which produces nitrilase enzyme, imparts resistance to plants against herbicide bromoxynil. Other genes, including tfdA for 2,4-D tolerance and GST gene for atrazine tolerance, have also been used.

2.1.4.1.2 Transfer of a Gene Whose Enzyme Product Becomes Insensitive to Herbicide (Target Modification) Using this approach, a mutated gene is introduced into the plant that produces a modified enzyme in the transgenic plant and that is not recognized by the herbicide; hence, the herbicide cannot kill the plant. For instance, a mutant *aro*A gene from bacteria *Salmonella typhimurium* has been used for developing tolerance to herbicide, glyphosate. The target site of glyphosate is a chloroplast enzyme 5-enol-pyruvylshikimic acid 3-phosphate synthase (EPSPS). Introduction of mutant *aro*A gene produces modified EPSPS, not recognizable to glyphosate. Likewise, sulfonylurea and imidazolinone herbicides inhibit acetolactate synthase (ALS) chloroplast protein. Tolerance to these herbicides has been achieved by engineering the expression of the mutant herbicide ALS gene derived from a plant.

2.1.4.2 Engineering for Insect Resistance

There have been two approaches to developing insect-resistant transgenic plants by transferring insect control protein genes.

2.1.4.2.1 Introduction of Plant Gene(s) Several insecticidal proteins of plant origin, such as lectins, amylase inhibitors, and protease inhibitors, can retard insect growth and development when ingested by insects at high doses [24, 25]. Some genes such as CpTi, PIN-1, PIN 11, αA-1, and GNA have been cloned from different plants and are being used in the genetic transformation programs aimed at developing insect resistance in plants [26–28].

2.1.4.2.2 Introduction of Bacterial Genes *Bacillus thuringiensis* synthesizes an insecticidal crystal protein, which resides in the inclusion bodies produced by the *Bacillus* during sporulation. This crystal protein when ingested by insect larvae is solubilized in the alkaline conditions of the midgut of the insect and processed by midgut proteases to produce a protease-resistant polypeptide that is toxic to the

insect. Lepidopteran-specific Bt gene from *B. thuringiensis* subsp. *kurstaki* has been widely and successfully used in tobacco, tomato, potato, cotton, rice, and maize for developing resistance against several lepidopteran insect pests [29–34]. The use of redesigned synthetic Bt gene has also been used in some of these crops and in several instances the synthetic versions have exhibited up to 500-fold increase in the expression [35]. Insect-resistant transgenic varieties/hybrids of several crops have been released for commercial cultivation in many countries [36].

2.1.4.3 **Engineering for Disease Resistance**

2.1.4.3.1 **Bacterial Resistance** Genetic engineering for bacterial resistance has met with relatively little success. The expression of a bacteriophage T_4 lysozyme in transgenic potato tubers led to increased resistance to *Erwinia carotovora*. Besides, the expression of barley α-thionin gene significantly enhanced the resistance of transgenic tobacco to bacterium *Pseudomonas syringae*. Advances in the cloning of several new bacterial resistance genes may provide better understanding in the area of plant–bacterial interactions. Further, RNAi technology has also been tried to control bacterial diseases. A crown gall disease management strategy [37] that targets the process of tumorigenesis (gall formation) by initiating RNAi of the iaaM and ipt oncogenes has been developed. Expression of these genes is a prerequisite for wild-type tumor formation. Transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* transformed with RNAi constructs, targeting iaaM and ipt gene(s), showed resistance to crown gall disease. Transgenic plants generated through this technology contained a modified version of these two bacterial gene(s) required to cause the disease and this was the first report to manage a major bacterial disease through RNAi. The extra genes recognize and effectively shut down the expression of the corresponding bacterial gene during infection, thus preventing the spread of infection. The incoming bacteria could not make the hormones needed to cause tumors and plants deficient in the silencing mechanism were hypersusceptible to *Agrobacterium tumefaciens* [38]. Successful infection relied on a potent anti-silencing state established in tumors whereby siRNA synthesis is specifically inhibited. The procedure can be exploited to develop broad-spectrum resistance in ornamental and horticultural plants that are susceptible to crown gall tumorigenesis. This approach can be advocated for the effective management of those pathogens that multiply very rapidly and result in tumor formation, such as *Albugo candida*, *Synchytrium endobioticum*, and *Erwinia amylovora*. The natsiRNA (nat-siRNAATGB2) was strongly induced in *Arabidopsis* upon infection by *P. syringae* pv. tomato and downregulated a PPRL gene that encodes a negative regulator of the RPS2 disease resistance pathway. As a result, the induction of nat-siRNAATGB2 increases the RPS2-mediated race-specific resistance against *P. syringae* pv. tomato in *Arabidopsis* [39]. Recently, the accumulation of a new class of siRNA, 30–40 nucleotides in length, termed as long siRNAs (lsiRNAs), was found associated with *P. syringae* infection. One of these lsiRNAs, AtlsiRNA-1, contributes to plant bacterial resistance by silencing AtRAP, a negative regulator of plant defense [40]. A *Pseudomonas* bacterial flagellin-derived peptide was found to induce the accumulation of miR393 in *Arabidopsis*.

The miR393 negatively regulated mRNAs of F-box auxin receptors, resulting in increased resistance to the bacterium (*P. syringae*), and the overexpression of miR393 was shown to reduce the plant's bacterial titer by fivefold [41].

2.1.4.3.2 **Virus Resistance** The genetic engineering of virus-resistant plants has exploited new genes derived from viruses themselves in a concept referred to as pathogen-derived resistance (PDR).

Coat Protein-Mediated Resistance (CP-MR) Introduction of a viral coat protein gene into the plant makes the plant resistant to virus from which the gene for the CP was derived. It was first demonstrated for TMV in tobacco [42]. Subsequently, virus-resistant transgenics have been developed in tomato, melon, rice, papaya, potato, and sugar beet [43]. A variety of yellow squash called "Freedom 11" has been released in the United States. Several CP-MR varieties of potato, cucumber, and tomato are under field evaluation. Transgenic papaya resistant to papaya ringspot virus (PRSV) has been developed and is being commercially grown in the United States [44].

RNAi Technology Antiviral RNAi technology has been successfully used for viral disease management in human cell lines [45–48]. The effectiveness of the technology in generating virus-resistant plants was first reported for PVY in potato, harboring vectors for simultaneous expression of both sense and antisense transcripts of the helper component of proteinase (HC-Pro) gene [49]. The P1/HC-Pro suppressors from the potyvirus inhabited silencing at a step downstream of dsRNA processing, possibly by preventing the unwinding of duplex siRNAs, or the incorporation into RISC, or both [50]. RNAi-mediated silencing of geminiviruses using transient protoplast assay where protoplasts were cotransferred with a siRNA designed to replicase (Rep) coding sequence of African cassava mosaic virus (ACMV) and the genomic DNA of ACMV resulted in 99% reduction in Rep transcripts and 66% reduction in viral DNA [51]. The siRNA was able to silence a closely related strain of ACMV but not a more distantly related virus. More than 40 viral suppressors have been identified in plant viruses [52].

A different siRNA vector has been used to target the viral suppressor of the cucumber mosaic virus (CMV), a suppressor that interacted with and blocked the slicer activity of AGO1 [53] that has also been shown to confer resistance to CMV infection in transgenic tobacco. A strong correlation between virus resistance and the expression level of the 2b-specific amiRNA was shown for individual plant lines. It is evident from the above-mentioned reports that the RNA components, such as single-strand template RNA, dsRNA, and/or siRNA of the silencing pathways, are the preferred targets of most viral suppressors. However, plant viruses are known to have evolved a counter-silencing mechanism by encoding proteins that can overcome such resistance [54, 55]. These suppressors of gene silencing are often involved in viral pathogenicity, mediate synergism among plant viruses, and result in the induction of more severe disease. Simultaneous silencing of such

diverse plant viruses can be achieved by designing hairpin structures that can target a distinct virus in a single construct [55].

2.1.4.3.3 **Fungal Resistance** Genetic engineering for fungal resistance has been limited. But several new advances in this area now present an optimistic outlook. Many reports have found positive results with transgenic plants expressing genes for disease resistance.

Antifungal Protein-Mediated Resistance Introduction of chitinase gene into tobacco and rice has been shown to enhance fungal resistance in plants [56, 57]. Chitinase enzyme degrades the major constituents of the fungal cell wall (chitin and α-1,3-glucan). Coexpression of chitinase and glucanase genes in tobacco and tomato plants confers a higher level of resistance than either gene alone. Use of genes for ribosome inactivating proteins (RIPs), along with chitinase, has also shown synergistic effects. A radish gene encoding antifungal protein 2 (Rs-AFP2) was expressed in transgenic tobacco and resistance to *Alternaria longipes* was observed [58]. Other pathogenesis-related proteins/peptides include osmotin, thionins, and lectins [59].

Antifungal Compound-Mediated Resistance The low molecular weight compounds, such as phytoalexins, possess antimicrobial properties and have been postulated to play an important role in plant resistance to fungal and bacterial pathogens [60]. Expression of a stilbene synthase gene from grapevine in tobacco resulted in the production of new phytoalexin (resveratrol) and enhanced resistance to infection by *Botrytis cinerea*. Active oxygen species (AOS), including hydrogen peroxide, also play an important role in plant defense responses to pathogen infection [61]. Transgenic potato plants expressing a H_2O_2 generating fungal gene for glucose oxidase were found to have elevated levels of H_2O_2 and enhanced levels of resistance to both fungal and bacterial pathogens, particularly to *Verticillium* wilt. Further, overexpression of defense response genes in transgenic plants has provided enhanced resistance to a variety of fungal pathogens [62]. For example, transgenic wheat lines carrying a barley seed class II chitinase exhibited enhanced resistance to powdery mildew [63, 64]. Varying amounts of resistance to powdery mildew were observed in transgenic wheat lines carrying a barley chitinase or a barley β-1,3-glucanase [65]. With respect to FHB, a transgenic wheat line carrying a rice thaumatin-like protein and a line carrying a combination of a wheat β-1,3-glucanase and chitinase exhibited delayed symptoms of FHB in greenhouse trials [66, 67]. In addition, transgenic *Arabidopsis* carrying an overexpressed *Arabidopsis* thionin has exhibited increased resistance to *Fusarium oxysporum* [68]. Transgenic wheat expressing the *Arabidopsis* NPR1 gene, which regulates defense responses, was shown to exhibit a high level of resistance to FHB in greenhouse evaluations [69].

The RNA-mediated gene silencing (RNA silencing) is being tried as a reverse tool for gene targeting in fungi. Homology-based gene silencing induced by transgenes (cosuppression), antisense, or dsRNA has been demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* [70], *Magnaporthe oryzae* [71], *Venturia inaequalis* [72], *Neurospora crassa* [73], *Aspergillus nidulans* [74], and *Fusarium*

graminearum [75]. Hairpin vector technology [72] has been used to trigger simultaneous high-frequency silencing of a green fluorescent protein (GFP) transgene and an endogenous trihydroxynaphthalene (THN) reductase gene in *V. inaequalis*. The GFP transgene served as an easily detectable visible marker, whereas the THN reductase gene played a role in melanin biosynthesis.

A protocol for silencing the mpg1 and polyketide synthase-like genes has been developed [75]. The mpg1 gene is a hydrophobic gene that is essential for pathogenicity as it acts as a cellular relay for adhesion and triggers the development of appressorium [76]. It was possible to successfully silence the genes at varying degrees by pSilent-1-based vectors in 70–90% of the resulting transformants. Ten to fifteen percent of the silenced transformants exhibited almost "null phenotype." This vector was also efficiently applicable to silencing a GFP reporter in another ascomycete fungus *Colletotrichum lagenarium* [75].

2.1.4.4 **Engineering for Improving Nutritional Quality**

There is now a growing interest in improving the nutritional quality of food crops. Genes cloned from plants and microbes are being introduced into crop plants to enhance their nutritional value. For instance, introduction of provitamin A and β-carotene genes has resulted in the production of "golden rice" [77–79]. Likewise, high-protein "phaseolin" and AmA1 genes have been introduced into heterologous systems. Introduction of AmA1 gene into potato [80, 81] has caused improvement in yield, protein content, and quality. Vitamin-producing transgenic plants have also been developed [82] and emphasis is being laid on multigene engineering [83]. The main objective of these crops is to add value to agri-foods [84].

2.1.4.5 **Engineering for Male Sterility**

The exploitation of heterosis (hybrid vigor) through the use of hybrid varieties is one of the major achievements of conventional plant breeding. However, in many crops, an efficient and economical method of producing hybrid seed is not available. To overcome these difficulties, genetic engineering of male sterility [85] and its restoration have emerged as tangible options for the development of male sterile and restorer lines for hybrid seed production. There are several biotechnological approaches to developing male sterile lines, but the barnase–barstar genes have been used with greater success. The barnase gene, from the bacterium *Bacillus amyloliquefaciens*, encodes the enzyme barnase (ribonuclease), which is produced in the transgenic plant/line during the development of anthers. Barnase destroys the tapetal tissues and prevents pollen production, conferring male sterility, whereas the introduction and expression of barstar gene, also from *B. amyloliquefaciens*, into another plant/line results in the development of restorer line. The hybrid plants derived from crosses of male sterile and restorer lines are fully fertile. This system has been commercially exploited in maize and oilseed rape [86–88]. Likewise, this method can be further extended to other crops for the production of hybrid seeds.

2.1.4.6 Engineering for Molecular Farming/Pharming

An additional goal for the production of transgenic plants is the use of living systems for the production of metabolites at the industrial scale, for example, specialty chemicals, antibodies, pharmaceuticals, edible vaccines, and so on. Cell's metabolic networks that evolved in nature are not optimized for industrial production of these metabolites. So, the performance of metabolic pathways is being manipulated, so that metabolites are overproduced. The introduction of heterologous genes and regulatory elements in the living systems is commonly called metabolic engineering. There are an increasing number of reports dealing with the production of specialty chemicals, biopharmaceutics, and edible vaccines that can be stored and distributed as seeds, tubers, or fruits [83, 89–93]. Solulin is a recombinant soluble derivative of human thrombomodulin [94]. To evaluate the production of pharmaceutical protein in plants, expression vectors were generated using four different N-terminal signal peptides. Immunoblot analysis of transiently transformed tobacco leaves showed that intact "solulin" could be detected using three of these signal peptides [95]. Furthermore, transgenic tobacco plants and BY2 cells producing solulin were generated. It has been demonstrated that plants and plant cell cultures can be used as alternative systems for the production of an active recombinant thrombomodulin derivative.

2.1.4.7 Engineering for Improving Postharvest Traits

The ripening of fleshy fruits involves changes in color, texture, and flavor. This process occurs through the action of enzymes. The activities of many of the key enzymes are regulated by the controlled expression of "ripening genes." In climacteric fruits such as tomato, the expression of ripening genes is stimulated by ethylene, which functions as a ripening hormone. Many ripening genes have now been cloned, including polygalacturonase and pectinesterase involved in cell wall softening, phytoene synthase required for carotenoid synthesis, and ACC synthase and ACC oxidase that catalyze the production of ethylene. A number of techniques are now available for overexpression of particular genes in fruits to change their physiological and biochemical properties. The techniques of gene silencing using either antisense genes or sense suppression have been used successfully to reduce or inactivate the expression of specific genes and determine their function. Ripening of fruits and senescence in flowers can be delayed by antisense expression of genes involved in pectin metabolism or ethylene biosynthesis. Genetically engineered tomatoes have low polygalacturonase activity that causes delayed ripening. Transgenic tomato variety "Flavr Savr" possessing longer shelf life was released in the United States, whereas reduction in ethylene synthesis has been shown to improve quality and storage life of tomato and melon. Fruit ripening was delayed by controlling ethylene production in apple [96, 97], grapes [98], and citrus [99]. Likewise, these approaches are now being used to introduce desirable traits, such as color, shape, plant architecture, and vase life to meet consumer demand for novelty. Transgenic carnations and geranium, exhibiting low ethylene production, show delayed petal senescence. Attempts are now being made to develop marker-free transgenic plants [100].

2.1.4.8 Engineering for Abiotic Stress Tolerance

Transfer of cloned genes has resulted in transgenics that are tolerant to some abiotic stresses [101]. For instance, for frost protection, an antifreeze protein gene from fish has been transferred into tomato and tobacco. Likewise, a gene coding for glycerol-3-phosphate acyltransferase from *Arabidopsis* has been transferred to tobacco for enhancing cold tolerance. Hal2 gene is being tried for developing salt tolerance. P5CS from *Vigna aconitifolia* was introduced through "particle gun" method of gene transfer into *Saccharum officinarum* under the action of AIPC promoter. Results indicated that stress-inducible proline accumulation in transgenic sugarcane plants under water-deficit stress acts as a component of antioxidative defense system rather than as an osmotic adjustment mediator [102]. TPS1–TPS2 fusion gene construct was introduced into *Arabidopsis* through *Agrobacterium*-mediated gene transfer under the control of CaMV 35S or stress-regulated Cd 29A promoter. No morphological growth alterations were observed in lines overexpressing the TPS1–TPS2 construct, whereas the plants overexpressing the TPS1 alone under the control of 35S promoter showed abnormal growth, color, and shape [103]. Thus, it can be concluded that engineering trehalose metabolism in plants can substantially increase their capacity to tolerate abiotic stresses. Late embryogenesis abundant (LEA) proteins are mainly low molecular weight (10–30 kDa) proteins that are involved in protecting higher plants from damage caused by environmental stresses, especially drought (dehydration) [104]. The OsLEA3-1 gene has been transformed into rice through the *Agrobacterium*-mediated gene transfer method under the control of different promoters. Constitutive and stress-inducible expression of OsLEA3-1 under the control of CaMV 35S and HVA1-like promoter, respectively, resulted in transgenic rice plants showing increased tolerance to drought under field conditions [105].

The gene regulating protein factors that regulate gene expression and signal transduction and function under stress responses may be useful for improving the abiotic stress tolerance in plants. These genes comprise regulatory proteins, that is, transcription factors (bZip, MYC, MYB, DREB, NACs, NAM, ATAF, CUC, etc.), protein kinases (MAP kinase, CDP kinase, receptor protein kinase, ribosomal protein kinase, transcription regulation protein kinase, etc.), and proteinases (phosphoesterase, phospholipase, etc.). Transgenic overexpression of HvCBF4 from barley in rice resulted in an increase in tolerance to drought, high-salinity, and low-temperature stresses without stunting growth. Using the 60K Rice Whole Genome Microarrays, 15 rice genes were identified that were activated by HvCBF4. When compared with 12 target rice genes of CBF3/DREB1A, 5 genes were common to both HvCBF4 and CBF3/DREB1A, and 10 and 7 genes were specific to HvCBF4 and CBF3/DREB1A, respectively [106]. Overexpression of stress-responsive gene SNAC1 (stress-responsive NAC1) significantly enhanced drought resistance in transgenic rice (22–34% higher seed setting than control) in the field under severe drought stress conditions at the reproductive stage while showing no phenotypic changes or yield penalty. The transgenic rice exhibited significant improvement in drought resistance and salt tolerance at the vegetative stage. Compared with wild

type, the transgenic rice was more sensitive to abscisic acid and lost water more slowly by closing more stomatal pores, yet displayed no significant difference in the rate of photosynthesis. DNA chip analysis revealed that a large number of stress-related genes were upregulated in the SNAC1-overexpressing rice plants. SNAC1 holds promise for improving drought and salinity tolerance in rice [107]. Several transcription factors have been used to develop transgenic plants tolerant to abiotic stresses [108–111].

2.1.5 Molecular Breeding

Development and utilization of molecular markers for detecting differences in the DNA of individual plants have many valuable applications to crop improvement. These differences are known as molecular markers because they are often associated with specific genes and act as "signposts" to those genes. Several types of molecular markers that have been developed and are being used in plants include restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNAs (RAPDs), sequence-tagged sites (STS), expressed sequence tags (ESTs), simple sequence repeats (SSRs) or microsatellites, sequence-characterized amplified regions (SCARs), and single nucleotide polymorphisms (SNPs) [112–114]. Such markers, closely linked to genes of interest, can be used to select indirectly for the desirable allele, which represents the simplest form of marker-assisted selection (MAS) that is now being exploited to accelerate backcross breeding and pyramid several desirable alleles [115]. Using molecular markers, genotypes can be distinguished at plant, tissue, or even at cellular level. These molecular markers are not stage specific. Selection of a marker flanking a gene of interest allows selection for the presence (or absence) of a gene in progeny. Thus, molecular markers can be used to follow any number of genes during the breeding program [116]. DNA markers are now extensively being used for gene mapping/tagging [117–125]. Further, these can also be used for dissecting polygenic traits into their Mendelian components or quantitative trait loci [126, 127]. Likewise, molecular markers are also extensively used to probe the level of genetic diversity among different varieties and related species. The applications of such evaluations are many, including DNA profiling, fingerprinting for patenting and IPR issues, efficiently managing genetic resources, and facilitating introgression of chromosomal segments from alien species. In addition, markers and comparative mapping of various species have been very valuable for improving the understanding of genome structure and function and have allowed the isolation of genes of interest via map-based cloning. Molecular maps [121] in several crop plants, for example, tomato, wheat, and rice, have been prepared using different molecular markers. The discovery of molecular markers has enabled dissection of quantitative traits into their single genetic components [128–131] and selection and pyramiding of QTL alleles through MAS [132–135]. A novel upland rice variety Birsa Vikas Dhan 111 (PY 84) has recently been released in the Indian state of Jharkhand [136]. It was developed

using marker-assisted backcrossing with selection for multiple QTL for improved root growth to improve its performance under drought conditions. It is an early-maturing, drought-tolerant, and high-yielding variety with good grain quality suitable for the direct seeded uplands and transplanted medium lands of Eastern India. It outyields the recurrent parent by 10% under rainfed conditions.

2.2 Conclusions

Climate change is now taking place, worldwide. Climate change is affecting agriculture directly or indirectly. It is evident that there is an increase in the average temperature, globally. The levels of greenhouse gases such as carbon dioxide, methane, and nitrous oxide have been increased. Maximum and minimum relative humidity levels have shown increasing trend, whereas high temperature and high humidity directly affect the crops, pathogens, insects, and weeds. Wheat and rice, which are the important cereal crops of the world, are being affected by elevated temperatures and humidity levels. Several new diseases such as powdery mildew and foliar blight of wheat have started appearing, and there is a risk of stem rust (black rust), caused by Ug99, which started from Uganda and has now spread up to Iran. Likewise, new insect pests such as black aphids and pink stem borer have started appearing on wheat. The horticultural crops, which require chilling for flowering, will not be getting minimum chilling hours, which may reduce their production. New technologies must be developed to accelerate breeding through improving genotyping and phenotyping methods and by increasing the available genetic diversity in crop breeding programs. Plant genetic engineering and DNA marker technologies have now become valuable tools in crop improvement for rapid precision breeding for specific purposes. Several climate resilient agricultural technologies such as zero tillage (no tillage), raised-bed planting, tensiometer, laser land leveling, happy seeder, and rotavator have been developed for conservation agriculture. Further, drip irrigation and fertigation, leaf color chart for need-based application of nitrogen, sensor-based yield monitors, nitrogen sensors/green seekers, special-purpose vehicles with sensor-based input applicators, integrated nutrient management systems, integrated pest management systems, integrated disease management systems, site-specific management systems using remote sensing, GPS, and GIS, and Web-based decision support systems for controlling diseases and insect pests have been developed and are being commercialized for precision farming and to mitigate the climate change.

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3
Genetic Engineering for Acid Soil Tolerance in Plants

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Abstract

Acid soils are characterized with the excessive occurrence of aluminum, manganese, and protons and are subsequently nutritionally deficient. Almost, 50% of the arable lands are affected mainly due to aluminum toxicity. Aluminum syndrome accounts for morphophysiological, biochemical, and molecular changes in plants. Extensive reports have been made in deciphering the components involved in aluminum toxicity, whereas the exact molecular mechanisms of aluminum menace still remain to be discovered. However, using genetic engineering approach, some success in generation of transgenic plants with enhanced aluminum tolerance abilities have been achieved. Emerging technologies explore for novel genes and their underlying involvement in Al tolerance mechanisms in plants. A breakthrough in this field can be achieved by unraveling the internal mechanisms of Al toxicity and tolerance. This chapter focuses on several aspects of Al interaction with plants in detail.

3.1
Introduction

Acid soil has been posing a constant threat to the agricultural productivity worldwide. It is naturally prevalent in the humid tropics and subtropics and almost half of agriculturally suitable land is under its negative impact [1]. Moreover, several additional factors like leaching, irrigational practices, and acid rain along with the extensive use of ammonia fertilizers have equally contributed to the increased rate of acidification of irrigated lands [2, 3]. Acid soil is mainly characterized by the excessive occurrence of minerals like aluminum and manganese that ultimately leads to the deficiency of phosphorous, calcium, magnesium, and molybdenum [1]. A minimum of 10 μM of aluminum in soil is sufficient to evoke primary and secondary responses in plants owing

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to its toxicity [4]. Among the practices followed to alleviate aluminum toxicity, liming has been the most widely used approach. It restores the agricultural importance of the acidified soil by increasing its alkalinity. But unfortunately, liming is a costly affair for poor farmers in underdeveloped areas and also seems impractical taking into consideration the vast Al-affected land resource. Furthermore, conventional surface liming is ineffective in reducing the subsoil acidity [5]. Conventional breeding manifests the future possibilities of generation of Al-tolerant species. However, this approach is limited due to genetic incompatibility, and absence of genetic variability among certain cultivars, and so on. Genetic engineering approaches explore the intrinsic molecular mechanisms involved in varied response plants to Al toxicity for development of crops tolerant to Al and other related stress.

Aluminum is the third most abundant mineral in the earth crust with a high reactivity potential, but its toxic effect becomes apparent only when the pH of soil is below 5.5. Aluminum owing to its complex chemistry exists in several forms and their availability is invariably dependent on soil pH. At low pH, aluminum occurs as octahedral hexahydrate, $Al(H_2O)_6^{3+}$, also known as Al^{3+}, a highly soluble phytotoxic form of Al. With the increase in pH, Al transforms into mononuclear forms like $AlOH^{2+}$ and $Al(OH)_2^{+}$. Interestingly, these cationic forms are observed to be highly toxic for certain dicotyledonous plants. At cytosolic pH, the insoluble form $Al(OH)_3$ is observed and at a relatively higher pH, the highly insoluble form of Al, that is, $Al(OH)_4^{-}$, predominates [6]. Again, the chemical interaction of these monovalent species of Al includes oxygen donor ligands, organic acids, inorganic phosphates, nucleotides, and sulfate to form low molecular complexes.

3.2
Phytotoxic Effect of Aluminum on Plant System

3.2.1
Al-Induced Morphophysiological Changes in Roots

The initial interaction of Al with plant begins at root level in the rhizosphere and, subsequently manifested by altered morphological changes in the root system. The root apices and lateral roots become swollen, thick, short, and fragile [7]. As a result, the water and mineral uptake capacity of plants is highly affected on account of damaged root system [8]. The root cap, meristem, and transition and elongation zones together constitute the root apex region, which is a prime target of Al toxicity [9]. More specifically, the distal region of transition zone of the root apex is attributed as the most sensitive region to Al toxicity [10, 11]. It accounts for high accumulation of Al and callose, a sensitive indicator for Al toxicity [11]. This observation has been in contradiction with Bennet and Breen [12] and in agreement with experimental evidence by Ryan *et al*. [9], with the former focusing only on the pivotal role of root cap in the signal cascade for aluminum stimulus, whereas the latter

indicating the elongation zone (0–3 mm behind the root cap) of the root apex. The reduced root growth was more pronounced in the Al-sensitive wheat variety Scout-66, visible within 1–3 h of Al treatment, than in Al-tolerant Atlas-66 variety. Furthermore, Sasaki *et al.* [13] showed longitudinal cell expansion and induced lateral cell swelling in the elongation zone of wheat roots. Combined together, plants experience root growth inhibition and reduced root cell elongation with the onset of Al toxicity.

3.2.2
Negative Influence of Al on Cytoskeletal Network of Plant Cells

The microtubules and microfilaments, components of the cytoskeleton, are associated with functions related to cell division, elongation, expansion of apical root cells, and cell wall biosynthesis [14–17]. The reduced root growth indicates the possible interference of Al with the cytoskeletal network of root cells. Alterations in stability and organization of microtubules and actin microfilaments upon Al stress have been studied extensively. Indirect immunofluorescence microscopy data has indicated altered microtubules and microfilaments in both stability and organization upon exposure to Al [18]. Root growth inhibition was observed in maize immediately within 1 h of exposure to Al followed by reorientation of microtubules in the inner cortex within next 3 hours. Subsequently, after 6 h, there was radial expansion of root due to abnormal isodiametric expansion leading to root swelling within the inner cortical cells. Schwarzerová *et al.* [19] showed similar results of disorientation of cortical microtubules after 3 h exposure of Al in tobacco cell suspension culture. Moreover, there have been contradictory reports regarding the depolymerization of cortical microtubules in wheat, maize roots, tobacco cell suspension culture, and *Arabidopsis* seedlings [20–22] with that of stabilization of microtubules by Al stress [18]. The distal part of the transition zone (DTZ) in Al sensitive variety of maize was found to be significantly affected with the depletion of MTs in the outermost cortical cell layer within 60 min of onset of Al stress and the increased exposure accounted for lesions to the MT cytoskeleton in its epidermal and outer cortical layers [20, 21]. Calcineurin or a calcineurin-type calcium-dependent phosphatase, a potential regulator of tension in the actin network, showed reduced activity in soybean suspension root culture exposed to Al [23]. Furthermore, the abnormalities in cytoskeletal organization caused by Al toxicity have been indicated for imbalance in Ca^{2+} homeostasis, Ca^{2+} calmodulin activity, and inhibition of phosphoinositide signaling [24]. Blancaflor *et al.* [18] also demonstrated the rigidity of the actin network in maize root cells with onset of Al stress. Furthermore, Amenós *et al.* [25] have demonstrated the impact of Al on the actin cytoskeleton and vesicle trafficking using maize cultivars differing in their sensitivities to Al. The interaction of Al ions in the nucleolar region has been related to the inhibition of the cell division as observed in *Triticum turgidum* root tips, soybean cells, *zea mays*-sensitive variety, and *Arabidopsis* [26–29].

3.2.3
Interaction of Al^{3+} Ions with Cell Wall and Plasma Membrane

Cell wall and plasma membrane display high negative surface charge that facilitates the binding of Al^{3+} ions. Numerous reports suggested that alteration in the mechanical stability of cell wall leads to impaired root growth. Liu *et al*. [30] demonstrated that in triticale cultivars, the involvement of oxidative stress and visible increment of hemicellulose component in inhibition of root elongation. Similar explanation has been presented in hydroponically cultured *Vicia faba* roots exposed to Al stress [31]. Lipid peroxidation was observed on account of the oxidative stress by Al^{3+} ions [32]. Plasma membrane depolarization was reported due to Al toxicity [22, 33]. Al changes the membrane potential toward more positive values, thus altering the ion transport processes [34]. Regulation of the PM membrane potential in plant cells is mainly mediated by H^+-ATPase, which influences cellular processes with the maintenance of electrochemical gradient across the membrane coupled with the secondary ion transport. Al toxicity is followed by the depolarization of plasma membrane in near-isogenic wheat lines ES8 (Al-sensitive) and ET8 (Al-tolerant) [35]. Earlier, Ahn *et al*. [36] correlated the decrease in H^+ ions pumping through PM in Al-sensitive plants with that of the membrane depolarization. PM repolarization was suggested with the accumulation of malate and reduced inhibition of H^+-ATPase activity. This observation was in agreement with Jones and Kochian [37], who demonstrated that reduction in binding affinities of Al with lipid can be achieved with high concentration of calcium and organic acid exudates a kind of tolerance mechanisms followed by most of the plants under Al stress. Similarly, upregulation of H^+-ATPase was coupled with the secretion of citrate from soybean roots [38]. Effect of Al on the closure of plasmodesmata, which mediates the apoplastic and symplastic transport of water molecules, nutrients, and signaling molecules–hormones across the plasma membrane of plant cells, has been investigated and concluded with the deposition of callose at the plasmodesmata occurring due to imbalance in calcium homeostasis in plants [39]. Furthermore, Al^{3+} ions were found to block the K^+ influx and efflux in the wheat protoplast [40].

3.2.4
Oxidative Stress Response upon Al Stress

Production of reactive oxygen species (ROS) in plants is generally observed during normal as well as under stress conditions. The destructive action of ROS on cellular metabolism is neutralized by the antioxidant enzymes. Aluminum-induced oxidative stress has been reported in *Arabidopsis* [41], maize [42], rice [43], pea [44], wheat [45], and ryegrass [46]. Consequently, the altered gene expression of the oxidative enzymes like superoxide dismutase (SOD), guaiacol peroxidase (GPOX), ascorbate peroxidase (APX), monodehydroascorbatereductase (MDHAR), dehydroascorbatereductase (DHAR), and glutathione reductase (GR) have also been indicated under Al toxicity (Figure 3.1) [43, 47].

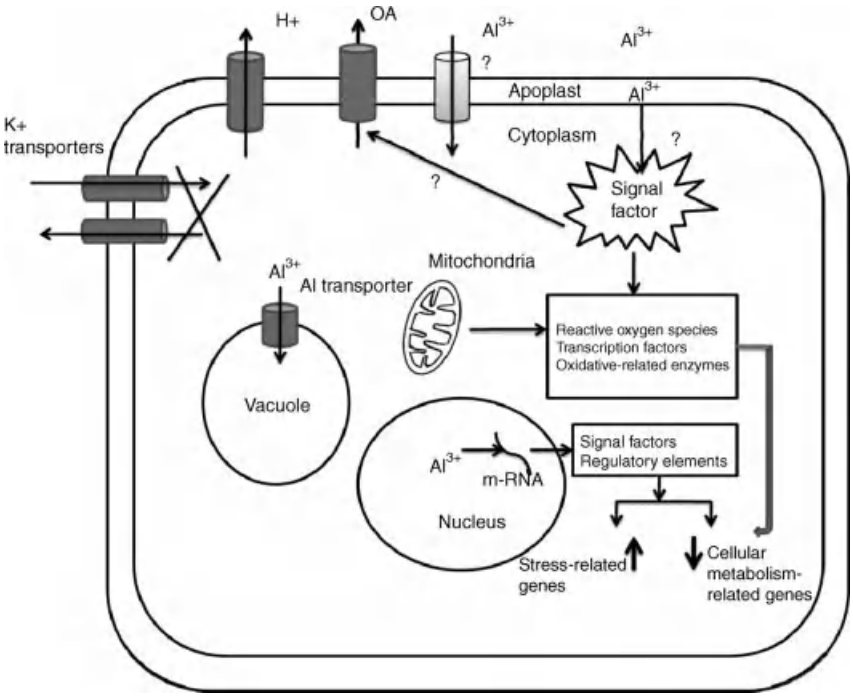

Figure 3.1 Probable activities in aluminum stress signaling pathway in plant cells.

3.3
Aluminum Tolerance Mechanisms in Plants

The potential inherent mechanism displayed by plants for their survival against environmental cues also classifies them into two main categories: the susceptible and the tolerant types. Plants follow different mechanisms to counter Al stress in soil which includes the organic exclusion from root apices, immobilization of Al^{3+} ions to cell wall, mucilage secretion, increasing pH condition in the rhizosphere, and internalization of Al^{3+} ions. These mechanisms shall be discussed in detail in the following section.

3.3.1
Preventing the Entry of Al into Plant Cell

Plant cell wall is the first barrier that any foreign component has to overcome to enter inside the cell. It has been reported that root mucilage secretion is mediated by plants to immobilize Al ions in cell wall [48, 49], thereby restricting their entry into the cell.

3.3.2 Role of Organic Acids in External and Internal Detoxification of Al

The most widely followed mechanism by plants is the secretion of organic acids (citrate, oxalate, and malate) to form insoluble and nontoxic Al–organic acid complex. These organic acids involved in the process are the intermediates of tricarboxylic acid (TCA) cycle. Plants differ in the type of organic acids for detoxification of Al. The exudation of an aluminum-chelating compound citrate in the rhizosphere has been elucidated in snap beans [50], maize [51], *Cassia tora* [52], and Stylosanthes [53]. It has been observed that the response solely depends on Al stress and is not due to phosphorous deficiency as observed in acid soil or due to the effect of Lanthanum (La^{3+}) or ytterbium (Yb^{3+}) that share the same ionic charge to that of Al^{3+}. Secretion of malate was also observed in case of isogenic wheat lines. The secretion of malate in case of Al tolerant wheat variety was 5–10-fold greater than the sensitive variety, suggesting the presence of altered mechanisms within same species against Al toxicity [54]. In addition, the inhibition of root elongation was alleviated with the chelation of Al ions with malate in the cortical cells [55]. Secretion of malate and citrate observed in triticale is regulated by the genes on the short arm of chromosome 3R [56, 57]. Taro (*Colocasia esculenta* L.), a nonaccumulating tropical plant, exudes oxalic acid in response to Al toxicity [58]. Oxalic acid secretion in the root apices of buckwheat occurs within 30 min of Al exposure and further increases linearly with the increase in Al ion [59]. Using the patch clamp technique, Pineros *et al.* [60] studied Al-activated anion channel activity in maize protoplasts suggesting the occurrence of an additional internal Al tolerance mechanism associated with Al-inducible changes in organic acid synthesis and compartmentation. Internal detoxification of Al with malic acid, in addition to secretion of malate and citrate, has been shown to the overall adaptation of eucalypt species to acid soil. Moreover, Al-accumulating plants like tea, *Richeria grandis*, hydrangea, buckwheat, and Melastoma are known to exhibit internal detoxification mechanism involving specific organic acids. As Al exhibits high affinity for cellular components, oxygen-donor compounds like Pi, ATP, nucleotides, RNA, DNA, proteins, phospholipids, polysaccharides, and anthocyanins, the toxicity of Al ions in symplasm is ameliorated by effective mechanisms by plants. High Al–citrate complex is observed in hydrangea leaves, which exhibits 3000 g/kg of Al on a dry weight basis [62]. Buckwheat that accumulates 450 g/kg of Al exhibits both external and internal detoxification strategies in order to overcome Al stress. The occurrence of Al in buckwheat leaves accounted for up to 90% of total Al in the plant cell. Interestingly, a hexacoordinated Al–oxalate complex (1 : 3 ratio) was detected in leaves of buckwheat due to the conversion of Al–oxalate complex into Al–citrate (1 : 1) complex in the xylem, and subsequently into Al–oxalate (1 : 3) complex during unloading from xylem to leaves. In addition to the formation of nontoxic complexes, sequestration of these Al^{3+} ions into vacuoles has been suggested as a possible mechanism for the high accumulation of Al in buckwheat [63–67]. The root tips of Al-sensitive cultivar of wheat (Scout 66) stained with eriochrome cyanine R solution in the presence and absence of Al as well as the Al–oxalic acid complexes

(1 : 1, 1 : 2, and 1 : 3 ratios) verified the presence of 1 : 1 Al–oxalate complex in the root apex. Similarly, in *Melastoma malabathricum*, a woody plant accumulating more than 10 000 mg/kg of Al in mature leaves, the translocation of Al–citrate complex from root to shoot and finally conversion into Al-oxalate complex for storage in leaves has been observed [68]. However, higher mobility of Al concurrent to more Al in young leaves as compared to mature ones was reported in buckwheat [69]. The above facts were correlated in tea and apparently that there exists a chain of conversions of Al–organic complexes occurring at different stages of transport in case of Al-accumulating plants. In tea roots, xylem sap, and leaves, occurrence of Al–oxalate, Al–citrate, Al–catechin were noted, respectively [70, 71]. Furthermore, the secretion of organic acids is charectorized as Pattern I wherein an immediate response to Al stimulus is generated by secretion of organic acids as in Al-tolerant wheat variety (Atlas 66, ET3) and buckwheat. Whereas, in pattern II a delayed response to the stimuli, indicating the activation of certain genes involved in the detoxification process. A delayed secretion of malic and citric acid vy 6-10 h has been observed in rye and triticale [56]. Ryan *et al.* [40] reported for the first time activation of an ion channel in the plasma lemma of wheat protoplasts by Al^{3+} ions. Subsequent studies categorized activation of an anion channel under Pattern I response. As the organic acids are present in highly deprotonated form in the cytoplasm and the equilibrium potential is much higher than the resting membrane potential, the activation of these channels results in the efflux of anions across the concentration gradient [72]. The marked difference in the activation of this anion channel was correlated with the variation in the level of secreted malate in case of Al-sensitive ES8 and Al-tolerant ET8 wheat variety. Further insight into the modified the OA secretion hypothesis by Kinraide *et al.* [73] raises our conern on the importance of protecting immature cortical cells displaying high binding affinity towards Al^{3+} ions as an alternative against root surface region for imparting tolernace against Al syndrome.

3.4 Aluminum Signal Transduction in Plants

Although a number of molecular mechanisms have been identified till date, the exact regulatory pathway still needs to be unraveled. The involvement of a signaling intermediate in Al induced cellular response is still under question and further investigation can provide insight to it. Al is beleived to induce a cascade of signals manifested in plant cells to overcome Al toxicity. Transcriptional factors are important components regulating gene expression in the cell. A C_2H_2-type zinc finger transcription factor ART1 (for Al resistance transcription factor 1) has been shown to regulate expression of genes involved in Al tolerance in rice. *ART1* was nuclear localized and showed constitutive expression in the root, irrespective of Al treatment. This observation suggested the occurrence of posttranscriptional modification required to activate it to functional form [74]. The gene pool regulated by *ART1* involves several categories comprising genes involved in cell wall

maintenance, root elongation, membrane proteins, metabolism, and internal as well as external detoxification of Al. Recently, functional characterization of a rice gene, *OsALS1*, encoding half-size ABC transporter regulated by *ART1*, elucidated its potential role in compartmentalization of Al into vacuoles [75]. The role of *cis*-acting element of *ART1*, GGN(T/g/a/C)V(C/A/g)S(C/G) identified by gel shift assays and transient expression analysis was verified by tandemly repeating as upstream element to study the expression of green fluorescence protein under a minimal 35S promoter in the presence or absence of *ART1* gene. The expression of green fluorescence protein corresponding to *ART1* expression confirmed the identified region as the target DNA binding domain of *ART1* [76]. A Cys_2/His_2 zinc finger-type transcription factor *STOP1* was found to be involved in the signal transduction pathway of Al^{3+} and H^+ response. The stop1 mutant of *Arabidopsis* was unable to activate *AtALMT1* and *ALS3* gene under acidic conditions emphasizing its potential role in Al tolerance mechanism [77, 78]. Further investigation on signal transduction of these transcription factors can promote a comprehensive understanding to the molecular mechanism of Al toxicity in plants. The oxidative stress response is one of the factors involved in Al signaling in plants. The 5′ UTR of glutathione S-transferase genes, *AtGSTl* and *AtGST11*, driving the GUS reporter was introduced into *Arabidopsis* ecotype Landsberg erecta. The time-dependent expression was observed with the *pAtGST1::GUS* gene being induced after a short Al exposure, while *pAtGSTII::GUS* gene took nearly 8 h for exhibiting maximum expression. The gus expression in leaves indicated the presence of a signaling system induced upon Al stress [79]. Recently, Kunihiro *et al.* [80] proposed an Al-signaling pathway involving the oxidative outburst in *Arabidopsis* cell suspension culture mediated by a NADPH oxidase encoded by *AtrbohD* gene (plant respiratory burst oxidase homologues, rbohs) and induced by salicylic acid. Thus, the increased ROS production is followed by subsequent events leading to cell death. The antioxidant role of nitric oxide (NO), a key signaling molecule, is known to protect plants against oxidative stress response mediated by Al. Results in Cassia tora revealed that NO promoted root elongation and a reduced Al accumulation in the root apex upon Al stress. Furthermore, the production of ROS like O_2^- and H_2O_2 along with lipid peroxidation is reduced by NO [81].

3.5
Genetic Approach for Development of Al-Tolerant Plants

The development of plants with enhanced aluminum tolerance through transgenic approach is most promising for sustainable agriculture in acidic soil. The expression of Al-induced plant genes encoding organic acids has resulted in significant enhancement of Al tolerance in plants. Overexpression of citrate synthase (CS) gene of *Pseudomonas aeruginosa* in transgenic tobacco and papaya plants resulted in enhanced Al tolerance [82]. Overexpression of *Arabidopsis* mitochondrial citrate synthase in carrot cells as well as in *Arabidopsis thaliana* conferred superior growth features in phosphorus-limiting soil through increased citrate synthase activity [83].

Transgenic canola lines overexpressing *At-mtCS* gene displayed enhanced transcript level of CS gene, reduced inhibition of root elongation, and increased Al tolerance level in comparison to the wild-type plants [84]. Engineering alfalfa with bacterial and *P. aeruginosa* CS presented enhanced plant growth due to higher exclusion of Al^{3+} from the root tips [85]. Overexpression of *Citrus junos* mitochondrial citrate synthase gene in *Nicotiana benthamiana* conferred aluminum tolerance [86]. Furthermore, transgenic tobacco expressing expression of a full-length citrate synthase gene of rice (*Oryza sativa* L.), showed increased the Al tolerance [87].

Similarly, overexpression of malate dehydrogenase in alfalfa reported a 7.1 fold increase of citrate, oxalate, malate, succinate, and acetate in root exudates, thereby resulting in an increased level of Al tolerance [88]. Apart from overexpression of the organic acids to alleviate Al toxicity, there has been an upsurge in the identification and characterization of genes encoding for transporters mediating the secretion of these organic acids. Sasaki *et al.* [89] were the first group to report the isolation and characterization of a *ALMT1* (aluminum-activated malate transporter) gene constitutively expressed in wheat. Later, citrate efflux was shown to mediate Al tolerance in wheat [90]. Hoekenga *et al.* [91] showed that *At ALMT1* expression only in root apices was critical in exhibiting Al tolerance in *Arabidopsis*. Similarly, Ligaba *et al.* [92] cloned two homologues of the *TaALMT1* gene, *BnALMT1* and *BnALMT2*, from rape. Heterologous expression of *TaALMT1*, *AtALMT1*, and *BnALMT* proteins separately in *Xenopous oocytes* and cultured tobacco cells lead to increased Al-induced malate efflux in each case. Furthermore, their heterologous expression enhanced the Al resistance of the transgenic tobacco cells. Furukawa *et al.* [93] were successful in identifying the gene named as *HvAACT1*, which is responsible for the Al-activated citrate secretion in barley. The gene was identified by fine mapping combined with microarray analysis, using an Al-resistant cultivar, Murasakimochi, and an Al-sensitive cultivar, Morex. *HvAACT1* belongs to the multidrug and toxic compound extrusion (MATE) family and was constitutively expressed mainly in the roots of the Al-resistant barley cultivar. Heterologous expression of *HvAACT1* in *X. oocytes* accounted for the efflux of citrate ion and increased Al resistance in transgenic tobacco plants. Immunostaining showed that *HvAACT1* was localized in the epidermal cells of the barley root tips. A gene belonging to the multidrug and toxic compound extrusion (MATE) family coding for Al-activated citrate transporter, at the Alt_{SB} locus, in sorghum was identified [94]. Transgenic barley expressing *ALMT1* conferred an Al-activated malate efflux and enhanced Al tolerance [95]. Overexpression of *TaALMT1* in wheat conferred Al tolerance [96]. The possible pathway for Al influx in *Arabidopsis* was suggested via endocytosis in root hair region [97] overexpression of F9E10.5 gene related to endocytosis located on chromosome 1 *A. thaliana* imparted higher tolerance level in the transgenic lines. *OsFRDL4* gene belonging to the MATE family in rice was functionally characterized as an Al-induced citrate transporter, regulated by *ART1*, and localized to plasma membrane root tip cells. Knockout of *OsFRDL4* showed decreased citrate

efflux and increased Al sensitivity [98]. The role of Mg in the alleviation of Al stress in plants was demonstrated in rice bean roots. The action of Mg in enhanced citrate exudation and restoration of the H^+-ATPase activity indicated its potent role in Al tolerance mechanism [99]. The overexpression of *AtMGT1*, a member of the *Arabidopsis* magnesium transporter family involved in Mg^{2+} transport, displayed increased level of Mg, Mn, and Fe and lower callose deposition. Root elongation was inhibited by 47% in transgenic plants compared to that of wild-type plants [100]. Under normal conditions, calcium is maintained at lower concentration in the cytoplasm. Imbalance in calcium homeostasis is a primary outcome of Al toxicity. It has been suggested in the calcium displacement hypothesis that the apoplasmic pectin-bound Ca^{2+} ions are displaced by Al^{3+} ions from the cell wall, thus altering its physiological properties. Recent findings by Li *et al.* [101] explain the role of Ca^{2+} transporters in alleviating Al toxicity in yeast. The yeast mutant, pmc1, lacking the vacuolar calcium ion (Ca^{2+}) high affinity pump Ca^{2+}-ATPase (*Pmc1p*) was found to be more sensitive to Al treatment than the wild-type strain. Furthermore, overexpression of either *PMC1* or an antiapoptotic factor, such as *Bcl-2*, *Ced-9*, or *PpBI-1*, decreased cytoplasmic Ca^{2+} levels and rescued yeast from Al sensitivity in both wild-type and pmc1 mutant. The importance of sequestration of Al ions is a prime requisite for the root cells to mediate internal detoxification. The meristematic and the distal portion of the transition zone exhibited high Al internalization [33]. Kollmeier *et al.* [10] suggested the existence of signaling pathway in the root apex mediating Al signal between DTZ and EZ through alterations in basipetal auxin transport. An Al-specific plasma membrane influx transporter, Nrat1 (Nramp aluminum transporter 1), in rice, was regulated by *ART1* and showed enhanced expression with Al stress. Knockout mutants of *Nrat1* exhibited decreased Al uptake and increased Al binding to the cell wall along with increased Al sensitivity. Furthermore, overexpression of Nrat1 resulted in enhanced Al uptake in transgenic rice confirming the role of Nrat1 in the internal detoxification of Al via their sequestration in vacuole [102, 103]. The ATP-binding cassette (ABC) transporters represent a large family in plants associated with a nucleotide binding domain (NBD) and a transmembrane domain (TMD). A bacterial type transporter comprising *OsSTAR1* (NBD) and *OsSTAR2* (TMD) was found to be an essential element for detoxification of Al in rice. Expression and localization study in onion epidermal cells, rice protoplast, and yeast demonstrated the association of *OsSTAR1* and *OsSTAR2* to form a functional complex localized to the root cells, except for the epidermal layer. Furthermore, its expression in *Xenopus laevis* oocytes revealed the transport of UDP-glucose indicating its role in the cell wall modification and in alleviation of Al toxicity [104]. *AtSTAR1* gene encoding an ATP binding domain of a bacterial type ABC transporter in *Arabidopsis* is an orthologue of rice *OsSTAR1*. The importance of *AtSTAR1* in Al tolerance was further supported with the findings that knockout of *AtSTAR1* resulted in increased sensitivity to aluminum. Expressional analysis detected the presence of *AtSTAR1* in both roots and shoots of *Arabidopsis* unlike *OsSTAR1* whose

expression was restricted to roots only. *ALS3* (an ABC transporter like protein) was observed to be associated with *AtSTAR1* for the proper functioning of the resulting complex [105]. Earlier, Larsen *et al.* [106] demonstrated the distribution of *AtALS3* in leaf hydathodes, root cortex, and phloem throughout the plant and related its function with the detoxification of Al from the sensitive tissues to promote their growth. ALS1 protein represents a half-type ABC transporter, primarily localized to the root tip and vasculature throughout the plant and expressed in an Al-independent manner as studied in the als1-1 mutant of *Arabidopsis*. The *ALS1::GFP* fusion accumulated in the vacuolar membrane of root cells suggesting its possible role in sequestration of Al in vacuoles [107].

Aluminum is known to induce oxidative stress in plants. Therefore, the importance of antioxidants in reducing the oxidative stress response was exploited. Overexpression of an *Arabidopsis* blue-copper binding protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*), and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) enhanced the aluminum tolerance in transgenic *Arabidopsis* ecotype Landsberg. Increased tolerance is attributed to the activity of these genes NtPox and parB against the oxidative stress [108]. Transgenic canola lines expressing a wheat manganese superoxide dismutase displayed lower root growth inhibition and accumulation of MDA, an indicator of lipid peroxidation under Al-stress condition [109]. Overexpression of enzyme dehydroascorbatereductase (*DHAR*) increased antioxidant capacity and Al tolerance in transgenic tobacco due to high level of ascorbic acid (AsA) and ascorbate peroxidase (APX) activity. However, similar results could not be obtained with overexpression of monodehydroascorbatereductase (*MDAR*), involved in the process [110]. Recently, overexpression of *PTrx* in barley displayed increased protection of transgenic barley against oxidative stress due to Al stress. *PTrx* gene belongs to the thioredoxin family known to react to oxidative stress [111].

3.6 Transcriptomics and Proteomics as Tools for Unraveling Al Responsive Genes

High-throughput technology such as microarrays and subtractive hybridization, have enabled identification of differentially expressed genes under Al stress. Transcriptome and proteome profiling of Al-sensitive and tolerant plants has enriched the repository of Al-induced genes. The upregulation of genes related to cell wall modification, oxidative stress-related genes, transporters, transcription factors, signaling elements, pathogen-related genes, and other stress-inducible genes along with novel ones were observed in almost all the cases, whereas, the downregulated genes comprised of those related to cellular metabolism.

The cell wall modifying gene pectin methyl esterase was found to be upregulated in sensitive maize variety L53, whereas downregulated in populus. Pectin methylesterase (PME) regulates the negative charge of pectin content in cell

wall by demethylation and, in turn, increases the sensitivity of plant by higher accumulation of Al^{3+} at the root region. Therefore, its lower expression reduces the chance of binding of Al^{3+} to pectin matrix, thereby limiting its accumulation in the apoplasm in tolerant species [112, 113]. Furthermore, the oxidative response in plants is a commonly known phenomenon in response to various stress conditions. The upregulation of several oxidative responsive genes like peroxidases and glutathione S-transferase were noted in maize [113], wheat [114], and *Arabidopsis* [115]. The transcriptome profiling obtained with the comparison of soybean cultivars Al-tolerant (PI 416937) and Al-sensitive (Young) indicated a myriad of genes, reportedly transcription factors, auxin downregulated-like protein (*ADR6*-like) and basic leucine zip-per (*bZIP* 94), sulfur transmembrane transport protein and lipid transfer protein, and several novel genes in the tolerant genotype compared to the sensitive variety. Furthermore, proteomic analysis of soybean roots under Al stress using a tandem combination of 2-D-DIGE, mass spectrometry, and bioinformatics tools indicated the upregulation of genes malate dehydrogenase, enolase, malate oxidoreductase, and pyruvate dehydrogenase, encoding citrate in PI 416937 but not in Young [116, 117]. Genes related to the biosynthesis of auxin, ethylene, and lignin were upregulated in the Al-sensitive maize genotype, indicating that these pathways might be associated with root growth inhibition [118]. In *Arabidopsis* interestingly, lack of abundance of transcripts encoding TCA cycle enzymes, except for malate dehydrogenase, suggested that synthesis of organic anions in response to Al may not be transcriptionally regulated. A similar result was obtained in Al-resistant bean genotype, which did not experience the upregulation of genes encoding enzymes involved in citrate metabolism, although an upregulation in ESTs belonging to the citrate transporter gene family (MATE) was observed [119]. Increased abundance of transcripts for several membrane receptor kinases and nonmembrane calcium response kinases suggested to be involved in transmission of Al-stress signals. Among Al responsive transcription factors, the most predominant families identified were *AP2/EREBP*, *MYB*, and *bHLH* [120]. Goodwin and Sutter [115] also identified several upregulated genes in *Arabidopsis* involved in vacuolar signaling, sorting, and docking, mainly Ras GTP-binding protein, ABC-cassette binding, and the *AtELP1* receptor genes.

3.7 Future Perspectives

Development of available land source toward agricultural sustainability is a prime necessity. Functional genomics and proteomics tools are promising for elucidation of novel genes that previously have not been identified, and that can further resurrect our approach toward overcoming Aluminum syndrome that takes a toll on the agricultural productivity. The identification of distinguishing factors prevailing in nature, that is, among the genetically varied plant species, analysis of single gene

with the help of mutants, and studying of loss and gain of function for related genes toward Al toxicity and tolerance mechanisms can provide intriguing success in future. The extent of success in this field has been possible with the identification of genes related to the secretion of OAs, transporters belonging to ALMT and MATE family, transcription factors, and oxidative stress agents. The main aim is to unravel the interaction of Al with plant at the molecular level that holds the key to regulate the time-dependent signal transduction occurring with the onset of Al exposure.

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4
Evaluation of Tropospheric O_3 Effects on Global Agriculture: A New Insight

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Abstract

Tropospheric ozone (O_3) is now considered as the most widespread and toxic gaseous pollutant in our environment. To date, unsustainable resource utilization has turned this secondary pollutant into a major component of global climate change, and a prime threat to agricultural production. The projected levels to which O_3 will increase are critically alarming, and have become a major issue of concern for global food production. Plants are "soft targets" for O_3. Ozone enters plants through stomata, where it can be dissolved in the apoplastic fluid. Ozone has several potential effects on plants: direct reaction with cell membranes; generation of ROS and H_2O_2 (which alters cellular function by causing cell death); induction of premature senescence; and up- or down-regulation of responsive components such as genes, proteins, and metabolites. In this chapter, we make an attempt to present an overview picture of agricultural crops and O_3 interactions. We summarize the vast number of available reports on plant responses to O_3 at the morphological, physiological, cellular, and biochemical levels, and also address effects on crop yield, and on "-omics" level.

4.1
Introduction

Agriculture plays a very important role in human welfare and provides the basis of human and animal nutrition. Past agricultural growth has been a precursor of industrial revolution across temperate and Asian countries generating agricultural surplus and enabling lower food prices. But, food security in the twenty-first century faces multifaceted challenges to feed the ever-increasing population with changing consumption patterns and changing climatic scenarios. Global climate change may alter many elements of the future crop production environment. Rise in concentrations of tropospheric ozone (O_3), carbon dioxide (CO_2), and other greenhouse gases may lead to changes in global climate. Tropospheric O_3 being a secondary pollutant is not emitted as such by any specific source; rather, it is

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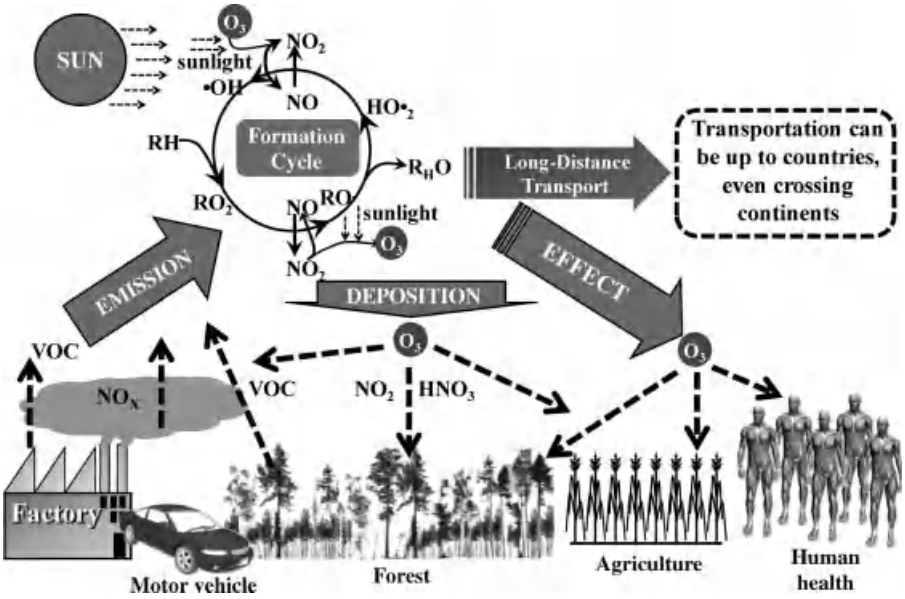

Figure 4.1 Atmospheric cycle of surface level/tropospheric O_3 and major emission sources of different precursor molecules.

formed during the atmospheric photochemical reactions involving oxides of nitrogen and reactive hydrocarbons emitted from automobiles [1] (Figure 4.1). Higher levels of surface O_3 may be found hundreds or thousands of miles away from the original sources, often affecting the remote rural areas, the active centers of agricultural production. Tropospheric O_3 is recognized as the most important air pollutant affecting plant productivity losses in most parts of the world [2–5] and hence is a major threat to global food security to feed the growing population in China, India, and the United States as they are the worst affected countries, bearing more than half of all global losses and threatened areas [2]. Food security in India is the most important issue of present time for the rapidly increasing population. Currently, we use 168 million ha of arable land for crop and animal production and there are not much prospects to expand the cultivated area further. We therefore need to produce 50–70% more food from the same or less land, water, and other natural resources.

Ozone is a potent phytotoxic air pollutant that reduces plant productivity. Ozone after entering through the stomata dissolves in the apoplast region of plant cells, where it generates different reactive oxygen species (ROS), which are capable of altering cellular functions that cause premature senescence, cell death, and up- or downregulation of specific genes [6]. This chapter focuses on the variations in cultivar-specific responses of agricultural crops to O_3 with special reference to mechanism of action, growth, physiology, and yield responses and crop improvement techniques to protect plants from O_3 or enhance the plant productivity by application of "-omics" approach.

4.2
Tropospheric O_3 Formation and Its Recent Trend

Ozone is produced in planetary boundary layer (PBL), free troposphere, and in the stratosphere. In the stratosphere, O_3 is produced due to photolysis of O_2 by ultraviolet radiation into atomic oxygen to form O_3. However, in the troposphere O_3 formation occurs due to photolysis of NO_2. In the free troposphere, O_3 formation depends on reaction of methane, carbon monoxide (CO), and nonmethane organic compounds with NO_x. These reactions are principally controlled by sunlight and temperature. Nitrogen dioxide diminishes when O_3 reaches its peak. Ozone concentration peaks during the late morning and early afternoon hours [7].

In the ambient air, O_3 precursors play important role during long-range transport downwind from the sources (Figure 4.1). Polluted air masses from urban and industrial areas can affect suburban and rural areas, even reaching to remote rural areas for considerable distances. High O_3 levels from one particular urban area can extend as far as 48–80 km [1]. Ozone formation also depends largely upon prevailing meteorological conditions of the area. Tiwari *et al.* [7] reported positive correlation between mean maximum temperature/sunlight and O_3 concentration.

Background O_3 concentrations have more than doubled in the past century [8] and there is an increase in annual mean values of O_3 ranging from 0.1 to 1 ppb/year [9]. In the northern hemisphere, O_3 is also influenced by the influx from the stratosphere [10, 11]. However, O_3 varies strongly with episodic peak concentrations during the warmest months in summer in the most polluted regions and maximum concentrations during spring prevailing at background sites [12]. In regions such as East Asia exposed to summer monsoon that transports oceanic air with less O_3, the seasonal patterns show a peak during pre- and post-monsoon periods [13]. During the day, O_3 concentration pattern depends on elevation and shows strong diurnal variations at lowland sites where its destruction dominates during the night and vertical mixing together with photochemical activity causes highest levels in the afternoon.

As sensitive crops mostly show visible injury/yield reductions above 40 ppb, a cumulative indicator of O_3 exposure above 40 ppb threshold (AOT40) was established by United Nations Economic Commission for Europe International Co-operative Programme (UN/ECE-ICP) [14]. AOT40 associated with a 5% yield reduction of wheat was suggested to be the most appropriate value for critical level for O_3. The critical level (AOT40) required for 5% reduction in yield for watermelon was 1.56 ppm h, for wheat 2.96 ppm h, for pulses 3.03 ppm h, for cotton 3.31 ppm h, for turnip 3.47 ppm h, for tomato 3.62 ppm h, for onion 4.13 ppm h, for soybean 4.31 ppm h, and for lettuce 4.63 ppm h [14]. These crops were categorized as sensitive crops as critical levels were less than 5 ppm h. For moderately sensitive crops, for example, sugar beet, potato, rapeseed, and tobacco, critical levels ranged between 5 and 10 ppm h. Rice, maize, grape, and broccoli were categorized as moderately resistant crops and critical levels ranged between 10 and 20 ppm h.

In rural areas of Europe, mean O_3 concentrations reach 40–50 ppb from spring to summer and variations in average levels of AOT40 calculated for May, June, and

July (from 1997 to 2001) ranged from less than 3 ppm h in northern Scandinavia to values >10 ppm h in southern Europe [15]. At individual sites, maximum values of around 30 ppm h (2003) were recorded in southern Switzerland and around 20 ppm h at sites in Italy [16]. In rural agricultural areas of the United States, mean ozone concentrations reach between 50 and 60 ppb (90th percentile) [17]. Concentrations over the mid- and high latitude of the Eurasian and North American continents were 15–25 ppb in 1860, but increased between 40 and 50 ppb even in remote areas and from 10–15 to 20–30 ppb over the mid- and high-latitude Pacific Ocean [18]. Measures taken to reduce O_3 precursor emissions led to changes in O_3 levels in many rural and urban areas of Europe, North America, and Japan; for example, the frequency of the highest values shows a declining trend, while lowest values are increasing [19]. The US EPA has reported that emission reduction in O_3 precursors has been substantial over the past 29 years [20]. The percent changes in emissions of nitrogen oxides (NO_x) and volatile organic compounds (VOCs) were 40 and 47%, respectively, for the period 1980–2008.

A major study, pooling together the monitoring data and modeling studies across the whole of Europe, showed that European emissions of O_3 precursors had decreased over the past three decades (between 20 and 40% for NO_x), with even larger reductions in Russia, and peak O_3 concentrations had also decreased but not in a linear fashion compared to emission reductions, although peak concentration of some sites had decreased between 1 and 1.5%/year [21]. In Southeast Asia, trends are different as a decrease in average concentration of O_3 was observed in the Yangtze Delta region of China, with an increase in the daily amplitude of the diurnal variation due to increasing frequencies at both high and low ends of the O_3 distribution due to higher NO_x concentrations [22].

In India, despite favorable climatic conditions for O_3 formation, very limited data from systematic monitoring of O_3 are available (Table 4.1 and Figure 4.2). It is clear from Table 4.1 that O_3 concentrations are continuously increasing from 1992 to 2008 with higher peaks in rural areas. In a field transect study at urban sites of Varanasi, O_3 concentrations varied from 6 to 10.2 ppb during 1989–1991 [23]. During the same period, daytime O_3 concentrations (9 h mean) were reported to vary from 9.4 to 128.3 ppb at an urban site in Delhi [24]. It was observed that 10 h ground level mean O_3 concentrations in Delhi varied between 34 and 126 ppb during the winter of 1993 [25]. At Pune, an annual average daytime O_3 concentration of 27 ppb and hourly concentration between 2 and 69 ppb were reported during August 1991 to July 1992 [26]. Lal *et al.* [27] studied the pattern of O_3 concentrations from 1991 to 1995 at an urban site of Ahmedabad (India), and reported that daytime mean O_3 concentrations exceeding 80 ppb were rarely observed. The monthly average O_3 concentrations ranged between 62 and 95 ppb in summer (April–June) and between 50 and 82 ppb in autumn (October–November) at New Delhi [28].

At suburban sites of Varanasi, 7 h O_3 concentrations varied from 23.4 to 62.4 ppb during 2002–2006. Seasonal variations in O_3 concentration showed maximum values in summer followed by winter and minimum in rainy season [7]. At a suburban site, mean O_3 concentrations (12 h) between 36.4 and 48 ppb from December to March during growth period of wheat [29] and at a rural site between 23.4 and

Table 4.1 Trends of tropospheric ozone concentration in India from 1989 to 2008.

City	Ozone concentration	Site	Month/Year	Reference
Varanasi	6.0–10.2 ppb	Urban	1989–1990	[23]
Delhi	4–128.3 ppb	Urban	1989–1990	[24]
Delhi	34–126 ppb	Urban	1993	[25]
Pune	2–69 ppb	Urban	August 1991–July 1992	[26]
Ahmedabad	62–95 ppb	Urban	1995	[28]
Varanasi	23.4–44 ppb	Rainy rural	July–October 2006	[29]
Varanasi	41.65–54.2 ppb	Rural	November 2006–March 2008	[30]
Varanasi	41.3–59.9 ppb	Rural	July–October 2007	[31]
Varanasi	45.3 ppb	Rural	December 2006–March 2007	[32]
Varanasi	47.3 ppb	Rural	December 2007–March 2008	[32]
Ahmedabad	70–100 ppb	Rural site	January 2001–2002	[33]
	65 ppb	Urban site		
Mt Abu		Remote mountain site	1993–2000	[34]
	46 ppb		Winter	
	34 ppb		Summer	
	36.3 ppb		Rainy	
Anantapur		Rural site	2002–2003	[35]
	46 ppb		Summer	
	22 ppb		Rainy	
	55 ppb		Winter	
Pune	33.1 ppb	Urban	2001–2005	[36]
	29.7 ppb	Rural	January 2001–December 2005	
	33.8 ppb	Remote rural	March–June 2005	
Chennai	30–69 ppb	Urban	March–October 2005	[37]
Delhi		Urban	1997–2004	[38]
	57.7 ppb		Summer	
	47.9 ppb		Winter	
	40.3 ppb		Rainy	

44.4 ppb from July to October during growth period of rice [39] were reported. O_3 monitoring conducted by Sarkar and Agrawal [32] at a rural site of Varanasi during 2007–2008 and 2008–2009 recorded mean O_3 concentrations of 45.3 and 47.3 ppb, respectively. Beig and Ali [40] reported O_3 levels reaching up to 45–80 ppb in the upper and middle Gangetic plains. Roy *et al.* [41] reported AOT40 value of 3.6 ppm h in Pune, which is almost 3.6 times higher during late winter to pre-monsoon season. It was also reported that AOT40 values were substantially higher throughout the year over Indo-Gangetic plains than the other regions of India. Emberson *et al.* [5] reported that large parts of South Asia experience up to 50–90 ppb mean

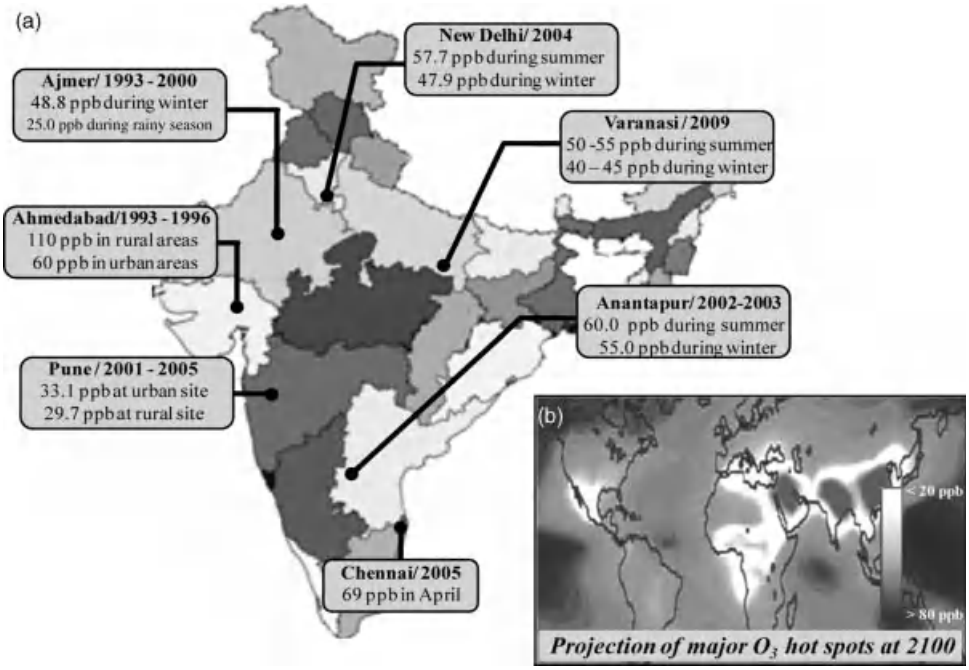

Figure 4.2 India: a future O_3 hot spot. (a) Major Indian cities with reported O_3 concentrations. (b) Global projection of O_3 for the year 2100 . (modified from Ref. [4], with kind permission of Elsevier, © 2009)

7 h (M 7) O_3 concentration. Mittal *et al.* [42] using the HANK model reported O_3 concentration varying from 25 to 100 ppb over the entire Indian region.

4.2.1 Projected Trends of Ozone Concentrations

Future trends of O_3 concentrations will depend on the anthropogenic emission path of precursors and on trends in temperature, humidity, and solar radiation, but the effects of both factors will vary spatially [43]. By 2030, average O_3 in surface air over much of the northern hemisphere may increase by 2–7 ppb across the range of IPCC SRES emission scenarios described by Nakicenovic and Swart [44]. By 2100, two more extreme scenarios projected baseline O_3 increases of >20 ppb, while the other four scenarios yielded changes of −4 to +10 ppb [45]. Although anthropogenic emissions cause the largest response in O_3, a major factor influencing future trend in O_3 is climate change. More recently, multimodel simulations for 2030 projected that with current air quality legislation implemented worldwide global surface O_3 would increase by 1.5 ± 1.2 ppb on average and for IPCCSRES A2 scenario by 4.3 ± 2.2 ppb [46], with the strongest increase in South Asia, Southeast Asia, and the Middle East [47]. Using 18 atmospheric models, Ellingsen *et al.* [48], for the CLE scenario (current legislation in place), obtained an increase in AOT40 by

21–38% by 2030 over the northern hemisphere, relative to 2000, and by 50% on the Indian subcontinent, but a decrease with current regional legislation in Europe. For the A2 scenario, the largest increase in AOT40 (80–100%) was found for India and Southeast Asia. With respect to the effect of climate change by 2030, the latter study provided inconclusive results because of the short time horizon. In the United Kingdom, the annual average O_3 concentrations are predicted to reach 30–40 ppb in rural areas leading to doubling of AOT40 (accumulated exposure over a threshold of 40 ppb) values by 2030 [49]. Global photochemical models project that under current legislation emission scenarios, parts of Asia will experience further significant increases in O_3 concentration up to 2030 [46]. Meehl *et al.* [8] projected an increase of 20–25% in O_3 concentrations between 2015 and 2050 and 40–60% by 2100 in Asia.

4.3
Mechanism of O_3 Uptake

The phytotoxic effects of O_3 depend on the ambient exposure pattern and the amount of O_3 diffusing into the leaves and then converting into the liquid phase within the cells and its reactivity with cellular constituents. Leaves are the primary route of uptake, which is controlled by the stomatal aperture and conductance to gas diffusion [50]. The absorption of O_3 is a consequence of chemical potential gradient between atmosphere and the site of deposition, either on the foliar surface or on the cells of the leaf interior [51].

Studies have shown that a major factor affecting plant response to O_3 appears to be stomatal conductance (g_s), and at higher concentrations of O_3, stomatal closure was reported in the majority of species [52, 53]. Efforts to understand the discrepancies between O_3 concentration in the air and variable plant response have focused on molecular and biochemical mechanisms involved in O_3 detoxification processes and certain interacting environmental factors affecting stomatal conductance (g_s) [54, 55]. The responses to O_3 concentration for a particular crop and cultivar depend upon genetic differences and environmental conditions experienced during the growing season. Stomatal conductance is typically higher in warm and humid environments that are likely to increase the risk of O_3 injury. Thus, crops in warm, humid environment are more likely under threat of O_3 damage compared to cool and dry environment. This approach holds promise as a relatively simple method for including environmental factors that influence g_s into O_3 exposure indices. The absorbed cumulative dose of O_3 is the most relevant in determining cause and effect relationships and quantifying dose responses. A modeling study based on multiplicative algorithm of stomatal conductance (g_s) developed by Emberson *et al.* [56] tested for wheat showed that 5% yield loss level was associated with approximate cumulative O_3 uptake of 0.3 $mmol/m^2/s$ and 1.6 $mmol/m^2/s$ for potato suggesting that cumulative uptake of O_3 in potato was larger than that in wheat.

Responses of stomata vary under O_3 exposure, such as partial opening/stomatal closure. Mechanisms suggested for ozone-induced stomatal closure are (1) reduced photosynthesis and increased guard cell CO_2 concentrations, (2) direct guard cell ion channel modulation, (3) altered plant calcium homeostasis, and (4) altered induction of signal transduction intermediates such as H_2O_2 and NO [57].

4.3.1
Mode of Action

Ozone that has entered through leaf internal air spaces dissolves in the aqueous layer surrounding the foliar cells. The breakdown of O_3 in pure water produces hydroxyl ($OH^{\bullet}$), peroxyl ($HO_2^{\bullet -}$), and superoxide ($O_2^{\bullet -}$) radicals, although the reactions proceed very slowly at neutral pH [58]. Ozone is known to react with a diverse set of molecules that would be encountered within the cell wall and on the plasma membrane surface. Initial targets for O_3 include plasma membrane lipids, susceptible amino acids in plasma membrane, proteins or apoplastic enzymes, and a variety of organic metabolites localized in the cell wall [59].

Both $O_2^{\bullet -}$ and H_2O_2 accumulated at the leaf margins of the sensitive white clover following O_3 exposure (150 ppb, 3 h), while only H_2O_2 accumulates in asymptomatic red clover [60]. The cytochemical localization of H_2O_2 by staining methodology revealed that H_2O_2 accumulation was one of the earliest detectable responses to O_3 in sensitive clone of poplar [61]. Levine *et al.* [62] reported that levels of H_2O_2 required to induce cell death are usually higher than those capable of inducing gene activation. Sarkar and Agrawal [31] also observed interveinal yellowing or chlorotic stippling in the leaves of mature rice plants under both ambient and elevated O_3 exposure conditions in open-top chambers (OTCs), and found that the magnitude of visible foliar injury formation depends on the cumulative effect of both concentration and duration of O_3 exposure.

A detailed study conducted by Ranieri *et al.* [63] on H_2O_2 localization and time course of production in sunflower leaves exposed to 150 ppb O_3 for 4 h showed that H_2O_2 progressively accumulates in the cells starting from 30 min of exposure mainly in the spongy mesophyll cells than in palisade parenchyma. Pellinen *et al.* [64] showed that when ROS formation exceeds the apoplastic antioxidant capacity perception, O_3-derived ROS induces an active additional ROS production, resulting in self-propagating secondary ROS generation that continues even after the end of O_3 exposure giving rise to a biphasic oxidative burst. Consequently, H_2O_2 accumulates in the cell wall and on the surface of the plasma membrane, and in the second time ROS production may occur in the cytoplasm, peroxisomes, and mitochondria.

4.3.2
O_3 Sensing and Signal Transduction

Ozone is known to induce deep changes in gene expression responsible for biochemical adjustments and metabolic shifts, ultimately leading to the overall

plant response [59]. Kangasjarvi *et al.* [65] proposed three mechanisms for O_3 perception, that is, by an apoplastic receptor, protein, and production of lipid-derived signal changes in the cell redox balance. It was found that following the apoplastic ROS increase over a threshold limit, a complex sequence of events takes place in the guard cells at both chloroplast and plasma membrane levels leading to endogenous ROS production and activation of MAP kinase cascade in the adjacent cells. MAPK activation in turn seems to be involved in the upregulation of the synthesis of ethylene (ET) that along with salicylic acid (SA) is needed for the development of O_3-induced lesions, whereas jasmonic acid (JA) acts antagonistically to contain the spread of cell death. The induction of ET biosynthetic gene is one of the earliest responses to O_3 [66]. It has been shown that lesion propagation and the extent of cell death are under the control of ET. A dramatic increase in ET has been observed in O_3-sensitive cultivar of tobacco (Bel W3) following O_3 exposure [67]. A functional ET perception is a fundamental step in triggering the signal transduction machinery and the consequent biological response [68]. A central role for SA has been demonstrated in lesion initiation and progression in response to O_3 [69]. Pasqualini *et al.* [70] reported high level of SA in highly O_3-sensitive tobacco cultivar Bel W3. In tomato, JA biosynthetic genes were induced during the recovery period [71].

4.3.3
ROS Detoxification Mechanisms: From Apoplast to Symplast

After O_3 exposure, there is a need to tune the level of ROS produced to achieve a positive cell reaction through the signaling cascade without inducing uncontrolled cell death. As ROS are physiologically generated from various sources during cell metabolism, plants have evolved very efficient enzymatic and non-enzymatic antioxidant defense systems, capable of detoxifying substantial amount of these reactive oxygen species. The antioxidant defense system plays a fundamental role in determining the cell fate, not only by keeping ROS level under control, but also by acting as a central component of the cell redox balance and of the signaling modulation.

The first line of defense against O_3-derived ROS is the apoplast, where ascorbate (ASC) is believed to provide important protection from the oxidative injury. The O_3-induced changes in apoplast ascorbate and redox state were first reported in 1996 [72]. Ranieri *et al.* [73] reported enhanced apoplast ascorbate level, while intracellular concentration did not vary markedly, supporting the hypothesis of an O_3-induced stimulation of ASC synthesis followed by active export to the apoplast in young asymptomatic and mature symptomatic leaves of pumpkin exposed to 150 ppb O_3 (5 h/day, 5 days). The protective role of ASC as ROS scavenger is also supported by the enhanced O_3 sensitivity shown by mutants deficient in ASC [74]. The importance of ascorbic acid is demonstrated in the VTC1 mutant of *Arabidopsis*, where low ascorbic acid (AA) content in leaf tissue was associated with increased O_3 sensitivity. There is also evidence that

O_3-tolerant genotypes have elevated AA content [75, 76]. Burkey and Eason [77] exposed three cultivars, that is, Tendrette and Provider (O_3-tolerant) and Oregon-91 (O_3-sensitive), and four experimental lines R123, R142, S144, and S156 of snap bean (*Phaseolus vulgaris* L.) to 75 ppb O_3 for 12 h and found higher levels of leaf apoplast ASC in O_3-tolerant genotypes relative to sensitive lines. Higher apoplast AA/ASC_T ratio was found in O_3-tolerant cultivars than sensitive lines suggesting greater capacity for transport of DHA (dehydroascorbate) from the apoplast into the cytoplasm.

Leaf ascorbic acid content and redox status were compared in O_3-tolerant (Provider) and O_3-sensitive (S156) genotypes of snap bean exposed to 71 ppb O_3 in open-top chambers for 10 days in mature leaves early in the morning (06:00–08:00 h) or in the afternoon (13:00–15:00 h) [78]. Results showed that total ascorbate content [AA + DHA] of leaf tissue was 28% higher in tolerant genotype compared to sensitive ones, exhibiting that tolerant cultivar (Provider) maintains total ascorbate content under O_3 stress and levels of apoplastic ascorbate were significantly higher in the afternoon than early morning for both genotypes. Rai and Agrawal [39] found higher ascorbic acid content in sensitive cultivar of rice NDR 97 compared to tolerant cultivar Saurabh 950 exposed to ambient O_3 concentration of 35.5 ppb grown in OTCs (Table 4.2). Higher ascorbic acid content was observed in tolerant soybean cultivar PK 472 compared to sensitive cultivar Bragg at 70 and 100 ppb O_3 for 4 h from germination to maturity. Feng *et al.* [79] showed that leaf apoplastic ascorbate content was 33.5% higher in tolerant wheat cultivar Y2 exposed to elevated O_3 concentration (83.8 ppb) that was 27% higher than the ambient O_3 concentration (66 ppb). Since ASC is synthesized inside the cells and the oxidized form must be transported back into symplast to be re-reduced, the transport rate across the plasma membrane must be taken into account when discussing the antioxidative capacity of apoplastic ASC in the detoxification of O_3. Burkey and Eason [77] showed that transport of DHA from the apoplast into the cytoplasm was higher in tolerant genotypes of snap bean than sensitive lines.

The antioxidant role played by ASC depends mainly on the cell ability to maintain it in a reduced state and it occurs at the cost of reduced glutathione (GSH) by monodehydroascorbate reductase (MDHAR) or dehydroascorbate reductase (DHAR). Glutathione is generated by glutathione reductase (GR) at the expense of NADPH oxidation in the Halliwell–Asada cycle. Among the tobacco cvs Bel B and Bel W3 known for their differential sensitivity to O_3, reduction in the chloroplastic GR mRNA was recorded in Bel W3 at exposure to 150 ppb to O_3 for 5 h [84]. Ascorbate may act as reducing substrate for ascorbate peroxidase (APX), which is one of the most efficient ROS scavenging systems. In sunflower, increased level of extracellular APX activity may contribute to avoid the buildup of toxic H_2O_2 concentrations [63]. The higher constitutive APX activity measured in a resistant white clover clone with respect to a sensitive clone was further enhanced following long-term exposure to O_3 (60 ppb for 5 h/day for 56 days). Sarkar *et al.* [82] reported increase in APX and GR activities in wheat cultivars exposed to elevated O_3 (Table 4.2).

Table 4.2 Impact of tropospheric O_3 on lipid peroxidation and antioxidative enzyme activities.

Plant	Ozone concentration	Percent change (increase/decrease)					Reference
		MDA	APX	SOD	POX	AA	
Clover	150 ppb for 3 h	—		—			[60]
T. repens			(−) 22.2		(−) 22.2		
T. pratense			(−) 27.3		(−) 33.3		
Clover	60 ppb for 7 h					—	[80]
NC-S		(+) 80	N.S.	(+) 75	(+) 350		
NC-R		(+) 12.5	(+) 116.6	(+) 16.4	(+) 133		
Wheat cv. M 234	42.2 ppb	(+) 41.6	—	—	(+) 38.1	(+) 11.2	[29]
Rice	38 ppb for 12 h		—				[39]
NDR 97		(+) 51.2		(+) 43.3	(+) 74.8	(+) 16.8	
Saurabh 950		(+) 69.2		(+) 43.6	(+) 36.6	(+) 6.4	
Soybean	70 and 100 ppb for 4 h	—	—	—			[81]
PK 472					(+) 34, (+) 47.7	(−) 18, (−) 19	
Bragg					(+) 29, (+) 41	(−) 3, (−) 8.5	
Wheat	47.3 ppb						[82]
Sonalika		(+) 24.1	(+) 30.4	(+) 35.6	(+) 25	(+) 23.1	
M 510		(+) 14.1	(+) 31.5	(+) 60.2	(+) 16.7	(+) 14.1	
Mustard	43.4 ppb						[83]
Kranti		(+) 54.9	(+) 20	(+) 9.2	(+) 34.1	(+) 8	
Aashirwad		(+) 74.6	(+) 70.5	(+) 7.2	(+) 14.3	(+) 25.9	
Vardan		(+) 41	(+) 11.1	(+) 10.2	(+) 17.5	(+) 43.1	
Wheat	66 ppb	—	—	—	—		[52]
Y2						(−) 88.8	
Y16						(−) 10	

(−) Decrease; (+) Increase; N.S.: not significant.

4.3.4 Physiological Responses

4.3.4.1 Photosynthesis

Tropospheric O_3 and their generated ROS are known to alter membrane properties and membrane-bound organelles such as chloroplast, which may lead to destruction of photosynthetic pigments, and thus ultimately affect photosynthetic activity. Accelerated chlorophyll destruction is reported due to induced metabolic changes within the plant cells caused by oxidative force of O_3 [85]. Several studies have suggested chlorophyll content of leaves as an indicator of stress under O_3 exposure [31, 86] (Table 4.3). Total chlorophyll content decreased significantly by 14, 32, 52, and 47% at elevated levels of O_3, that is, 74, 86, 100, and 124 ppb, respectively, in maize plants [86]. In a study with 20 cultivars of wheat, Biswas *et al.* [87] found 24–35% reduction in total chlorophyll content in recent cultivars and 3–12% reduction in older cultivars of wheat exposed to 82 ppb O_3 for 7 h/day over 21 days in OTCs, suggesting that recent cultivars are more sensitive than older ones. Similar finding was also reported by Pleijel *et al.* [88] with two wheat cultivars, one modern cultivar "Dragon" and another 100-year-old "Lantvete," when exposed to 57 ppb O_3 (nonfiltered chamber receiving elevated O_3) compared to 9 ppb O_3 (filtered chamber). It was found that O_3 induced decline in flag leaf chlorophyll content, which proceeded at a faster rate in "Dragon" compared to Lantvete. Reduction in chlorophyll content is known to reflect the activation of leaf senescence. The degradation of chloroplastic absorbing pigments might be an adaptive response to limit the production of ROS mainly driven in chloroplasts by excess absorption in photosynthetic apparatus [89].

Carotenoids are vital photoprotective agents, which prevent photooxidative chlorophyll destruction [81]. Carotenoid content also reduced due to oxidative destruction under O_3 stress, leading to a decreased capacity to protect photosystems against photooxidation [81]. Hence, the loss of chlorophyll and carotenoids can produce a decrease in the light absorbing capacity to develop thermal dissipation energy under O_3 exposure [81].

Several studies have indicated that an early or primary response to O_3 in leaves is an interference with photosynthesis, carbohydrate metabolism, partitioning of photosynthetic products between mobile and stored pools in the leaf, and/or the translocation of photosynthate within the plants. Reductions in photosynthesis have been widely reported under ambient field conditions at higher concentrations of air pollutants [52, 82, 95]. Metadata analyses of wheat, soybean, and rice varieties showed varying degrees of negative response of photosynthesis under O_3 exposure [96]. Tropospheric ozone also reduces assimilation by decreasing leaf longevity and increasing senescence in wheat plants grown in NFCs compared to FCs [82]. Loss of assimilation capacity was attributed to reduced carboxylation efficiency, which can be directly related to loss of Rubisco activity. Ozone affects the synthesis as well as leads to the degradation of Rubisco due to its oxidation [97]. Nondenatured Rubisco has a large number of free sulfhydryl (–SH) residues and these groups are responsible for maintaining the correct structural conformation of Rubisco. Ozone-induced oxidation of SH groups in Rubisco could alter the

Table 4.3 Impact of tropospheric O_3 on photosynthetic pigments and chlorophyll fluorescence kinetics.

Plant	Ozone concentration	Percent change (increase/decrease)		Reference
		F_v/F_m	Total chlorophyll	
Lettuce	39 and 83 ppb for 12 h			[90]
Valladolid		(−) 2.5, (−) 6.4	(−) 20.8, (−) 60	
Morella		(−) 2.6, (−) 3.4	(+) 30.8, (−) 8.3	
Soybean	58–60 ppb for 7 h			[76]
Essex		—	(+) 2.5	
Forrest		—	(+) 7.4	
Mustard	47.9 ppb	(−) 9.4	(−) 11.3	[30]
Soybean	70 and 100 ppb for 4 h			[81]
PK 472		(−) 16, (−) 16.9	(−) 26.6, (−) 34	
Bragg		(−) 2.5, (−) 5.9	(−) 19.2, (−) 37	
Clover				[91]
NC-R		(−) 12.1	(−) 23.6	
NC-S		(−) 5.1	(−) 32	
Tomato	150 ppb for 3 h			[92]
Cuor di Bue		(−) 2	—	
93.1033/31		(−) 8.9	—	
Clover	60 ppb for 7 h			[80]
NC-S			(−) 10	
NC-R			(−) 36.1	
Rice	42.7 ppb for 7 h			[93]
SY63		—	(−) 46.3	
WYJ3		—	(−) 25.7	
Cotton	100 ppb for 7 h			[94]
Romanos		—	(−) 71	
Allegria		—	(−) 75	
Wheat				[79]
Y19		(−) 6	—	
Y2		(−) 2	—	
Wheat	42.2 ppb for 7 h	(−) 4.9	(−) 39.2	[29]
Rice	38.8 ppb for 12 h			[39]
NDR 97		(−) 16.7	(−) 22.9	
Saurabh 950		(−) 19.5	(−) 27.2	
Wheat	47.3 ppb			[32]
Sonalika		(−) 5	(−) 12.5	
M510		(−) 11.1	(−) 23.1	
Wheat cv. M533	45.1 ppb	(−) 14	(−) 37	[85]

(−) Decrease; (+) Increase.

structural conformation of this enzyme, resulting in reduced catalytic activity and increased vulnerability [97].

Ozone caused reduction in the level of RNA transcript for the small subunit (rbcS) of Rubisco and also decreased the expression of photosynthetic genes for Rubisco and Rubisco activase [98]. Ozone led to reductions in mRNA levels of both small (rbcS) and large (rbcL) subunits of Rubisco in wheat [89]. In a proteomic analysis conducted under *in vivo* conditions on rice seedlings exposed to O_3 (40, 80, and 120 ppb for 6 h/day for 9 days), reductions in expression of Rubisco large subunit (LSU) and small subunit (SSU) were reported [98]. Agrawal *et al*. [97] found that O_3 imposes a negative effect on energy metabolism by altering gene expression of enzymes involved in energy metabolism, that is, fructose bisphosphate aldolase, chloroplast P, and ATP synthase beta subunit. This leads to reduction in ATP production through photophosphorylation and thus affects the Calvin cycle in photosynthesis. Similar findings of reductions in expression of large subunit and small subunit of Rubisco were observed in rice cultivars Shivani and Malviya dhan 36 grown in NFCs at a rural site of Varanasi at 20 ppb above ambient O_3 level (51 ppb) under natural field conditions [31].

An analysis of the documented work from 2000 to 2010 on changes in rate of photosynthesis and stomatal conductance due to O_3 is given in Table 4.4. Sarkar *et al*. [82] reported more reductions in photosynthesis in sensitive cultivar of wheat than tolerant cultivar, which also showed higher reduction in g_s suggesting more stomatal closure to avoid O_3 uptake. Response of rice cultivars showed a contrasting trend as sensitive cultivar NDR 97 showed higher photosynthesis rate and more reductions in g_s compared to Saurabh 950, a tolerant cultivar [39].

Biswas *et al*. [87] and Pleijel *et al*. [88] found that modern or cultivated species demonstrated higher O_3 flux as shown by increased g_s resulting in higher relative reduction in photosynthesis than wild/old species of wheat. Two cultivars of clover exposed to 150 ppb for 3 h showed 37% reduction in photosynthesis and 38% reduction in g_s in *Trifolium repens*, a sensitive cultivar, while tolerant cultivar *T. pratense* did not show any change in photosynthesis and g_s suggesting that tolerant cultivar performed better due to better ability of photosynthetically active mesophyll cells to cope up with photooxidative stress [104]. Similar findings were also recorded in two tomato genotypes 93.1033/1 and Cuor di Bue exposed to O_3 (150 ppb for 3.5 h) [101]. Among bush bean cultivars exposed to 160 ppb O_3 for 3 h, higher reduction in photosynthesis was recorded in sensitive cultivar (36%), while no change was recorded in tolerant cultivar [102]. Ozone at 60 and 100 ppb concentrations reduced photosynthesis rate by 27.6–39.9% in Sufi and Bijoy wheat cultivars of Bangladesh suggesting no variation in sensitivity [95]. Feng *et al*. [52] exposed wheat cultivars Yangmai 16 (Y16) and Yangfumai 2 (Y2) after flag leaf development to 27% higher ambient O_3 (52.1 ppb for 7 h) and found significant reductions in photosynthesis rate and stomatal conductance in Y2.

The reduction in photosynthesis may also occur due to structural damage of thylakoids, which affects the photosynthetic transport of electron, indicated as reduction in F_v/F_m ratio. Reduction of F_v/F_m ratio indicates an alteration of photosystem II (PS II) photochemistry associated with a sign of photoinhibition, making plants more sensitive to light. Lowering of F_v/F_m ratio is observed in lettuce cvs Valladolid

Table 4.4 Changes in rate of photosynthesis (Ps) and stomatal conductance (g_s) of selected plants at different concentrations of O_3 (studies from 2000 to 2011).

Plant	O_3 concentration	Percent change (increase/decrease)		Reference
		Ps	g_s	
Tomato	150 ppb for 3 h			[92]
Cuor di Bue		(−) 17.6	(−) 7.6	
93.1033/31		(−) 31.2	(−) 33.3	
Barley cvs Haider 93, Haider 91, Jou 87, Jou 85	71 ppb for 6 h	(−) 13–21	(−) 6–12	[99]
Soybean cv. S156	60 ppb	(−) 38	(−) 52.6	[100]
Tomato	150 ppb for 3.5 h			[101]
93.1033/1		(+) 19.8	(−) 26.5	
Cuor di Bue		(−) 26.9	(−) 43.7	
Wheat, 20 cultivars	82 ppb for 7 h	(−) 24	(−) 8	[87]
Rice	35 ppb for 12 h			[39]
Saurabh 950		(−) 28.3	(−) 36.6	
NDR 97		(−) 18.3	(−) 52.2	
Soybean	58–60 ppb for 7 h			[76]
Essex		(−) 2.8	(+) No change	
Forrest		(−) 3	(−) 14.5	
Bush bean	165 ppb for 3 h			[102]
Camellino		(−) 36	(−) 26	
Top crop		N.S.	N.S.	
Soybean	70 and 100 ppb for 4 h			[81]
PK 472		(−) 19.8, (−) 40.4	(−) 21, (−) 26	
Bragg		(−) 25.6, (−) 31.6	(−) 61, (−) 66	
Wheat	145 ppb for 4 h			[103]
Yannog 19		(−) 30	(−) 27.1	
Nongda 311		(−) 24	(−) 16.6	
Cotton	100 ppb for 7 h			[94]
Romanos		(−) 20	(−) 69	
Allegria		(−) 33	(−) 60	
Rice	42.7 ppb for 7 h			[93]
SY 63		(−) 27.1	(−) 33	
WYJ3		(−) 14.8	N.S.	
Mustard	47.9 ppb	(−) 20	(−) 27.2	[30]
Wheat	47.3 ppb for 12 h			[82]
M 510		(−) 31	(−) 9.5	
Sonalika		(−) 15.5	(−) 12	
Wheat	60 and 100 ppb for 7 h			[95]
Sufi		(−) 27.7, (−) 37.4	(+) 31.8, (+) 1	
Bijoy		(−) 5.2, (−) 39.9	(+) 2.4, (−) 10	
Wheat	56.4 ppb for 7 h			[52]
Y2		(−) 66.6	(−) 50	
Y16		(−) 50	(−) 16	

(−) Decrease; (+) Increase; N.S.: not significant.

(2.5%) and Morella (2.6%) at mean O_3 concentration of 60 ppb [90]. F_v/F_m ratio reduced by 12% in white clover sensitive clone (NC-S) at 200 ppb O_3 for 5 h/day [91], by 9.3% in snap bean cv. S156 at 60 ppb O_3 [100], and by 5.4% in wheat cv. M 234 at mean O_3 concentration of 42.4 ppb [29] (Table 4.2). Ishii *et al.* [105] also found lowering of F_v/F_m ratio in rice cvs MR 84 and MR 185, at low, medium, and high O_3 doses of 27, 55, and 87 ppb, respectively.

The reduction in F_m under ambient O_3 levels is ascribed to decline in the ability to reduce the primary acceptor Q_A and associated increase in nonphotochemical quenching. Reductions recorded in variable fluorescence (F_v) are more strongly correlated with lowering of F_m, suggesting impairment of an electron transport, which involves a recombination reaction between P680 and reduced phaeophytin ($Phaeo^-$) within PS II or directly affecting a PS II antenna system [105]. Degl'Innocenti *et al.* [104] exposed *T. repens* and *T. pratense* to 150 ppb O_3 for 3 h and maximum reductions in F_m and F_o were recorded in sensitive cultivar *T. pratense* (28 and 13.2%). But after fumigation, recovery in F_v/F_m ratio was observed in *T. repens*, a resistant clone, and no recovery in *T. pratense*. Under O_3 exposure, there are several reports for increase in F_o and a parallel decrease in F_m in wheat [29] and rice [39] suggesting impairment of PS II activity due to the inability of the reduced plastoquinone acceptor Q_A to oxidize completely because of retardation of the electron flow through PS II or due to the separation of light harvesting chl a/b protein complexes. This effect may be due to the inhibition of Calvin cycle activity as indicated by the reduction in CO_2 assimilation rates, signifying that O_3 increased excitation pressure on PS II reaction centers and thus decreased the possibility of e^- transport from PS II to PS I [106].

4.3.5
Cultivar Sensitivity in Relation to Growth and Yield

Tropospheric O_3 was found to adversely affect the growth and yield of a variety of agricultural plants. Tropospheric O_3 reduced the marketable yield of a range of crop species even in the absence of visible injury, primarily through its effects in reducing photosynthesis rates and accelerating leaf senescence [107]. Ozone exposure reduces root biomass as reported in many agricultural crops such as rice [3], wheat [32], and mustard [30]. Root biomass shows a greater sensitivity to elevated O_3 than shoot biomass, leading to reduced root–shoot ratio. The possible cause is reduced photosynthesis in the leaves of lower canopy, the major source of assimilate to roots, as these older leaves are more susceptible to accelerated senescence under elevated O_3 [108], leading to reduced C availability to roots. Wilkinson and Davies [57] proposed that reductions in root biomass could also be ethylene based as ethylene has been known to reduce root growth and its elongation, and under O_3 exposure ethylene emissions increase. Reductions in shoot and leaf biomass are also observed under O_3 exposure due to reduction in carbon assimilation and/or assimilate diversion in producing proteins involved in detoxification of ROS.

Cultivar sensitivity was evaluated on the basis of experiments conducted in open-top chambers [16] and FACE experiments [109]. In Pakistan, 29–47% yield

reductions were reported for six varieties of wheat [110, 111], 28–42% for two varieties of rice [112], and 37–46% for two varieties of soybean [113] due to different pollutants in the ambient air. Exposure to O_3 at 80 ppb concentration for 1.5 h daily for 30 days showed yield reductions of 29.5% in *Vicia faba*, 20.6% in *Oryza sativa*, 13% in *Panicum miliaceum*, and 9.7% in *Cicer arietinum* [114]. Various studies conducted worldwide on crop yield response to O_3 are presented in the table. Differential responses were recorded among different crops and their cultivars. Maximum reductions were found in soybean (40–60%) followed by wheat (20–40%), rice (10–20%), and minimum in barley [96]. Same trend of sensitivity, reporting legumes to be most sensitive and barley to be most resistant under O_3 exposure, has been reported [5, 14, 95].

In open-top chamber studies, wheat and soybean cultivars were studied extensively. Ozone exposure of 70 and 100 ppb for 4 h/day for 70 days led to reductions in yield by 13.9 and 10 and 33.5 and 25% in soybean cvs PK 472 and Bragg [115]. The yield reductions in wheat cvs HP 1209 and M 234 at O_3 concentrations of 70 and 100 ppb for 4 h daily for 70 days were 8 and 4.7 and 17 and 15.5%, respectively [114]. Rai *et al.* [29] found 20.7% reduction in yield of wheat cv. M 234 grown in chambers ventilated with ambient air (40.6 ppb) as compared to filtered chamber. Analyzing the cultivar sensitivity response, Sarkar and Agrawal [32] found reductions of 7, 16.7, and 22% in wheat cv. Sonalika and 8.4, 18.5, and 25% in cultivar HUW 510 grown in NFCs (45.3 ppb), NFCLOs (50.4 ppb), and NFCHOs (55.6 ppb) compared to FCs. Rai and Agrawal [39] reported yield reductions of 10 and 14% in rice cultivars Saurabh 950 and NDR 97 at ambient O_3 concentration of 35.5 ppb grown in open-top chambers (Table 4.4). Among soybean cultivars, highest reduction in yield was recorded in Forrest under O_3 exposure as compared to Essex [76]. In SoyFACE experiment, 10 soybean cultivars were exposed to ambient (46.3 and 37.9 ppb) and elevated (82.5 and 61.3 ppb) O_3 concentrations in 2007–2008 [116]. Yield reductions varied from 11.3 to 36.8% in 2007 and from 7.5 to 16% in 2008 at ambient and elevated O_3 levels and the yield response relationships also indicated that Loda and Pana were tolerant and IA 3010 was sensitive [116]. Zhu *et al.* [109] exposed four winter wheat cultivars (Yannog 19, Yangmai 16, Yangmain 15, and Yangfumai 2) under elevated O_3 using a FACE system with 7 h mean O_3 levels of 56.9 ppb for 7 h in 2006–2007, 57.6 ppb in 2007–2008, and 57.3 ppb in 2008–2009. The grain yield reductions recorded in most sensitive cultivar Y19 were 18.7, 34.7, and 10.1% in three consecutive years of O_3 exposure from 2006 to 2009.

Morgan *et al.* [117] showed that a 23% increase in O_3 from an average daytime ambient level of 56–69 ppb will lead to 20% more reduction in soybean yield. Feng and Kobayashi [96] calculated that at projected O_3 concentration (51–75 ppb), the yield losses would be 10% more for soybean, wheat, and rice and 20% more for bean than at present ambient level of O_3 (41–40 ppb), thus predicting that future rise in O_3 is a significant threat to food production in the world.

The different studies conducted worldwide using study approaches such as OTCs and FACE showed economic losses varying from US$ 24 to 1573 in major crops as shown in Table 4.5. Rai *et al.* [118] reported economic loss of US$ 25–623 ha^{-1} for major agricultural crops wheat, rice, mustard, urd, soybean, pea,

Table 4.5 Estimates of economic loss for different crops due to tropospheric O_3.

Study site	Crops and cultivars	O_3	Economic loss (US$/ha)	Reference
Pakistan	Wheat	71 ppb		[120]
	Inqilab 91		297	
	Punjab 96		603	
	Pasban 90		576	
Pakistan	Barley	72 ppb		[99]
	Haider 93		124	
	Haider 91		267	
	Jou 87		224	
	Jou 83		205	
Urbana-Champaign, USA	Soybean	AOT 4017 ppm h	47	[121]
Urbana-Champaign, USA	Soybean			[122]
	2002	AO_3 (62 ppb), EO_3 (75 ppb)	1573–1337	
	2003	AO_3 (50 ppb), EO_3 (63 ppb)	966–724	
India	Wheat M 234	42 ppb	487	[29]
China	Rice			[123]
		32 ppb	111	
		62 ppb	191	
		85 ppb	330	
China	Rice	56 ppb		[124]
	WJ 15		55	
	YD6		33	
	SY 63		287	
	LYPJ		424	
India	Soybean			[81]
	PK 472	70 ppb	145–303	
	Bragg	100 ppb	121–269	
India	Rice	35 ppb		[39]
	NDR 97		87	
	Saurabh 950		40	
India	Mustard cv. Kranti	48 ppb	153	[83]
India	Wheat	47 ppb		[87]
	Sonalika		110	
	M 510		287	
Japan	Wheat	AO_3 (44 ppb), EO_3 (57.3 ppb)		[52]
	Y2		457	
	Y15		91	
	Y19		46	
	Y16		526	
Japan	Wheat			[95]
	Sufi	60 ppb	221–853	
	Bijoy	100 ppb	915–1257	

Minimum support price (2010) as on 10/6/2010: wheat US$ 0.23 per kg, mustard US$ 0.38 per kg, rice US$ 0.22 per kg, barley US$ 0.16 per kg, and soybean US$ 0.49 per kg. EO_3: elevated O_3; AO_3: ambient O_3.

and mung bean grown at ambient O_3 using different study approaches like in Indo-Gangetic plains of India. Van Dingenen *et al.* [4] using a global chemistry transport model calculated economic losses of approximately US$ 14–26 billion due to O_3 at world market prices for the year 2000. Emberson *et al.* [5] conducted modeling-based studies to assess the extent and magnitude of O_3 risk to agriculture and suggested that yield losses of 5–20% for important crops may be common in areas experiencing elevated O_3 concentrations. It was also concluded that Asian wheat and rice cultivars are more sensitive to O_3 than the North American cultivars. The economic loss for 23 horticultural and agricultural crops due to O_3 was estimated to be 3% (€ 6.7 billion) for the base year 2000 in Europe [119]. But with the scenario of implementation of current legislation, the overall loss of all crop species is estimated to be 2% (€ 4.5 billion) for 2020 [119]. The scenario is, however, entirely different for Asia due to tremendous increase in anthropogenic activities and rapid expansion of economy, leading to an increased emission of O_3 precursors.

4.4
Looking Through the "-Omics" at Post-Genomics Era

4.4.1
Evolution of Multi-Parallel "-Omics" Approaches in Modern Biology

We are running through the golden era of *genomics* (study of whole "genome" is called "genomics"), for both plants and animals, and are also in position to use multiple parallel approaches for the functional analysis of genomes in a high-throughput manner. These parallel approaches surely result in an exceptionally swift and effective system for the analyses and deductions of gene(s) function in a wide range of plants, at the level of transcript (*transcriptomics*), protein (*proteomics*), and metabolite (*metabolomics*) (Figure 4.3). Altogether these four approaches are commonly referred to as the multi-parallel "-omics" approaches in modern biology.

The integration of multiple "-omics" techniques such as *proteomics* and *transcriptomics* with *metabolomics* would also help further elucidate the biochemical and common metabolic mechanisms used by plants not only under normal growth and development, but also in response to environmental stresses. Herein, bioinformatics and systems biology will play a crucial role in our quest for understanding the plant and its molecular components in response to the various environmental stresses, especially O_3.

4.4.2
"*-Omics*" Response in Ozone-Affected Crop Plants: An *In Vivo* Assessment

Over the years, many integrated and individual studies on O_3 stress responses have been reported in several plant species, and such studies have used typical research approaches. Although the demonstration of complicated mechanisms of O_3 response in plants has been attempted, much work still remains to be

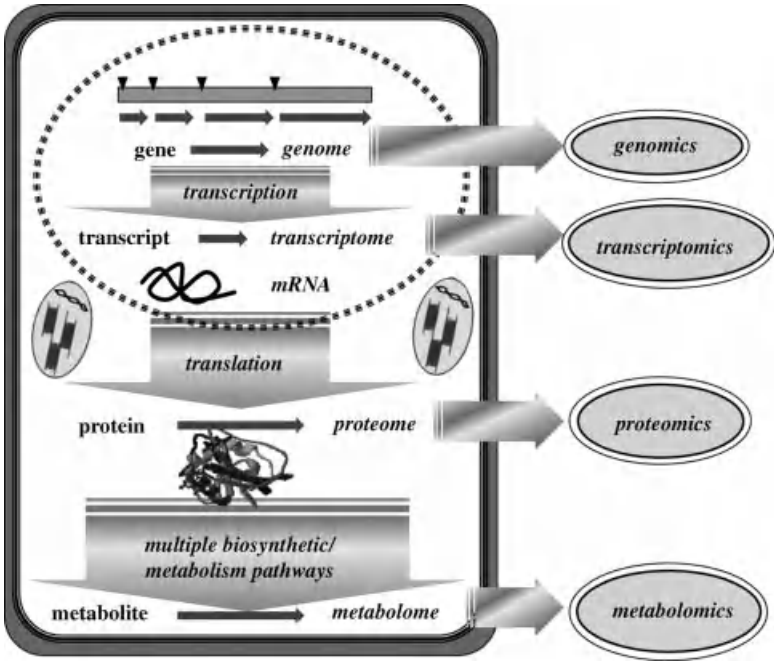

Figure 4.3 Evolution of multi-parallel "-omics" approaches in modern biology.

done in this area. In recent years, analyses have been performed to obtain information on O_3-triggered responses in plants; to this end, many high-throughput "-omics" approaches were performed in *Arabidopsis*, bean, maize, pepper [125–128], rice [6, 97], and wheat [82]. However, as this chapter mainly deals with the agricultural crops, in the following section we will discuss about the "-omics" responses of some important crops under O_3 stress.

4.4.2.1 Case Studies in Major Crop Plants

4.4.2.1.1 Rice (*Oryza sativa* L.) Among all the major crops, rice (*O. sativa* L.) has been studied most for its response to O_3 stress [6, 97, 98, 129]. Agrawal *et al.* [97] first reported a detailed combined transcriptomics and proteomics response of rice plants under elevated O_3 exposure. Two-week-old rice (cv. Nipponbare) seedlings were exposed to 200 ppb O_3 for 3 days in a controlled fumigation chamber. A drastic visible necrotic damage in O_3-exposed leaves and consequent increase in ascorbate peroxidase protein(s) accompanied by rapid changes in the immunoblotting analysis and 2-DE protein profiles was observed. They also reported nearly 52 differentially expressed proteins, among which O_3 caused drastic reductions in the major leaf photosynthetic proteins, including the abundantly present ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and induction of various defense/stress-related proteins. Most prominent change in the rice leaves,

within 24 h post-treatment with O_3, was the induced accumulation of a pathogenesis-related (PR) class 5 protein (PR5), three PR10 class proteins, ascorbate peroxidase(s), superoxide dismutase, calcium binding protein, calreticulin, a novel ATP-dependent CLP protease, and an unknown protein. Feng *et al.* [98] also followed similar experimental model with 2-week-old rice seedlings exposed to 0, 40, 80, and 120 ppb O_3 for 9 days. A drastic damage in the photosynthetic proteins, mainly large and small subunits of Rubisco, and primary metabolism-related proteins, but an induced expression of some major antioxidants such as glutathione S transferase and Mn superoxide dismutase and defense/stress-related proteins such as PRPR5 and two PR10 proteins OsPR10/PBZ1 and RSOsPR10 was reported. Feng *et al.* [98] also confirmed that the damage in rice proteome is strictly O_3 dose dependent. In another independent study, Cho *et al.* [6] also checked the expression profiles of genes in leaves of 2-week-old rice seedlings exposed to 200 ppb O_3 for 1, 12, and 24 h using a 22K rice DNA microarray chip. A total of 1535 genes were differentially expressed more than fivefold over the control. Their functional categories suggested that genes involved in transcription, pentose phosphate pathway, and signal transduction at 1 h and genes related to antioxidant enzymes, ribosomal protein, post-translational modification (PTM), signal transduction, jasmonate, ethylene, and secondary metabolism at 12 and 24 h play a crucial role in O_3 response [6]. Recently, Frei *et al.* [129] have tried to identify the possible mechanism of O_3 response in rice seedlings by characterizing two important quantitative trait loci (QTL), in two different chromosome segment substitution lines (SL15 and SL41), and demonstrated that the activity of some major antioxidant genes might contribute significantly in the response strategy of rice plant under higher O_3 stress.

In contrast with the above laboratory-based experimental models, Sarkar and Agrawal [31] had applied "field-based integrated -omics" approach to understand the background of O_3 response in two high-yielding cultivars (Malviya dhan 36 and Shivani) of mature rice plants under natural conditions (Figure 4.4) and found dependable phenotypical response, in the form of foliar injury, followed by definite changes in leaf proteome. Major damage in the photosynthetic proteins such as large and small subunits of Rubisco and primary metabolism-related proteins, but an induced expression of some antioxidants and defense/stress-related proteins in rice leaf proteome was reported. As illustrated in Figure 4.4, this "field-based integrated -omics" approach mainly differs from the previous laboratory-based approach in terms of the exposure setup and age of the plants. Sarkar and Agrawal [31] had used open-top chambers for exposing the rice plants against O_3.

4.4.2.1.2 **Wheat (*Triticum aestivum* L.)** Wheat (*T. aestivum* L.) is the third most important crop around the globe, and nearly two-thirds of the world population depends on this crop for their primary nutrition supplement. Sarkar *et al.* [82] recently employed "field-based integrated -omics" approach (Figure 4.4) to understand the background of O_3 response in two wheat cultivars (cvs Sonalika and HUW 510) against elevated O_3 concentrations (ambient + 10 and 20 ppb) under

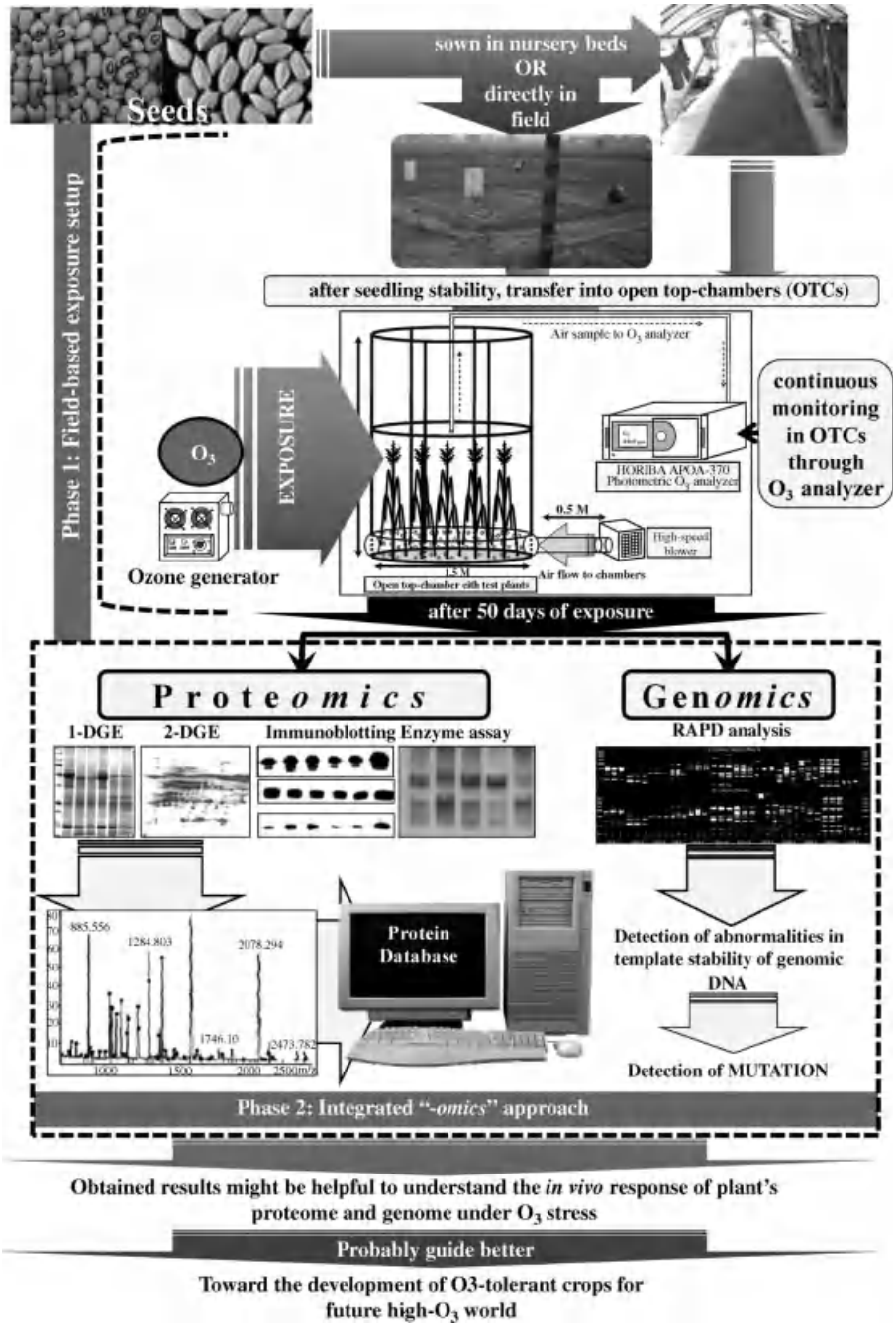

Figure 4.4 Detailed diagram of field-based "-omics" approaches for assessing O_3 effects in modern-day agricultural crops.

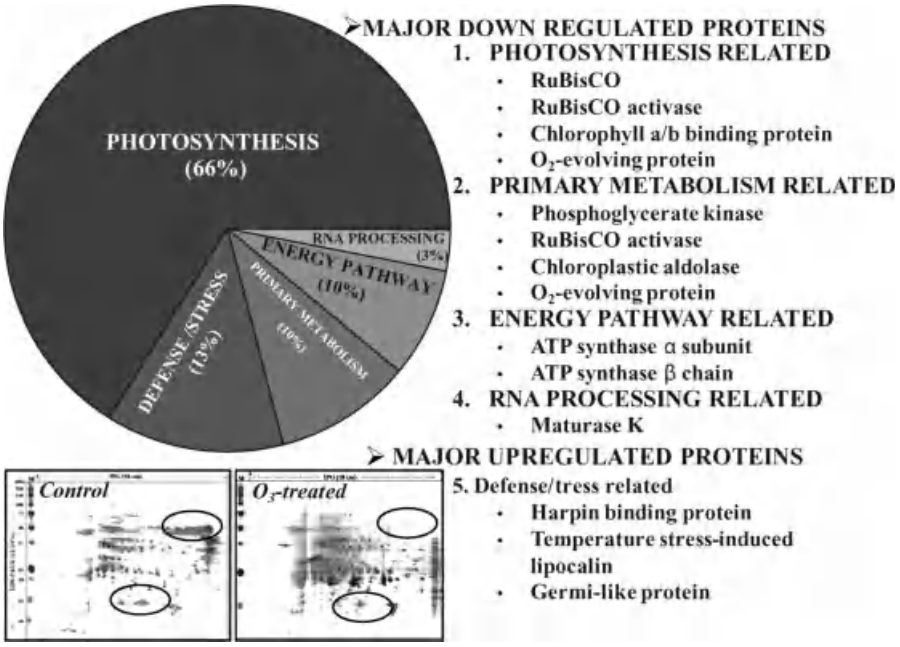

Figure 4.5 Response of wheat proteome under elevated O_3 stress.

near-natural conditions using OTCs. Results of their study showed drastic reductions in the abundantly present Rubisco large and small subunits. Western blot analysis confirmed induced accumulation of antioxidative enzymes such as superoxide dismutase and ascorbate peroxidase protein(s) and common defense/stress-related thaumatin-like protein(s). 2-DGE analysis revealed a total of 38 differentially expressed protein spots, common in both the wheat cultivars (Figure 4.5). Among those, some major leaf photosynthetic proteins (including Rubisco and Rubisco activase) and important energy metabolism proteins (including ATP synthase, aldolase, and phosphoglycerate kinase) were drastically reduced, whereas some stress/defense-related proteins (such as harpin binding protein and germin-like protein) were induced.

4.4.2.1.3 **Maize (*Zea mays* L.)** Maize (*Z. mays* L.) is another important crop at global context. Being a C_4 crop, its response to climate change has been always bit different from the others. Torres *et al.* [127] have done detailed investigation of O_3 response in maize (cv. Guarare 8128) plants through gel-based "-omics" approaches. In that experiment, 16-day-old maize plants (grown in controlled environment at greenhouse) were exposed to 200 ppb O_3 for 72 h, and then the response was compared with a controlled plant (grown under filtered pollutant-free air). Results showed that nearly 12 protein spots were differentially expressed under O_3 exposure, and can be exploited as marker proteins. Expression levels of

catalase (increased), SOD (decreased), and APX (increased) were drastically changed by O_3 depending on the leaf stage, whereas cross-reacting heat shock proteins (HSPs; 24 and 30 kDa) and naringenin-7-*O*-methyltransferase (NOMT; 41 kDa) proteins were strongly increased in O_3-stressed younger leaves. The study also enumerated leaf injury as biomarker under O_3 stress in maize leaves.

4.4.2.1.4 **Bean (*Phaseolus vulgaris* L.)** Torres *et al*. [127] also conducted a study on response of cultivated bean (*P. vulgaris* L. cv. IDIAP R-3) against O_3 stress using the same experimental protocol, and the effects were evaluated through integrated "-omics" approach using gel-based proteomics followed by MS and immunoblotting. Results showed that in bean leaves two SOD proteins (19 and 20 kDa) were dramatically decreased, while APX (25 kDa), small HSP (33 kDa), and NOMT (41 kDa) were increased after O_3 fumigation.

4.4.2.1.5 **Pepper (*Capsicum annuum* L.)** cDNA microarray technique was applied to monitor the transcriptome of ozone stress-regulated genes (ORGs) in two pepper cultivars (*Capsicum annuum* cv. Dabotop (O_3-sensitive) and cv. Buchon (O_3-tolerant)) and results showed that O_3 stress up- or downregulated 180 genes more than threefold with respect to their controls [127]. Transcripts of 84 ORGs increased, transcripts of 88 others diminished, and those of 8 either accumulated or diminished at different time points in the two cultivars or changed in only one of the cultivars. Sixty-seven percent (120) of the ORGs were regulated differently in O_3-sensitive and O_3-tolerant pepper cultivars, most being specifically upregulated in the O_3-sensitive cultivar.

4.4.2.1.6 **Linseed (*Linum usitatissimum* L.)** The response of linseed plants under elevated O_3 stress through combined genomics and proteomics approaches was analyzed by Tripathi *et al*. [130]. The results showed that 10 ppb elevation over ambient O_3 concentration can cause 50% damage in the genome stability of linseed plants. In line with the genome response, leaf proteome also got severely affected under O_3 stress, and the damages were mainly found on the photosynthetic and primary metabolism-related proteins.

4.5 Different Approaches to Assess Impacts of Ozone on Agricultural Crops

Application of chemical oxidants for protection of vegetation from O_3 has been extensively studied over the past four decades. A large number of chemicals mainly synthetic antioxidants, fungicides, insecticides, herbicides, nematocides, growth regulators, antitranspirants [131], and fertilizers [30] are used for the protection of plants. Some antioxidants were found to protect sensitive crops from O_3-induced injury with varied effectiveness, while others were ineffective or produced unacceptable side effects. The most efficient and the most studied synthetic protectant is ethylene diurea (EDU; *N*-[2-{2-oxo-1-imididazodinyl}ethyl]-*N*-phenylurea),

which has also been used extensively to detect plant injury caused by ambient O_3 under bioindicator programs [132]. EDU was also potentially used as a research tool for O_3 injury survey work and plant response assessment in remote areas, particularly in developing regions where electricity and funding are limited [133]. EDU protects plants from premature senescence and pigment degradation and helps in maintenance of higher nutrient levels to allow successful growth and reproduction [134]. Studies using EDU as a chemical protectant to O_3 have shown that at seasonal mean O_3 concentration of 60 ppb for 6 h daily during the growth period, yield reductions of up to 40% in mung bean and 37% in pea were recorded in rural areas of Varanasi [135]. Singh and Agrawal [136, 137] used EDU for screening cultivar sensitivity of wheat and soybean. Among five wheat cultivars (M 234, M 468, M 510, PBW 343, and Sonalika), maximum yield reductions were recorded in M 510 (20.5%) and minimum in PBW 343 (2%) at 400 ppm EDU at 43 ppb mean 8 h O_3 concentration. Soybean cultivars Pusa 9814 and Pusa 9712 showed yield reductions of 28.2 and 29.8% at 52 ppb and 29 and 33% at 72 ppb, respectively. Variability in response of EDU was recorded at different exposures of O_3: EDU showed maximum protection at higher O_3 levels. EDU treatment showed improvement in total biomass and its accumulation in leaves during vegetative period and translocation of more photosynthates toward reproductive parts, which resulted in yield enhancement in black gram (*Vigna mungo* L.) cultivars Barkha (36.4%) and Shekhar (35.6%) while no changes in TU-94-2 [138]. Singh *et al.* [139] evaluated the response of EDU against negative impact of O_3 on two cultivars of clover *T. repens* L., Vardan and Bundel, at two EDU concentrations of 150 and 300 ppm and found Vardan relatively sensitive to O_3 and 300 ppm as the most effective dose of EDU to alleviate O_3 stress. A metadata analysis conducted by Feng *et al.* [140] to estimate the effects of EDU treatment on plants as a proportionate change relative to non-EDU-treated plants using 50 publications revealed that EDU application significantly reduced foliar injury against ambient O_3 by 76%. The largest increase due to EDU was found in wheat (19.5%) and soybean (19.4%). EDU significantly decreased visible injury by 77, 73, and 82% for crops, grasses, and trees, respectively.

The influence of NPK fertilizer on the sensitivity to O_3 has been studied in many crops [30, 83]. Higher N supply may contribute to delay in leaf senescence under O_3 stress by maintaining higher protein levels. Nitrogen is one of the most important nutrients for crop production as it affects dry matter production by influencing leaf area development and maintains the photosynthetic efficiency under O_3 stress. Singh *et al.* [83] reported that application of 1.5 times higher NPK (1.5 RNPK) caused lower magnitude of oxidative stress in mustard cultivars (Kranti, Aashirwad, and Vardan) than recommended NPK level grown under ambient O_3 (43.4 ppb) and hence showed lower activities of antioxidative enzymes such as peroxidase, superoxide dismutase, and ascorbate peroxidase compared to recommended dose of NPK (RNPK). The level of NPK showed a significant positive correlation with total chlorophyll and carotenoids in all cultivars of mustard suggesting higher production or less destruction of photosynthetic pigments at 1.5 RNPK under O_3 stress. Higher levels of carotenoids at 1.5 RNPK may have provided protection to chlorophyll molecules against ROS generated by O_3. Even more K availability may have

enhanced the uptake of nutrients such as SO_4^{2-}, Fe^{2+}, and Mg^{2+} that are associated with the synthesis of chlorophyll.

The other antioxidants/plant metabolites displaying potential to mitigate O_3-induced injuries are (i) ascorbic acid, (ii) phytohormones, (iii) flavonoids, and (iv) polyamines. The protective effects of exogenous ascorbic acid against O_3 are ascribed to its antioxidant potential that inhibits stimulation of ethylene production by O_3 [141], and also ameliorates CO_2 [142].

Zheng *et al.* [142] reported that foliar spray of ascorbic acid (10×10^{-3} ppm) before O_3 exposure (40 ppb for 7 h) in *Plantago major* had caused no change in stomatal conductance but photosynthesis rate increased by 31% over those not sprayed with ascorbic acid. Exogenous ascorbic acid and its salt were shown to reduce acute O_3-induced foliar necrosis, biomass reduction, membrane damage, and alteration of mitochondrial respiration in sensitive plants, but no change in resistant plants [143]. Phytohormones have been widely used in modern agriculture as growth regulators and antisenescence agents. Apart from their effects on plant development, some phytohormones, that is, cytokinins, gibberellins, salicylic acid, and abscisic acid, were shown to enhance tolerance of crops to O_3 stress [143]. Some phytohormones such as cytokinins, gibberellins, and abscisic acid were showed to be antioxidants [144].

4.6
Tropospheric O_3 and Its Interaction with Other Components of Global Climate Change and Abiotic Stresses

UV-B, CO_2, and O_3 are major components of the global climate and slight changes in their levels have been shown to adversely affect the growth and productivity of crop plants.

4.6.1
Elevated CO_2 and O_3 Interaction

Exposure to O_3–CO_2 interaction in most of the studies has shown that CO_2 enrichment protects plants from O_3 stress. Meta-analysis conducted by Ainsworth [145] on rice using Web of Science database and the Agricola database for all the peer-reviewed literature on rice photosynthesis, biomass, and yield responses at elevated CO_2/O_3 from 1980 to 2007 showed that rise in CO_2 concentration in future may ameliorate the detrimental effects of elevated O_3. Booker *et al.* [146] showed in soybean that inhibitory effects of O_3 (72 ppb) on photosynthesis rate were generally attenuated by elevated CO_2 (173 ppm above ambient CO_2) while stomatal conductance reduced under elevated CO_2 and O_3 as well. Booker and Fiscus [147] reported 24% increased biomass production of soybean under elevated CO_2 and $CO_2 + O_3$ treatment and reductions by 28% were recorded in elevated O_3 treatment. Increase in ascorbic acid content was also recorded in $CO_2 + O_3$ treatment in soybean as compared to elevated CO_2/O_3 treatments. Feng and Kobayashi [96] conducted a

meta-analysis of Web of Science reviewed literature between 1980 and 2007 on wheat, rice, soybean, and barley and showed that elevated CO_2 ameliorated the detrimental effects of elevated O_3 in wheat and soybean. Soybean cv. Pioneer 93B15 grown at elevated CO_2 (650 ppm), elevated O_3 (90 ppb), and elevated $O_3 + CO_2$ showed a 60% increase in total antioxidant capacity but dampened and delayed transcriptional response [148]. Heagle *et al*. [149] found that elevated CO_2 was much less protective against O_3 stress in highly O_3-sensitive snap bean cultivar S156 under severe O_3 suppression of 24% pod yield.

4.6.2
O_3 and Drought Interaction

O_3 episodes and incidences of drought often occur together during the reproductive stage of crops [150]. It is widely accepted that drought may reduce O_3 injury in crop plants through drought-induced suppression of stomatal conductance under elevated O_3. Wilkinson and Davis [151] were the first to show that ethylene-dependent reductions in stomatal sensitivity to abscisic acid under O_3 stress lead to higher stomatal conductance under soil moisture deficit and elevated O_3; hence, plants continue to lose water and O_3 flux increases [57]. Elevated O_3 concentration also upregulates ethylene emissions. In response to direct application of ABA (abscisic acid), O_3 repressed stomatal closure even in response to soil drying treatments (drought) where plants synthesize ABA endogenously. The O_3 response is also characterized by an induction of ROS, including H_2O_2 in the form of an oxidative burst [71]; hence, O_3 and ROS such as H_2O_2 induce upregulation of NO production associated with guard cells. Higher concentrations of NO inhibit ABA-induced K^+ efflux at the guard cells and prevent stomatal closure. This result conflicts with predictions that drought may offer some protection against O_3 damage by inducing stomatal closure and restricting O_3 uptake [152]. Biswas and Jiang [153] exposed primitive *Triticum turgidum* L. (O_3-tolerant) and modern wheat (*T. aestivum* L. cv. Xiaoyan 22, O_3-sensitive) to O_3 (83 ppb for 7 h/day) and drought (42% soil water capacity) from flowering to grain maturity to assess drought-induced modulation of O_3 tolerance. It was reported that the primitive wheat, more tolerant to O_3, showed greater O_3-induced reduction in photosynthesis (37%) due to higher loss of Rubisco and carboxylation efficiency in drought-stressed plants than in modern species that showed only 29% reduction in photosynthesis rate. Yield and yield attributes did not vary significantly in O_3–drought interaction suggesting primitive wheat lost its tolerance against O_3 in combination with drought, and modern species though sensitive against O_3 showed increased level of tolerance to O_3.

4.6.3
O_3 and UV-B Interaction

There has been an average of 7% increase in biologically active UV-B radiation in northern mid-latitudes due to depletion of the stratospheric O_3 layer, induced by anthropogenic emissions in the past two decades [154]. Field experiments

conducted to assess the growth, physiological, and biochemical responses of wheat plants exposed to supplemental UV-B (7.1 kJ/m^2) and O_3 (70 ppb) showed that enhanced UV-B and O_3 reduced ascorbic acid content and catalase activity [154]. Photosynthesis rate declined significantly in O_3 by 13% and 22.1% in UV + O_3 treated plants and even higher reductions were observed in stomatal conductance. Similarly, chlorophyll content, biomass accumulation, and yield were lower in all the treatments, but more pronounced in combined treatments, suggesting that interactive effects were less than additive.

Impact of ambient O_3 (60 ppb) and supplemental UV-B (ambient + 7.2 kJ/m^2/day) on linseed showed that in combined treatment, symptoms were of low intensity compared to the individual treatments [130]. The treatment of plants with sUV-B and O_3 led to enhanced lipid peroxidation and reduction in plant biomass. Antioxidative enzymes increased significantly in O_3 followed by sUV-B + O_3 and minimum in sUV-B treatment. Hence, the interactive study between sUV-B and O_3 was more synergistic.

4.7 Conclusions

The projected levels of O_3 are critically alarming, and have become a major issue of concern for food security. Scientific evidence clearly indicates that crop plants are sensitive to O_3 in different ways. Plant resistance to O_3 involves a wide array of responses ranging from the molecular and cellular level to the whole plant level. Significant effects of O_3 are early leaf senescence, decreased photosynthetic assimilation, altered stomatal behavior, decreased growth and productivity, reduced carbon allocation to roots, and changes in metabolic pathways. Genotype differences in response to O_3 are related to stomatal behavior, antioxidative potential hormonal regulation, and carbon allocation during reproduction affecting the yield responses. Detailed understanding of genotypic response is crucial in predicting the long-term impacts of O_3 on agriculture in global context, including the breeding of resistant cultivars. Several potential O_3 biomarkers have been identified, which may be exploited to screen and develop O_3-tolerant varieties in future.

The studies on interactions of different factors of climate change and abiotic stresses with O_3 are also needed to predict crop responses under futuristic scenario of multifactorial stresses such as drought, heat, CO_2, and UV-B. Estimating the interactive effects of rising O_3 concentration along with drought, CO_2, heat, and so on is especially important for agriculture in view of their common occurrence in tropical countries such as India. Studies are also needed on interactive effects of macro- and micronutrients and O_3 on crop plants in view of changing nutrient availability in agricultural soil. The experiments using "-omics" approach on O_3 response were mostly performed under controlled conditions and hence more studies are required on molecular characterization of biomarkers under natural field conditions.

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Part II
Methods to Improve Crop Productivity

Improving Crop Productivity in Sustainable Agriculture, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, and Renu Tuteja.

5
Mitogen-Activated Protein Kinases in Abiotic Stress Tolerance in Crop Plants: "-Omics" Approaches

Monika Jaggi, Meetu Gupta, Narendra Tuteja, and Alok Krishna Sinha

Abstract

Plants, in order to grow and survive, need to counter a plethora of stresses, both biotic and abiotic. During the process of evolution, plants have developed sophisticated mechanisms to perceive these stresses and transduce them down to the nucleus for appropriate cellular adjustment. Phosphorylation of proteins is one of the important mechanisms for controlling many fundamental cellular processes in all living organisms. A network of mitogen-activated protein kinases (MAPKs) is an evolutionarily conserved phosphorelay cascade among animals, plants, and yeasts that transduces a variety of signals from cell surfaces to the nucleus. This unique protein cascade is also involved in the development and survival of the plants. This cascade consists essentially of three components, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK connected to each other by the event of phosphorylation. Signaling through MAP kinase cascade can lead to cellular responses including cell division and differentiation as well as responses to various stresses. In plants, MAP kinases are represented by multigene families and are involved in efficient transmission of specific stimuli and also involved in the regulation of the antioxidant defense system in response to stress signaling. In this chapter, we summarize and investigate the participation of MAPKs as possible mediators of various abiotic stresses in plants. We also focus on recent progress in integrated transcriptomics, proteomics, and metabolomics analyses of MAPK signaling pathway that regulates plant physiological processes during abiotic stress responses. We also deal with the limitations and future prospects of these "-omics" approaches.

5.1
Introduction

Plants have developed a very fine integrated signaling network that mediates the perceptions of environmental cues for their survival, growth, and development. Because plants are sessile, they are particularly sensitive to environmental changes.

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To sustain their normal growth and development, plants must respond and adapt to continuously changing environmental conditions. Analyses of plant responses to abiotic stresses are simpler than studies of biotic or host–pathogen responses in a way that it is comparatively easy to give an abiotic stress treatment. The term "abiotic stress" includes various stresses present in the environment, for example, light, wound, UV irradiation, drought, salinity, temperature, and so on. These stresses greatly affect plant growth and productivity, sometimes leading to great losses to the crops. Therefore, it is very important to deeply understand the regulatory mechanism underlying the abiotic stress responses to improve crop productivity under unfavorable or stressful conditions.

Before the advent of the genomics era, a gene-by-gene approach was used to identify the genes involved in abiotic stress response. The application of molecular biology approach revolutionized our understanding of plant stress regulation. A lot of information was gathered using both forward and reverse genetic approaches on the functional aspects of various genes involved in gene regulation, signal transduction, and stress tolerance [1]. Many abiotic stress-inducible genes were isolated and their functions were deciphered in transgenic plants. Thus, these studies broadened and deepened our knowledge of abiotic stress responses and tolerance in plants. In the genomics era, the availability of the complete genome sequences facilitated access to essential information for all genes, for example, gene products and their functions, transcript levels, putative *cis*-regulatory elements, and so on. In the post-genomics era, comprehensive analyses using functional genomics technologies such as transcriptomics, proteomics, and metabolomics have increased our understanding of the complex regulatory networks associated with stress adaptation and tolerance.

As environmental conditions begin to change, plants sense this and alarm a signal that is relayed via several signal transduction cascades. Post-translation modifications of proteins also played important role in different signaling cascades. The phosphorylation of proteins by specific protein kinases modulates the activity of specific signaling molecules, resulting in signal amplification. Different types of kinases have been identified from plants and classified into different groups. One of the largest and most important categories is the mitogen-activated protein kinases (MAP kinases). These are present in both the cytoplasm and nucleus and are involved in different signal transduction pathways such as osmoregulation, cell growth, and differentiation. These are also known as a universal signal module that helps in mediating plant responses to various environmental stresses. In a general model, stimulated plasma membrane receptors activate MAPK kinase kinases (MAP3Ks, MAPKKKs, or MEKKs) or MAPK kinase kinase kinase kinases (MAP4Ks). These activated MAP3Ks then subsequently mediate the phosphorylation of MAPK kinases (MAP2Ks, MAPKKs, or MEKs) that in turn phosphorylate MAPKs. MAPKs then target various effector proteins in the cytoplasm or nucleus, which include other kinases, enzymes, or other transcription factors (Figure 5.1). These kinases are functionally interlinked and have been found to play a central role in signal

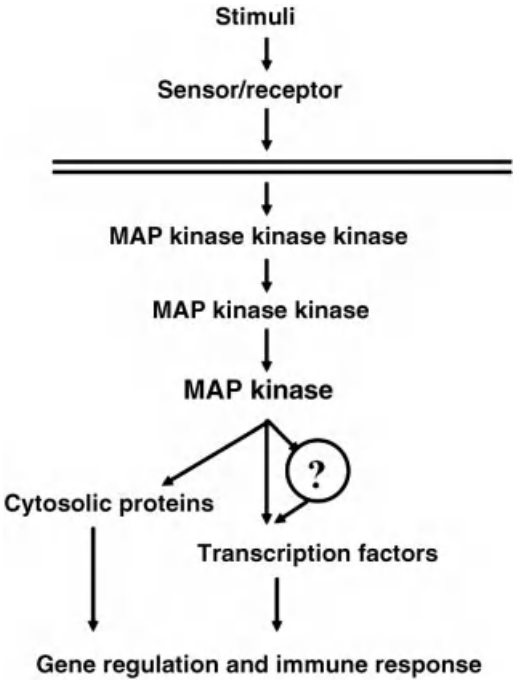

Figure 5.1 A typical MAP kinase cascade. "?" denotes an unknown component between MPK and transcription factor.

transduction mechanisms. Stressor-specific induction of MAPK genes and increased MAPK kinase activity have been detected when plants are exposed to different abiotic stresses, for example, touch, cold, salinity, genotoxic agents, UV irradiation, ozone, and oxidative stress. In plant systems, the MAP kinase genes were first reported from pea in 1993 and now the cDNA clones of MAPKs are reported from many plants (see Ref. [2]). The major milestones in the discovery of MAP kinases with special emphasis on plant MAP kinases are described earlier [2]. The biochemical assay of MAP kinase activity is based on the ability of this enzyme to phosphorylate a protein containing a consensus sequence, P-x-S/T-P (proline–X–serine/threonine–proline). Myelin basic protein (MBP) is the widely used substrate for MAP kinase assay. A routine technique involves the incorporation of MBP into a polyacrylamide gel over which cellular proteins are separated. The kinasing reaction is carried out in the presence of γ^{32P}-ATP, followed by autoradiography (see Ref. [2]).

In this chapter, we focus on recent progress in integrated transcriptomics, proteomics, and metabolomics analyses of MAPK signaling pathway that regulates plant physiological processes during abiotic stress responses. We also deal with the limitations and future prospects of these "-omics" approaches.

5.2 MAPK Pathway and Its Components

MAPK cascades are conserved signaling modules found in all eukaryotes that transduce environmental and developmental cues into intracellular responses. A MAPK cascade is minimally composed of MAP3Ks/MAPKKKs/MEKKs, MAP2Ks/MAPKKs/MEKs/MKKs, and MAPKs/MPKs [2, 3]. During stress, stimulated plasma membrane activates MAP3Ks or MAP4Ks [4]. MAP4Ks may act as adaptors linking upstream signaling steps to the core MAPK cascades. MAP3Ks are serine/threonine kinases phosphorylating two amino acids in the S/T-X_{3-5}-S/T motif of the MAP2K activation loop. MAP2Ks phosphorylate MAPKs on threonine and tyrosine residues at a conserved T-X-Y motif [5]. MAPKs are serine/threonine kinases able to phosphorylate a wide range of substrates, including other kinases and/or transcription factors. The formation and integrity of a specific MAPK cascade can be mediated by scaffold proteins, shared docking domains, and adaptor or anchoring proteins [6–8]. MKPs (MAPK phosphatases) are involved in the time-dependent controller in the shutdown of the pathway after signaling [9].

In the first reports of MAPKs in 1986, Sturgill and Ray discovered microtubule-associated protein–protein kinase (MAP-2 kinase) in animal cells [10] and renamed it as mitogen-activated protein kinase [11]. In plants, the MAPK genes were first identified as MsERK1 in alfalfa [12] and D5 kinase in pea [13]. Complete sequencing of the genome of various genera revealed a large number of genes coding for MAPK-related kinases. *Arabidopsis* possesses genes encoding 20 MAPKs, 10 MAP2Ks, and 80 MAP3Ks [14]. A similar repertoire of genes was observed in the sequenced genomes of other plants such as rice (*Oryza sativa*), poplar (*Populus trichocarpa*), grapevine (*Vitis vinifera*), or sorghum (*Sorghum bicolor*).

5.2.1 MAP3Ks

MAPKKKs form the upstream component of the MAPK module and are often activated by G proteins. However, there are also reports of their activation by MAP4Ks. In *Arabidopsis*, at least 10 genes encoding MAP4K are related to yeast Sterile (Ste20) or mammalian p21-activated protein kinases (PAKs), but none of these plant kinases has been shown to regulate a MAPK pathway [15].

MAPKKKs form the largest group in this cascade with putative numbers being 80 in *Arabidopsis* [14, 15] and 75 in rice [16]. This largest group is further subdivided into three major subtypes, namely, Raf, MEKK, and ZIK [16, 17] (Figure 5.2). The Raf subfamily consists of 43 members from rice and 48 members from *Arabidopsis*, MEKK had 22 from rice and 21 from *Arabidopsis*, while 10 from rice and 11 from *Arabidopsis* fall in ZIK family. Number of annotated MAPKKKs is 40 in *Sorghum* and 60 in *Populus*. Interestingly, 8–10 MAPKKKs are reported in algal systems *Chlamydomonas* and *Volvox* [3].

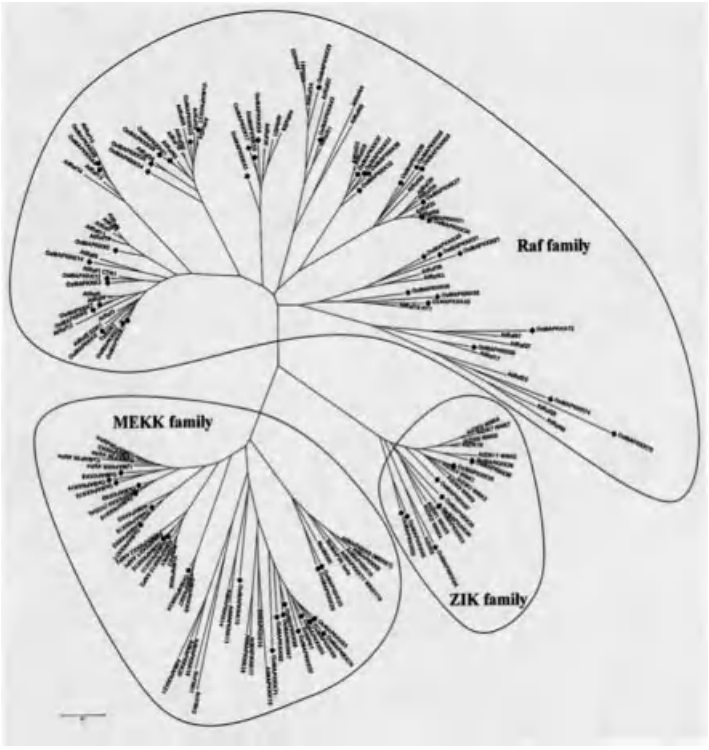

Figure 5.2 Phylogenetic tree of MAPKKKs from different plant species. All 80 *Arabidopsis*, 75 rice, and 14 MAPKKKs from other plants were considered for the generation of phylogenetic tree classified into three distinct groups. *Source*: Ref. [16]. Reproduced with permission of Oxford University Press, © 2010.)

The Raf family of MAPKKKs possesses a specific signature motif GTXX(W/Y) MAPE. This kinase domain of human B-Raf has high affinity and activates MEK [18, 19]. In *Arabidopsis*, a member of Raf subfamily, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), acts as negative regulator of ethylene signaling [20, 21], while ENHANCED DISEASE RESISTANCE 1 (EDR1) acts in defense response to powdery mildew attack [22, 23]. Both these genes have close homologues in *Physcomitrella* and *Selaginella*. Intriguingly, the participation of both CTR1 and EDR1 in canonical MAPK cascade has not been confirmed, making it difficult to assign their biochemical function.

The characteristic conserved signature of ZIK family consists of GTPEFMAPE (L/V/M)(Y/F/L) across the membrane. There are 10 and 11 members of MAPKKKs in rice and *Arabidopsis*, respectively. An *Arabidopsis* ZIK family member, WNK1, has been shown to phosphorylate a protein involved in circadian rhythms [24].

Among the three subfamilies, MEKK is relatively well characterized. ANP1 in *Arabidopsis* was found responsive to oxidative stress and is involved in negative

regulation of auxin signal transduction pathway [25, 26]. ANP1 along with ANP2 and ANP3 from *Arabidopsis* is reported to be involved in plant cytokinesis [27, 28]. MAPKKKα is involved in defense response [29], while YODA was characterized in stomatal development [30]. Similarly, the MAP3Ks are reported from tobacco, *Solanum chacoense*, *Medicago sativa*, and rice. Twenty-two MAPKKKs from rice and 21 from *Arabidopsis* belong to this subfamily. G(T/S)PX(F/Y/W)MAPEV forms a conserved signature catalytic domain of this family.

5.2.2 MAP2Ks

Plant MAP2Ks have the S/T-X_5-S/T as the phosphorylation site and a putative MAPK docking domain K/R-K/R-K/R-X_{1-6}-LX-L/V/I [31]. They are classified into four different groups (A–D), which are known from *Arabidopsis*, rice, and *Populus* [32] (Figure 5.3). Interestingly, the numbers of MAP2Ks are almost half of the numbers of MAPKs in these plants. There are 10, 8, and 11 members of MAP2Ks reported from *Arabidopsis*, rice, and *Populus*, respectively. Group A includes *Arabidopsis thaliana* MKK1 and MKK2 that act upstream of the MAPK MPK4 [33]. Group B includes MKK3 in *Arabidopsis* that has orthologues in rice, *Selaginella*, *Physcomitrella*, and *Chlamydomonas*. Kinases in this group are characterized by a nuclear transfer factor (NTF) domain [32] that enhances the nuclear import of cargo proteins indicating their involvement in cytoplasmic/nuclear trafficking [34]. Group C comprises of MKK4 and MKK5 and rest MKKs from *Arabidopsis* belong to group D. Functional evidence for the role of MAP2Ks is available for *Arabidopsis* MKK1, MKK2, MKK4, MKK5, and MKK6, *Medicago* PRKK and SIMKK, tobacco MKK2 and MKK4, parsley MKK5, and rice MEK1 [35].

5.2.3 MAPKs

In all plant species, MAPKs carry either a TEY or TDY phosphorylation motif at the active site similar to animal ERK kinases. In contrast to TEY MAPKs, all TDY MAPKs have long C-terminal extensions. *A. thaliana* MAPKs have also been subdivided into four groups (A–D) (Figure 5.4) [14]. TEY MAPKs contain groups A, B, and C, whereas group D comprises TDY MAPKs. Group A consists of *A. thaliana* MPK3 and MPK6 and their homologous sequences in tobacco, alfalfa, rice, and poplar. They are involved in developmental processes and are activated in response to biotic and abiotic stresses [36–38]. Group B MAPKs are related to AtMPK4 and are involved in pathogen defense and abiotic stress response [39–43]. Group D MAPKs are identified by a C-terminal common docking domain that may act as a docking site for MAP2Ks [44]. Recent investigations have confirmed major roles of defined MAPK pathways in developmental, cellular proliferation, and hormone physiology, as well as in biotic and abiotic stress signaling.

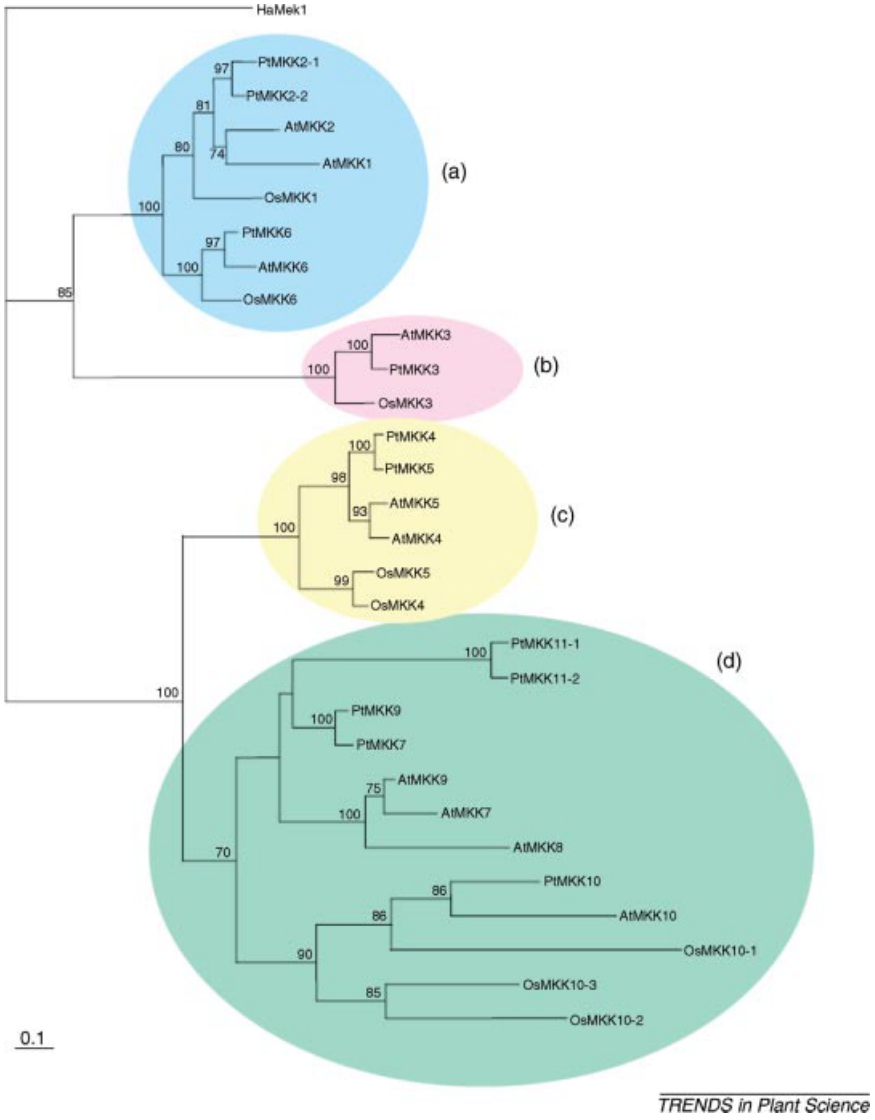

Figure 5.3 Phylogenetic relationships of 10 *Arabidopsis*, 11 poplar, and 8 rice MAPKK genes. MAPKKs are divided into four different groups. *Source*: Ref. [32]. Reproduced with permission of Elsevier, © 2006.)

5.3
Plant MAPK Signaling Cascade in Abiotic Stress

Signaling pathways are induced in response to environmental stresses, and recent molecular and genetic studies have revealed that these pathways involve a host of diverse responses [45, 46]. It has been well established that abiotic stress response

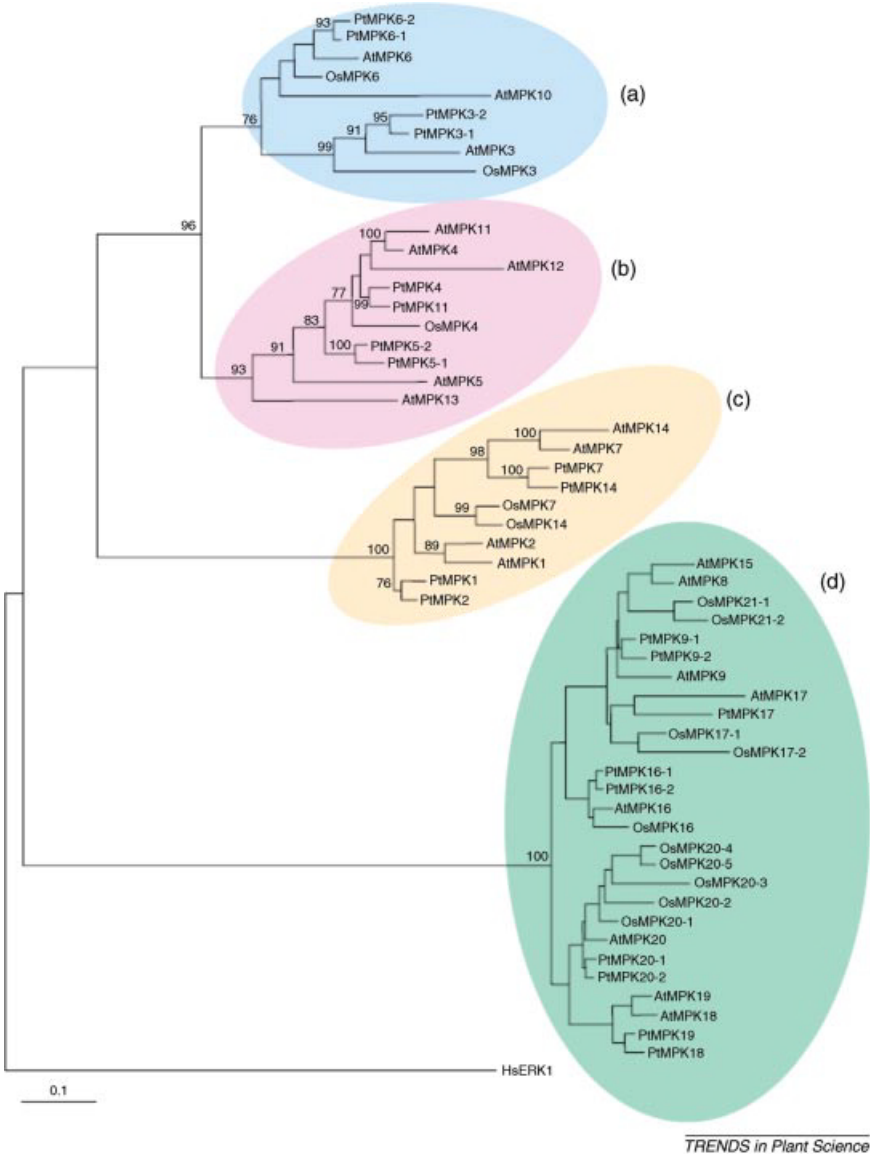

Figure 5.4 Phylogenetic relationships of 20 *Arabidopsis*, 21 poplar, and 15 rice MAPK genes. MAPKs are divided into four different groups. *Source*: Ref. [32]. Reproduced with permission of Elsevier, © 2006.)

is a complex trait governed by multiple genes. In the past two decades, basic biological research has taken a big leap from studying the expression of single genes or proteins to focusing on a large number of genes or gene products simultaneously, enabling genome-wide expression strategies for better understanding of these complex traits.

One of the signaling pathways involved in abiotic stress response is the MAPK cascade. This signaling module links external stimuli with several cellular responses and is evolutionarily conserved among eukaryotic organisms [15, 47]. In several species, including *Arabidopsis*, MAPK cascades have been shown to be involved in signaling pathways activated by abiotic stresses such as cold, salt, touch, wounding, heat, UV, osmotic shock, heavy metals, and so on (Table 5.1). Latest insights and findings are discussed in the context of MAPK pathways in plant abiotic stress signaling.

5.3.1
MAPK Cascades under Salt Stress

Salt stress afflicts plant agriculture in many parts of the world, particularly irrigated land [80]. In *Arabidopsis*, previous study has demonstrated that the MEKK1 (a MAPKKK) mRNA accumulated in response to environmental stresses, including high salinity [54]. Yeast two-hybrid analyses showed protein–protein interactions between MEKK1 and MKK2/MEK1 (MAPKKs), between MKK2/MEK1 and MPK4 (a MAPK), and between MPK4 and MEKK1 [81]. Further study demonstrated that environmental stress signals are transmitted to at least two MAPK cascades. One is the MPK4 cascade (MEKK1–MEK1/MKK2–MPK4) and the other involves MPK6 and p44MAPK [57]. Under salt or cold stress, MAPK pathway involves MEKK1 as an upstream activator of MKK2 and the downstream MAPKs MPK4 and MPK6 [58]. MKP1 plays a negative role in salt stress signaling through MAPKs (MPK6 and MPK4) [9].

In alfalfa, a 46 kDa SIMK (salt stress-induced MAPK) was activated by salt [48]. Yeast two-hybrid analysis identified an upstream activator kinase SIMKK that interacts specifically with SIMK and enhanced the salt-induced activation of SIMK *in vivo* as well as *in vitro* [36]. It was also reported that tobacco protoplasts exposed to salt and osmotic stress showed enhancement of a 48 kDa kinase, the SIPK (salicylic acid-induced protein kinase) [75]. Osmotic stress reportedly activated the expression of AtMPK3, AtMPK4, and AtMPK6 in *Arabidopsis* [40, 55]. Recently, three salt stress-induced MAPKs, ZmMPK3, ZmMAPK5, and ZmSIMK1, have been identified in *Zea mays* [62, 66, 82]. Overexpression of OsMAPK5 in rice transgenic plants increased tolerance, while suppression led to hypersensitivity to various stresses including salt [70]. Salt stress also activated expression of CbMAPK3 [59] and GhMPK7 [61].

5.3.2
Drought Stress-Induced MAPKs

Among the stresses, drought is a major environmental factor limiting productivity and distribution of plants [83]. When soil moisture is continuously low, water extraction by root and water transport within the plant is reduced and a drought-like situation prevails. Drought is a major constraint to increase yield in crop plants. Many stress-responsive genes have been identified and their altered gene

Table 5.1 List of MAPKs from different plant sources involved in abiotic stresses.

Plant	Component of MAPK cascade	Remarks	References
Alfalfa	SIMK	Activated by hyperosmotic conditions and metal stress	[48, 49]
	MMK2, MMK3, SAMK	Activated by heavy metal stress	[49]
	P44MKK4	Activated by cold and drought	[50]
	MMK4/MKK4	Wound stress	[51]
	OMTK1	Oxidative stress	[52]
	HAMK	Heat stress	[53]
	SAMK	Cold stress	[50]
Arabidopsis	AtMEKK1, AtMPK3	Touch, cold, and salt stress	[54]
	AtMPK3, AtMPK6	Hypoosmolarity, ozone	[55, 56]
	AtMPK1	Salt stress	[57]
	AtMPK4,6	Low temperature, dehydration, touch, wounding, hyperosmotic stress	[57]
	MAPKK, MKK2	Cold and salt stress	[58]
C. bungeana	CbMAPK3	Cold, salt	[59]
Cotton	GhMAPK	Wounding, cold, salinity	[60]
	GhMPK7	Salt, wounding	[61]
Maize	ZmMPK3	Activated by cold, drought, UV, salinity, heavy metals, wounding	[62]
	ZmMPK7	H_2O_2, osmotic stress	[63]
	ZmMAPK5	H_2O_2, PEG, NaCl, $CdCl_2$, cold, wounding, UV	[64]
	ZmMPK5	Low-temperature stress	[65]
	ZmSIMK1	Salt stress	[66]
Pea	PsMPK2	Wounding, ABA, H_2O_2	[67]
Potato	StMPK1	Wounding, heat	[68]
Rice	OsMSRMK2	Wounding, UV, metal, salt, drought, ozone, high and low temperatures	[69]
	MAPKK4,6	Cold and salt	[31]
	MAPKK1	Salt, drought	[31]
	MAPKK10-2	Cold	[31]
	OsMAPK5	Wounding, drought, salt, cold	[70]
	OsBWMK1	Mechanical wounding	[71]
	DSM1 (MAPKKK)	Drought	[72]
S. brachiata	SbMAPKK	Dehydration, cold, salt	[73]
Soybean	MAPK	Activated by light, wound stress	[74]
Tobacco	SIPK	Salt and osmotic stress, ozone	[75, 76]
	NtWIPK1	Wounding	[77]
	NtMPK4	Wounding, ozone tolerance	[78]
Tomato	tMEK2	Wounding	[79]

expression plays an important role in plant drought resistance [80, 84, 85]. *In gel* kinase assays followed by immunoprecipitation with specific peptide antibodies raised against different alfalfa MAP kinases showed that alfalfa p44MKK4 (MAP kinase kinase) gene expression and kinase activity got activated under drought conditions in an ABA-independent manner [50]. Research in *Arabidopsis* found that the expression of AtMEKK1 and AtMPK3 could be induced by drought [54]. Drought stress resulted in the activation of OsMSRMK2 and OsMAPK5 in rice plants [69, 70]. Overexpression of DSM1 (a putative rice MAPKKK gene in rice) increased the tolerance to dehydration stress [72]. Peng *et al.* [86] investigated the expression patterns of MaMAPK and showed that activity of MAPK might be one of the molecular mechanisms of different drought tolerance in *Malus*. ZmMPK3 also plays an important role in response to environmental stresses including drought stress [62].

5.3.3
Temperature Stress Response and MAPK Cascades

Plants exhibit a range of responses to the temperature of their environment. They depend on the perception of both high and low temperatures, both for their survival and for the regulation of key developmental events. Although environmental change is expected to increase average temperatures, this will also have important consequences for the way in which plants perceive low temperature. A lot of studies have been carried out in *Arabidopsis* that indicated the role of MAPKs in temperature stress. AtMEKK1 and AtMPK3 are transcriptionally induced by cold stress [54], while AtMPK4 and AtMPK6 are also activated by cold stress [57]. *Arabidopsis* MAPKK, MKK2, also got upregulated in response to cold stress [58]. Yeast two-hybrid as well as protein kinase assays revealed that AtMPK4 and AtMPK6 were direct and specific substrates of MKK2 [87]. Functional and interaction analyses in yeast suggested that MEKK1 functions upstream of MKK1, MKK2, and MPK4 [81] and a role for the MAPK module consisting of MEKK1–MKK2–MPK4/6 has now been confirmed in cold stress. The transcript level of ZmMPK3 increased markedly within 30 min and remained high during a 4 h period [62]. Cold stress also induced the expression and activity of ZmMAPK5 [65, 88]. Recently, a lot of information has been gathered where cold stress led to the activation of MAPKs in different plant genera, for example, *Chorispora bungeana* [59], *Gossypium hirsutum* (GhMAPK) [61], and *Salicornia brachiata* (SbMAPKK) [73].

Not only low temperature but also high temperature leads to the activation of MAPKs. The sudden increase in ambient maximum temperature, in a matter of few days, by 5–7 °C with corresponding increase in the minimum temperature, creates heat stress on plants. The normal physiology of the plant gets affected and the plant maturity is accelerated. In practical agriculture, such heat stress inflicts enormous crop losses. In the European heat wave of 2003, crop production was reduced by around 30% [89]. Due to global weather change, the frequency of heat stress is predicted to increase in different parts of the world. Sangwan *et al.* [53] identified the first plant heat shock-activated MAPK (HAMK) from alfalfa cells. In tomato photoautotrophic cell cultures, a partially purified heat-activated MAPK was shown

to phosphorylate HsFA3 transcription factor [90]. Another report was published on *Solanum tuberosum* where heat treatment to potato tubers elevated StMPK1 transcript levels [68]. In rice, changes in temperature affected the transcript levels of OsMSRMK2 [69]. High temperature (37 °C) resulted in a considerable decrease in its transcript level at 30 min, whereas at 25 °C an increase was observed at 30 min, which drastically decreased with time. Interestingly, at low temperature (12 °C), the OsMSRMK2 transcript started to accumulate only around 60 min, reaching a maximum at 90 min, followed by a slight decline at 120 min. Thus, rapid induction of OsMSRMK2 mRNA at 37 °C suggests its role in sensing high temperatures [69].

5.3.4 Activation of MAPKs by Oxidative Stress

Oxidative stress is a term used to describe the effect of oxidation in which an abnormal level of reactive oxygen species (ROS), such as the free radicals (e.g., hydroxyl, nitric acid, superoxide) or the nonradicals (e.g., hydrogen peroxide, lipid peroxide), leads to damage (called oxidative damage) to specific molecules with consequential injury to cells or tissue. Most types of abiotic stresses such as drought, salinity, heat, and cold stresses disrupt the metabolic balance of cells, resulting in enhanced production of reactive oxygen species [91]. Removal or neutralization of ROS is achieved with antioxidants, endogenous (e.g., catalase, glutathione, superoxide dismutase) or exogenous (e.g., vitamins A, C, and E, bioflavonoids, carotenoids). Plants overcome oxidative stress with the production of scavenger enzymes such as catalases, which decompose H_2O_2. For example, *A. thaliana* CAT1 is regulated by ABA, and Xing *et al.* [92] found that the MAP2K inhibitor PD98059 hindered ABA-mediated CAT1 expression. In addition, the *A. thaliana* mkk1 and mpk6 mutants were altered in their responses to ABA and desiccation stress. These results, together with the lack of ABA-mediated activation of MPK6 in mkk1 mutants, suggested that MKK1–MPK6 regulate H_2O_2 metabolism through CAT1 [93]. In contrast with CAT1, the closely related CAT2 expression seems to be regulated by MEKK1 and MPK4 [94], which are involved in plant defense and SA accumulation. The MEKK1–MPK4 cascade playing an important role in ROS metabolism was first demonstrated by Nakagami *et al.* [95]. In addition, other MAPKKKs are activated in *A. thaliana* protoplasts by H_2O_2 that include ANP1, which may cause the downstream activation of MPK3 and MPK6 [26]. These findings imply that multiple MAPK modules mediate oxidative stress responses and that MAP kinase cascades not only are induced by ROS but may also regulate ROS levels by affecting catalase activity. Notably, ROS homeostasis is a convergence point that indicates plant stress status because oxidative stress is a common response to biotic and abiotic stresses. A recent review compiled ROS-mediated MAPK signaling literature [94]. The continued examination of available *A. thaliana* mutants and other *in planta* studies of stress-specific protein interactions will help dissect the roles of MAPK modules. An important issue that has emerged in this field is how cellular redox status determines cell growth and differentiation and, thus, development. In alfalfa, a novel MAPKKK, OMTK1

(oxidative stress-activated MAP triple kinase 1), was identified that further activated downstream MAPK, MMK3 [52]. H_2O_2-induced activation of plant MAPK has also been reported in various genera, for example, maize [63, 82] and pea [67].

5.3.5 Ozone-Induced MAPKs

Ozone is a strong oxidant and atmospheric pollutant and is known to activate MAPK signaling pathway. MAP kinases in plants are also activated by exposure to ozone [76]. A 46 kDa MBP kinase activity immunoprecipitable with anti-SIPK is induced in tobacco leaves and cells after ozone treatment. Ozone treatment also triggers the accumulation of H_2O_2, superoxide anion, and hydroxyl radicals that ultimately cause an oxidative burst in cells [96]. Ozone also showed a dramatic increase in the transcript level of OsMSRMK2 gene (MAP kinase) in rice [69]. In *A. thaliana*, MPK3 and MPK6 were activated by ozone exposure [56], and plants lacking these kinases became hypersensitive to ozone [97]. Similarly, NtMPK4-silenced tobacco plants showed enhanced sensitivity to ozone [78]. In poplar, ozone treatment activated two MAPKs and activation of these MAPKs was dependent on the production of reactive oxygen species, the influx of calcium ions via membrane channels, the activation of an upstream, membrane-localized component, and a cognate MAPK kinase [98]. Recently, a MKP2 was identified as an important regulator for controlling both ozone-induced MPK3 and MPK6, and MKP2 RNAi plants were shown to exhibit hypersensitivity to ozone [99].

5.3.6 Wounding-Induced MAPKs

Wounding in plants is typically caused by physical injury and herbivore or insect attack. When wounded, plants express several sets of defense-related genes that are involved in healing damaged tissues and protecting against pathogen infection and insect attack [100, 101]. These genes are activated through signaling pathways that include various protein kinases. Many plant species demonstrate an increase in MAPK levels after being wounded. First report of the activation of a MAP kinase in response to wounding was published in tobacco [77] and named as WIPK (wound-induced MAP kinase). Bogre *et al.* [51] demonstrated that wounding alfalfa leaves specifically induced the activation of MMK4 (MAPK). AtMPK4 and AtMPK6 are also shown to get rapidly activated by wounding [57]. NtMPK4, a tobacco homologue of AtMPK4, revealed wound-induced activation along with two other wound-responsive tobacco MAPKs, WIPK and SIPK [78]. Molecular characterization of StMPK1 (potato MAPK) revealed its transcriptional upregulation upon wounding [68]. During the past 10 years, several wound-activated MAPKs have been identified in various plant species, for example, rice [69–71], tomato [79], soybean [74], cotton [60, 61], pea [67], and maize [62, 82].

5.3.7
MAPKs in Heavy Metal Signaling

Heavy metal ions are essential in many physiological and developmental processes of plants but higher dose of these heavy metals adversely affects plant growth and development. The presence of enhanced level of heavy metal ions triggers a wide range of cellular responses. In plants, higher amount of copper, cadmium, and mercury ions resulted in the activation of a novel MAPK gene *OsMSRMK2* from japonica-type rice (cv. Nipponbare) [69]. Yeh *et al*. [102] confirmed the activation of a MAPK gene and MBP kinases in rice in response to cadmium stress. Exposure of *Medicago* seedlings to excess copper or cadmium ions resulted in a complex activation pattern of four distinct MAPKs: SIMK, MMK2, MMK3, and SAMK (stress-activated MAPK) [49]. In protoplasts, the *Medicago* MAPKK, SIMKK, only conveyed activation of SIMK and SAMK, but not of MMK2 and MMK3. Furthermore, SIMKK only mediated activation by copper but not by cadmium ions. Gupta *et al*. [103] reported the activation of MAPK activity in response to As(III) treatment indicating role of this important cascade in transducing As(III)-mediated signals. Recently, activation of MAPKs by heavy metals was demonstrated in maize [62, 82]. These data show that MAPK cascades are involved in signaling activated by different heavy metals.

5.4
Crosstalk between Plant MAP Kinases in Abiotic Stress Signaling

The term "crosstalk" is used generally to refer to situations where different signaling pathways share one or more intermediates/components or have some common outputs. Various abiotic stresses result in both general and specific effects on plant growth and development. Based on the presence of general and specific abiotic stress tolerance mechanisms, it is logical to expect plants to have multiple stress perception and signal transduction pathways, which may crosstalk at various steps in the pathways. As discussed above, MAP kinases play a central role in transduction of different types of signals. Perhaps some of the strongest evidence for crosstalk during abiotic stress signaling in plants comes from studies of MAPK cascades. The *Arabidopsis* genome contains approximately 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs that offer scope for crosstalk between different stress signals. MAPKs are involved in developmental, hormonal, biotic, and abiotic stress signaling [3]. Members of MAPK cascades are activated by more than one type of stress (Figure 5.5); for example, AtMPK6 is involved in O_3, H_2O_2, ethylene, ABA, and JA signaling pathways, and also in important developmental processes such as epidermal patterning, and anther and embryo development. The functional interaction of MPK6 has been demonstrated by a wide set of MAP2Ks such as MKK2 [58], MKK3 [104], MKK4, MKK5 [105], and MKK9 [44]. Thus, it suggests that MAPK cascades act as points of convergence in stress signaling.

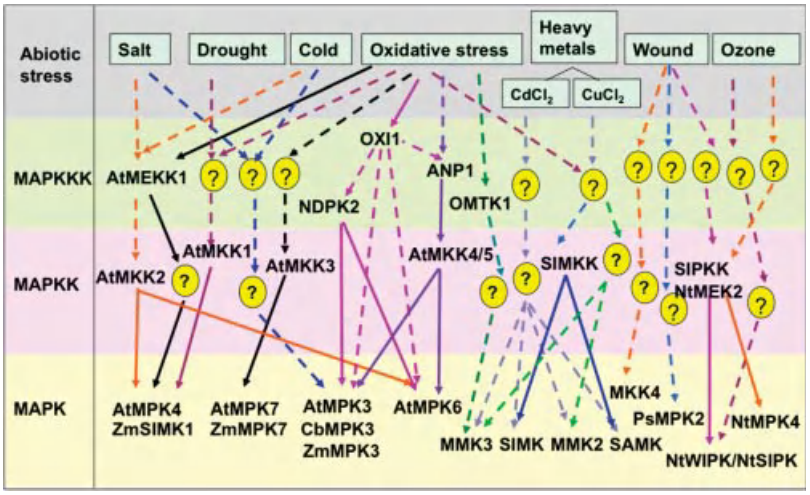

Figure 5.5 Schematic representation of crosstalk among different plant MAP kinase signaling components. The scheme of general signal transduction is shown on the left. The homologues in *Arabidopsis* (At), tobacco (Nt), maize (Zm), pea (Ps), and *C. bungeana* (Cb) are shown. Broken arrows indicate hypothetical pathways; question marks indicate unknown cascade components.

5.5 "-Omics" Analyses of Plants under Abiotic Stress

One purpose of studying abiotic stress responses in plants is to improve the abiotic stress tolerance of crops by means of genetic manipulation. The results of basic research using *Arabidopsis* have been applied to improve stress tolerance in other plant species, including crop plants. These studies have been summarized very well in several recent reviews [106–108]. The complete genome sequence of rice and *Arabidopsis*, and emerging sequence information for several other plant genomes, such as *Populus*, *Medicago*, lotus, tomato, maize, and chickpea, has given rise to the use of tools that can aid in the determination of the function of any genes simultaneously. Thus, an era has arisen in which the data generated can be used as a resource to answer many biological problems, which may thus provide rapid systematic ways to identify genes for crop improvement. Transcriptomics, proteomics, metabolomics, bioinformatics, and high-throughput DNA sequencing have enabled active analysis of regulating networks that control abiotic stress responses. Such analyses have markedly increased our understanding of global plant systematic responses and adaptation to stress conditions.

Transcriptomics is often considered as a step next to genomics in the study of biological systems. The transcriptome is a set of all mRNA molecules or transcripts produced in one organism or cell type under a given set of conditions. It is more complex than genomics, mostly because an organism's genome is rather constant, while a transcriptome differs from cell to cell and constantly changes with

changing environmental conditions. Thus, the transcriptome reflects the genes that are being relatively expressed at any given time, with the exception of mRNA degradation phenomenon such as transcriptional attenuation. Microarray technologies are powerful tools for the global analyses of transcripts. The use of microarrays to study global gene expression profiling in response to abiotic stress in rice was first reported by Kawasaki *et al*. [109]. Global gene expression analysis of rice in response to drought and high-salinity stresses in shoot, flag, and panicle has also been performed using microarrays [110]. In *Arabidopsis*, the AtGenExpress project (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) has collected thousands of transcript profiles on the basis of the Affymetrix ATH1 GeneChip that are now publicly available. This contribution has enabled the discovery of candidate genes on the basis of expression profiles in various tissues, developmental stages, and environmental conditions [111–113]. Transcriptome analysis technologies have advanced to the point where high-throughput DNA sequencers and high-density microarrays such as tiling arrays are readily available [114, 115]. These studies revealed whole-genome transcriptomes of plants exposed to abiotic stresses such as dehydration, cold, heat, high-salinity, and osmotic stresses, as well as ABA treatment [116, 117]. These technologies provide new opportunities to analyze noncoding RNAs and also to clarify aspects of epigenetic regulation of gene expression.

The systematic analysis of the entire protein complement or proteome is referred to as "proteomics." Analysis of the proteome provides a direct link of genome sequence with biological activity [118, 119]. Analysis of the proteome includes knowledge of the entire protein repertoire as well as studies on other aspects, such as expression levels, post-translational modifications, and interactions, to understand the cellular processes at the protein level [120]. The combination of mass spectrometry (MS) with two-dimensional gel electrophoresis (2DGE) in the 1990s proved to be useful for proteome analysis. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and electrospray ionization (ESI) are the two most commonly used MS techniques [121, 122]. Proteome analysis of rice was performed by Salekdeh *et al*. [123] to study changes in response to drought stress. Bae *et al*. [124] used 2DGE and MALDI-TOF-MS to study the *Arabidopsis* nuclear proteome and changes in the nuclear proteome in response to cold stress. Proteome analysis was performed to study the effect of cold stress on rice anthers at the young microspore stage [125]. The rice root proteome was also studied to identify salt stress-responsive proteins [126]. The study of tobacco leaf apoplast proteome in response to salt stress identified 20 proteins whose expression changed in response to stress. These included several well-known stress-associated proteins, together with chitinases, germin-like protein, and lipid transfer proteins [127]. Recently, Popescu *et al*. [128] identified several MKK/MPK/substrate signaling pathways by employing *Arabidopsis* protein microarrays.

Protein–protein interactions are at the core of all cellular processes. Proteins function by interacting with other biomolecules such as other proteins, lipids, nucleic acids, and several low molecular weight compounds. Therefore, a comprehensive knowledge of protein interactions is an important source of information to

understand the cellular processes on a genome-wide level. The collection of all protein interactions in an organism is typically referred to as an interactome [129]. One of the major goals in the post-genomics era is to analyze the complete protein linkage map, that is, interactome of an organism to understand the signaling pathways operative inside the cell that help it in responding and adapting to the fluctuating environmental conditions. The yeast two-hybrid method is the most commonly used technique to study protein–protein interactions. A large-scale yeast two-hybrid analysis was performed by Cooper *et al.* [130] to identify rice proteins involved in stress and development. In 2009, Ding *et al.* [64] generated a rice kinase–protein interaction map and reported a protein interaction map of 116 representative rice kinases and 254 of their interacting partners. Similarly, a directed protein–protein interaction screen between all the *Arabidopsis* MAPKs and their upstream activators MAPKKs was carried out to gain insight into their potential relationships [131]. A large number of bioinformatics tools are available for plant proteome analysis. These include the Proteins of *A. thaliana* Database (PAT) (http://www.pat.sdsc.edu/), MIPS *A. thaliana* Database (MAtDB) (http://mips.gsf.de/proj/thal/db), and Rice Proteome Database (RPD) (http://gene64.dna.affrc.go.jp/RPD/main_en.html).

The metabolome represents the collection of all metabolites in a biological organism, which are the end products of its gene expression. Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of the cell. The word was coined in analogy with transcriptomics and proteomics as like the transcriptome and the proteome, the metabolome is dynamic, that is, changing every second. Gas chromatography–mass spectrometry (GC–MS), gas chromatography–time-of-flight mass spectrometry (GC–TOF-MS), and liquid chromatography–mass spectrometry (LC–MS) are currently the standard mass spectrometry methods for metabolite analyses. The metabolic changes in plants in response to environmental stress factors have been extensively analyzed using several MS technologies and bioinformatics [132]. Integrated metabolome and transcriptome analyses of model plants have markedly increased our understanding of plants' responses to various stresses. Metabolite profiling has been used to characterize stress responses to abiotic stresses such as water deficit (dehydration and high salinity) [133, 134] and extreme temperature (cold and heat) [135–139] for comprehensive analyses of the final steps of stress signal transduction pathways. Thus, metabolomics represents an important addition to the tools currently employed for crop improvement.

Phylogenomics refer to analyses involving genome data and phylogenetics to predict the biological functions of members of large gene families by assessing the similarity among gene products. This approach is especially helpful in studying large gene families because redundancy limits the ability to assess the function of individual genes experimentally. Recently, Jung *et al.* [140] described the application of phylogenomics to elucidate the functions of individual members of the large rice kinase gene family. The authors developed rice kinase database for 1508 rice kinases [141] and also identified the functions of MAPKs, MAP2Ks, and six MAP3K

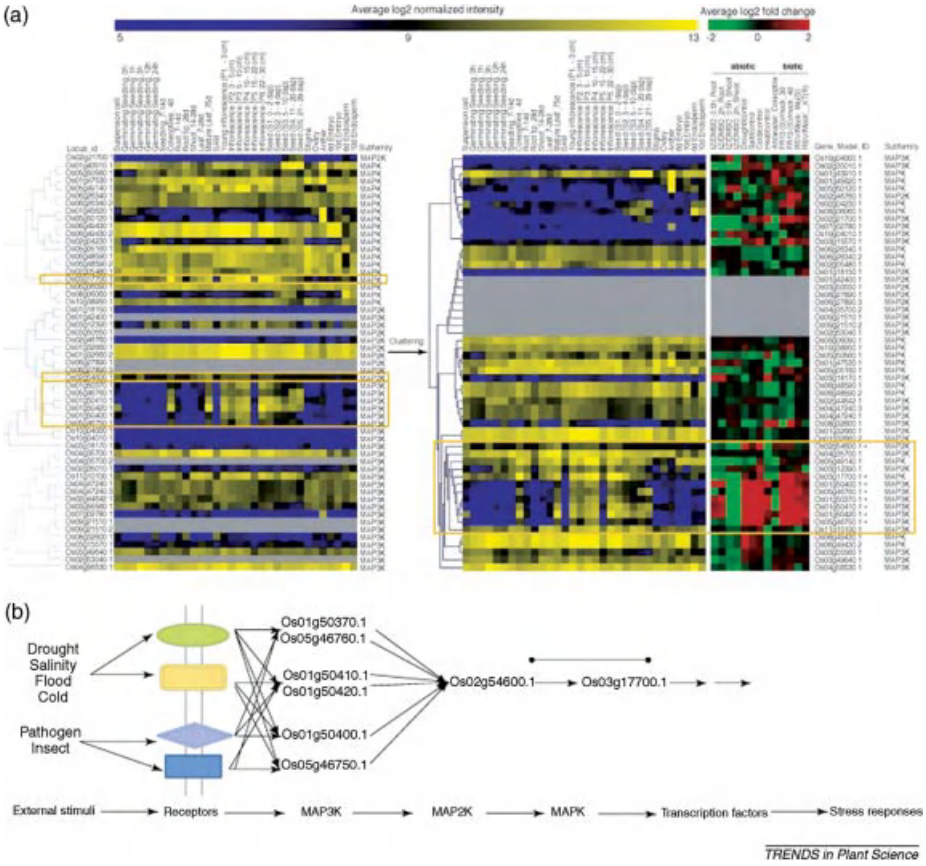

Figure 5.6 Identification of eight MAPK genes predicted to function in the same signaling cascade. (a) The left panel shows phylogenomics data for each MAPK, MAP2K, and MAP3K subfamilies and the right panel shows hierarchical clustering (HCL) analysis of normalized Affymetrix gene expression data for all subfamilies in 32 types of anatomically or development-related tissues or organs. Corresponding Affymetrix differential expression data following exposure to various biotic or abiotic stresses are also shown. Asterisks indicate eight genes grouped by HCL analysis, using normalized expression levels, including one MAPK, one MA2PK, and six MA3PKs. These genes are similarly regulated under various biotic or abiotic stresses. (b) A proposed regulatory model for the six MAPKs, MAP2Ks, and MAP3Ks identified in (a). Biotic or abiotic stresses are perceived by receptors represented by four color-filled boxes. Stress perception is transferred from the receptors to downstream responses via a MAPK cascade. Lines with two dark-filled circles indicate the protein–protein interactions confirmed by yeast two-hybrid screening assays. (Adopted from Ref. [140].)

genes playing important roles in a broad range of stress responses. Based on the expression pattern of all rice MAPKs during development, a cluster was developed. This along with protein–protein interaction data identified a set of MAPK components that appear to function in the same signaling cascade (Figure 5.6).

Integrating the orthologous gene information from other recently sequenced crop plants with rice database will enable the prediction of gene function in these species.

5.6 Conclusions and Future Perspectives

Plants must respond and adapt to abiotic stresses to survive under various environmental conditions. Plant growth and productivity are greatly affected by environmental stresses such as dehydration, temperature, and salinity and thus it is important to understand plants' stress responses to improve crop productivity under stressful conditions. Both forward and reverse genetic approaches have elucidated genes and gene products that are involved in gene expression, signal transduction, and stress tolerance [1]. MAP kinase modules have evolved in eukaryotes as efficient modulators in signaling pathways. MAPK signaling cascade forms complex interconnected networks within cells [142]. Traditional genetic and biochemical methods have identified MKKK/MKK/MPK signaling modules with overlapping roles in controlling cell division, development, hormone signaling and synthesis, and response to abiotic stress and pathogens. For example, for *Arabidopsis*, the MEKK1–MKK1/2–MPK4/6 module was activated by various stress treatments [57, 58] and the YODA–MKK4/5–MPK3/6 cascade was established as a key regulator of stomatal development and patterning [143]. A detailed biochemical analysis of single and multiple MAP kinase mutants will facilitate a better understanding of the role of MAP kinase signaling. Identification of MAP kinase partners, various substrates, and negative regulators of all the components of this cascade will help in understanding the MAP kinase pathway in plants.

During the past decade, comprehensive analyses using functional genomic technologies such as transcriptomics, proteomics, and metabolomics have increased our understanding of the complex regulating networks associated with stress adaptation and tolerance. The global profiling of mutants and wild-type plants at different developmental stages after exposure to stresses is currently generating useful information. Deep sequencing and global transcriptome profiling also increase the resolution with which we can follow metabolic and physiological processes from the perspective of gene expression. Database mining is revealing candidate interaction partners and signaling modules based on coexpression. Such bioinformatic studies will help assign novel functions for differentially expressed kinases at the tissue and developmental levels [144]. Recently, the overexpression of CsNMAPK, encoding a mitogen-activated protein kinase of cucumber, in tobacco has been shown to enhance seed germination as well as stress tolerance under salt and osmotic stresses [145]. The knowledge gained so far about plant MAP kinases has built a strong case for further studies toward careful analysis of the genes involved for elucidating their biological functions using genetic, genomic, and proteomic approaches. Further insights are also expected from three-dimensional structural

studies of individual components of MAP kinase cascade and their scaffold/anchoring proteins.

Although the whole genome sequence is available for a limited number of plant species, such as *Arabidopsis*, rice (*O. sativa*), poplar (*P. trichocarpa*), and grapevine (*V. vinifera*), it is important to decode the gene functions of other crops such as soybean and potato and industrial and medicinal plants such as rubber tree and artemisinin-producing plants. "-Omics" analyses are crucial to understand the whole processes of molecular networks in response to abiotic stress. Thus, a systems biology approach based on construction of mathematical models using large "-omics" data sets should lead to better understanding of hidden networks of molecular elements (genes, transcripts, proteins, and metabolites) in plant life [146, 147], which will eventually be applicable to biotechnology in the future, when plant biotechnology might come to have a more indispensable role in our lives. Recently, it has been shown that MPK2 from *Reaumuria soongorica* (a stress-tolerant woody shrub) is involved in the regulation of the antioxidant defense system in response to stress signaling, which suggests that MAPKs also function as possible mediators of abiotic stresses [148]. In conclusion, we can say that in plants MAP kinase cascades are not linear pathways but complex networks, which are necessary for many fundamental physiological functions such as stress signaling, hormonal responses, cell cycle regulation, and defense mechanisms. This network is also known to be involved in plant innate immunity, conferring resistance to fungal and bacterial pathogens. Overall, MAPK kinase cascades are highly conserved and have emerged as universal signal transduction components that link different receptors/sensors to cellular and nuclear responses.

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6
Plant Growth Promoting Rhizobacteria-Mediated Amelioration of Abiotic and Biotic Stresses for Increasing Crop Productivity

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Abstract

Several microbes with ability to promote plant growth and their products that can stimulate plant growth have been commercialized. This chapter is mainly restricted to the bacteria that are derived from plant rhizosphere and demonstrate their beneficial effects on the root. Such group of bacteria is generally designated as PGPR (plant growth promoting rhizobacteria). Beneficial effects of this group of rhizobacteria on plant growth can be demonstrated by both direct and indirect mechanisms. Various abiotic and biotic stresses negatively influence survival, biomass production, and crop yield. This chapter begins with the description of various abiotic and biotic stress factors affecting plant growth. Tolerance to these stresses is achieved by both physiological and molecular level adaptation. To exert the beneficial effects, bacteria must colonize the root surface efficiently as the roots are the first underground part of the plant system that experiences adverse conditions. Mechanisms of abiotic and biotic stress amelioration in the rhizosphere by PGPRs are subsequently described. PGPRs often have more than one mechanism for enhancing plant growth and experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms of action that may be activated simultaneously. Thus, to fulfill the need of food production for ever-increasing global population under extreme environmental conditions or with limited sources, PGPRs are surely a major target to be developed for the improvement of tolerance to stresses.

6.1
Introduction

Human population is increasing as never before in the human history and is expected to reach 7 billion within 25 years and over 10 billion in the year 2050. As the world population is outstripping food supply, one of the major challenges for twenty-first century will be an environmentally sound and sustainable crop

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production from the available resources that are subjected to limitations such as desertification and salinization of agricultural lands. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most of the major crop plants by more than 50%. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable land by the year 2050 [1]. These stresses cause considerable losses in crop yields worldwide, while the demand for food and energy is on the hike [2]. Thus, one way of addressing food insecurity is to increase food productivity in fields and environment continuously exposed to a myriad of abiotic and biotic stresses such as drought, flooding, heat, radiation, salinity, chilling, and freezing. Easier said than done, improving crop productivity in stressed environments is an awful challenge since biotic stresses are rarely singular. More likely, they affect plant populations as a set of interacting multiple stresses present in a given environment, including the biotic stresses such as disease, pests, and so on. Conventional breeding [3, 4], trait-based approaches [5], and wide crossing [6, 7] have achieved significant impacts. With respect to molecular technologies, marker-assisted selection (MAS) is routinely applied for genetically simple traits indirectly related to abiotic and biotic tolerance [8, 9]. Transgenic and genomic technologies have generated substantial information on the molecular basis of abiotic stress adaptation and are a relatively fast and precise means of achieving improved stress tolerance. Many organisms have evolved traits that endue them to survive in extreme environments, and the gene(s) that confer these properties can potentially be acquainted into higher plants that may soon deliver impacts [10–12].

For several years, microorganisms have been used as a model to characterize the properties of stress-induced genes and their products. There is a long list of studies that demonstrate the beneficial effects of microorganisms, reporting that they promote significant improvement in plant growth and in the yield of several types of crops under stress conditions.

6.2 Factors Affecting Plant Growth

Plants in natural environment are continuously exposed to several biotic and abiotic stresses. These stresses adversely affect plant growth and productivity and are considered a severe threat to sustainable crop production under changing climatic conditions. The global temperatures are predicted to increase by 2.5–4.3 °C by the end of the century with significant effects on agriculture production. Extreme environmental conditions such as prolonged droughts, intense rains and floods, heat waves, and frost are likely to increase in future due to climate change. In 2009–2010, heat waves affected wheat (*Triticum aestivum*) production in Central Asia, while extreme floods affected agricultural production in South Asia [13].

Soil environment, in which the plants are rooted, impose tremendous positive or negative effects on their growth. Soil biota consist of beneficial as well as antagonist

organisms affecting plant growth. Antagonists, as biotic stress, impose negative effect on plant growth. The rapid change in environmental conditions mainly originated from anthropogenic activities causing soil and air pollution, and thus plants are exposed to climatic or edaphic stresses. These abiotic and biotic stresses are important limiting factors in agricultural productivity and how they affect plant growth is discussed here.

6.2.1 Biotic Stress

Biotic stresses caused by living organisms including herbivores (insects, nematodes), pathogens such as virus, bacteria, and fungi, and parasitic weeds create several constraints to crop productivity, which influence the total agricultural production. Herbivory is the prime biotic environmental stress factor for a lot of plants [14]. However, maximum loss of plant productivity is because of viral diseases. Due to vast economic losses caused by biotic stresses, it has become a major concern for agricultural research. The relationship between biotic stress and plant yield affects economic decisions, crop yield, population dynamics, plant–stressor coevolution, and ecosystem nutrient cycling.

6.2.2 Abiotic Stress

Abiotic stresses consist of nonliving factors imposing negative impact on living organisms in a specific environment. The nonliving variables beyond their normal range adversely affect the population performance [15]. Abiotic stresses affect both plants and animals, but being sessile, plants are especially affected by environmental factors, and they are the most harmful factor concerning the growth and productivity of crops worldwide and result in yield losses ranging from 50 to 82%, depending on the crop [16]. Reports have also shown that abiotic stressors are most harmful when they occur in combination and they include heat, chilling, excessive light, drought, waterlogging, wounding, ozone exposure, UV-B irradiation, osmotic shock, and salinity [17].

It has been estimated that only 10% of arable land comes under the nonstress category, which implies that crops grown on the other 90% of arable land experience one or more environmental stresses. Drought, salinity, and high temperature are among the important abiotic stresses that cause severe loss in crop production [18]. Drought stress is a major constraint to yield stability and crop production in drought-prone areas, particularly arid and semi-arid areas. Increasing crop production under drought conditions requires an integrated approach in which infrastructure development, crop and resources management, and plant breeding are essential elements. Soil salinity is another major factor that is an enormous worldwide problem for agriculture especially for crops that are grown under irrigation

because salt inhibits the growth of a large number of crop plants [19]. It is of major concern that about 20–50% of the land devoted to irrigated crops is adversely affected by salinity [20]. In many semi-arid and arid regions of the world, crop yield is limited due to increasing salinity of irrigation water as well as soil salinity. Reduced leaf growth rate due to decreased water uptake restricts photosynthetic capacity under high salinity. Soil salinity also affects total nitrogen uptake and soil nitrogen contribution [21]. High salinity exposure results in imbalance of ion homeostasis, mainly Cl^- and Na^+, and because of excess Na^+, membrane dysfunction and inhibition of metabolic activity have been reported [22]. Salt stress also affects the balance of other ions such as K^+, Ca^{2+}, and NO_3^- and physiological processes such as vascular K^+ circulation, translocation of Ca^{2+} in root and shoot, and transition metal exchange in stress response of the plants.

Heat or high-temperature stress often is defined as high temperatures for sufficient time to cause irreversible damage to plant function or development. High day temperatures have both direct and indirect damaging effects associated with hot tissue temperatures, plant water deficits due to high transpiration, and low plant water potentials [23]. High day temperatures also cause reduction in the rate of photosynthesis and carbon assimilation compared to optimum temperatures because of damage to components of photosystem II [24]. Comparison of contrasting species in response to heat indicates that photosystem II in wheat is more sensitive to heat than rice and pearl millet, which are warm season species adapted to much higher temperatures [24]. It is also expected that with the decrease in the ozone layer, UV exposure will become an important stress for the cropping system [25].

6.3 Plant-Mediated Strategies to Elicit Stresses

Tolerance and yield stability are complex genetic traits of a crop. A crop can face stress in a continuous or an intermittent manner. Plant responses to stresses and its adaptation include both physiological and molecular aspects. Dehydration, salinity, temperature, and other abiotic stresses lead to metabolic toxicity, membrane disorganization and generation of ROS, retardation of photosynthesis, reduced nutrient acquisition, and altered hormone levels. Ubiquitous occurrence of the genetic determinants helps us to further dissect the abiotic stress tolerance. Plant physiological responses involve avoidance, escape, and tolerance by osmotic adjustment and water use efficiency, whereas accumulation of osmoprotectants, production of scavengers, exclusion or compartmentalization of ions, and production of specific enzymes involved in the regulation of plant hormones are some of the molecular mechanisms that plants have evolved for adaptation to abiotic stresses [26]. Salt tolerance of plants depends primarily on three characteristics: physical uptake, transport and compartmentalization, and physiological and metabolic events that counteract the presence of salt at the cellular level, which depends on sensitivity/tolerance level of the plant [27]. Salt-sensitive plants restrict the uptake of salt and synthesize compatible solutes (e.g., proline, glycine betaine, and soluble sugars) to adjust their osmotic pressure [28], while salt-tolerant

plants sequester and accumulate salt into the cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells [29]. Drought tolerance in plants is mostly mediated by avoidance through morphological adaptations and tolerance by low water use efficiency and osmoregulation [30]. Being a multigenic as well as a quantitative trait, it is a challenge to understand the molecular basis of abiotic stress tolerance as compared to biotic stresses. These characteristics suggest that crops with inbuilt capacity to withstand abiotic stresses would help to stabilize the crop production. This is well explained by the response of a photosynthetic apparatus of homochlorophyllous desiccation-tolerant and poikilochlorophyllous desiccation-tolerant plants in response to drought [31]. Knowledge of stress-induced functional changes has added one step ahead in the development of methods to isolate genes involved in the metabolic pathways or their associated physiology for the development of stress-tolerant transgenic crops [32].

6.3.1 Osmoadaptation

Osmotic adjustment is the net increase in intercellular solutes in response to water stress, which allows turgor maintenance at lower water potential [33]. Accumulation of nitrogenous compounds such as amino acids, amides, imino acids, proteins, quaternary ammonium compounds, and polyamines in response to abiotic stresses has been reported frequently [30]. Proline has been proposed to act as a compatible osmolyte and its role as a ROS scavenger and chaperone and in the maintenance of cytosolic pH and regulation of redox potential has been reported [34]. Up to 80% proline accumulation of the total amino acids by inhibition of proline degradation under stress conditions has been observed. Although the regulation and function of proline accumulation are not yet completely understood, engineering of proline metabolism could open new avenues to improve plant tolerance under environmental stresses [35].

6.3.2 Antioxidative Enzyme Production

Exposure of plants to stresses such as temperature extremes, heavy metals, drought, or salt stress can increase the production of ROS, for example, $^1O_2$, O_2^-, H_2O_2, and OH^-; to protect themselves against these toxic oxygen intermediates, plant cells employ antioxidant defense systems [36]. Detoxification of excess ROS in plant, induction of antioxidant enzymes, and accumulation of antioxidants such as ascorbic acid and glutathione for the alleviation of salinity stress have been studied [37].

6.3.3 Effect of Stress on Plant Nutrient Uptake

Stress affects nutrient availability to plants, which is governed by root and shoot. The overall response of plants to nutrient uptake is determined by stress and the

ability of the root system to adjust nutrient acquisition capacity to meet variations in shoot demand caused by environmental stresses. This further depends on the soil nutrition conditions, exogenous addition of which alleviates the adverse effects of stress. Nitrogen and phosphorous are the major nutrient components playing important role in stress adaptation [38].

6.4
Plant Growth Promoting Rhizobacteria-Mediated Beneficiaries to the Environment

Plant roots are surrounded by a thin layer of soil known as rhizosphere that serves as an active area for root activity and metabolism. It is a narrow zone of soil whose physical, chemical, and biological properties change due to root growth and activity. Among the microbes surrounding the rhizosphere, bacteria are the most abundant. Plant growth promoting rhizobacteria (PGPR) having positive correlation with a plant root influence the plant growth from direct mechanisms to indirect effects. Advancement in the concept of rhizosphere as an ecosystem has gained importance in functioning of the biosphere. Microorganisms could play a significant role in this respect, if we can exploit their unique properties of tolerance to extremities, their ubiquity, their genetic diversity, and their interaction with crop plants, and develop methods for their successful deployment in agriculture production. Use of these microorganisms can alleviate stresses in crop plants, thus opening a new and emerging application in agriculture [39]. These microbes also provide excellent models for understanding the stress tolerance, adaptation, and response mechanisms that can be subsequently engineered into crop plants to cope with stresses induced by climate change.

6.4.1
PGPR as Abiotic Stress Ameliorating Agent

PGPRs help in imparting tolerance to the plants against various abiotic stresses. Drought stress limits the growth and productivity of crops, particularly in arid and semi-arid areas. The mechanisms involved are expression of drought-responsive genes and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Under stress conditions, including drought, the plant hormone ethylene endogenously regulates plant homeostasis and results in reduced root and shoot growth. However, degradation of the ethylene precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth [40]. The ethylene produced under high salt stress is reduced by ACC deaminase activity of bacteria. Interestingly, some of the volatile organic compounds (VOCs) that are emitted from *Bacillus subtilis* GB03 are bacterial determinants involved in tolerance against stress conditions [41]. The introduced PGPRs in the rhizosphere also enhance soil aggregation by production of exopolysaccharide (EPS), thereby improving the water availability to plants, and form biofilms in the rhizosphere that protect plants from surrounding harsh environments [42]. They induce the synthesis of heat shock

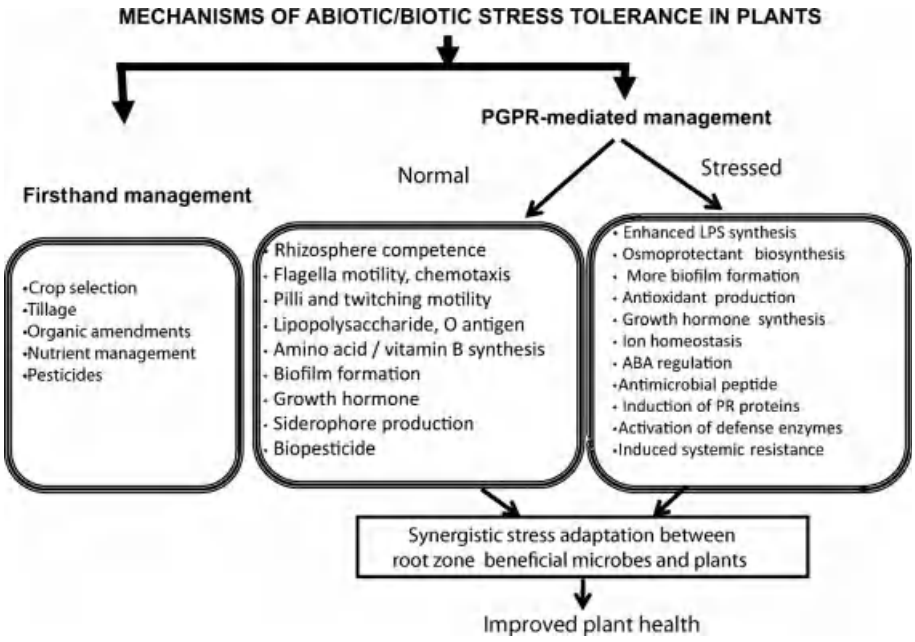

Figure 6.1 Simplified overview of the mechanisms involved in plants in response to abiotic and biotic stresses. (From author's laboratory.)

proteins and osmoprotectants such as proline and glycine betaine and help in maintaining cell membrane integrity, all of which contribute to improved stress tolerance in plants. Proline synthesis has been shown to be increased in abiotically stressed plants in the presence of beneficial bacteria such as *Burkholderia* [43], as well as *Arthrobacter* and *Bacillus* [44]. Bacterial inoculation alters the selectivity for Na^+, K^+, and Ca^{2+}, resulting in higher K^+/Na^+ ratios, and helps in maintaining the ion homeostasis (Figure 6.1). Khan *et al.* [45] reported the multifarious role of *Paenibacillus lentimorbus* B-30488r as a potent plant growth promoting and bioremediation agent useful in Cr-contaminated rhizosphere soil by enhanced biofilm formation using sodium alginate and $CaCl_2$. Biofilm acts as a protective covering around the root and protects the plant from a major metal pollutant, Cr.

6.4.2
PGPR Action against Multiple Pathogens

New species of bacteria may provide potentially new biological control agents with novel mechanisms of disease suppression active in a range of environments. The rich diversity of the microbial world from stressed environments provides a seemingly endless resource for disease suppression [46]. These beneficial microbes can be exploited as biological pesticides either alone or in combination with chemicals to lower the doses of chemicals needed to obtain a profitable crop yield. DasGupta *et al.* [47] reported three plant growth promoting

strains of *Bacillus*, for example, *B. lentimorbus* B-30486 (B-30486), *B. subtilis* B-30487 (B-30487), and *B. lentimorbus* B-30488 (B-30488), which were isolated from the milk of Sahiwal cows.

PGPR-induced systemic resistance (ISR) is a widespread phenomenon of plants against pathogens by using salicylic acid (SA)-dependent signaling pathway in response to infection [48, 49]. PGPR-induced systemic protection against tomato late blight under *in vitro* conditions has been reported by Park *et al.* [50]. Compared to systemic acquired response (SAR), induced by the pathogen, beneficial PGPRs induce ISR in a jasmonic acid-dependent manner.

The introduction and augmentation of microbial antagonist to seeds and other plant-associated bacteria are common approaches to biocontrol of plant diseases. In particular, there has been much success in obtaining biocontrol of pathogens using seed bacterization [51]. These bacteria are mostly *Pseudomonas fluorescens* [52], *P. putida*, and *Bacillus* sp. [53, 54]. Single bacterium may have resulted in inadequate colonization, limited tolerance to changes in environmental conditions, and fluctuations in the production of antifungal metabolites. Several approaches have been used to overcome these problems, including combined application of two or more biocontrol strains to enhance the level and consistency in disease control. Multiple strain mixture of microbial agents has been employed with some success against plant pathogens including mixtures of fungi, bacteria, and bacteria and fungi [55]. Enhancing biocontrol activity by using mixtures of antagonists may have the following advantages: (i) it may broaden the spectrum of activity, (ii) it may enhance the efficacy and reliability of the biocontrol, and more importantly (iii) it may allow the combination of various traits without employment of genetic engineering [56] (Figure 6.1). Moreover, the designing of strain combinations and making use of multiple antifungal traits exhibited by them may prove to be advantageous by ensuring that at least one of the biocontrol mechanisms will be functional under the unpredictable field conditions faced by the released PGPR strains [57]. Several potent strains from different species of *Bacillus* have been tested on a wide variety of plant species for their ability to control several diseases. There are a number of reports on the potential of *B. subtilis* as a biological control agent against fungal pathogens [58, 59]. *Bacillus* species produce 167 biological compounds active against bacteria, fungi, protozoa, and viruses [60]. *P. fluorescens* CHA0 produces antibiotics using GacS/GacA system for the biological control of root pathogenic fungi [61, 62].

6.4.3
Determinants of PGPR Colonization in Stressed Environment

Soil microorganisms are getting affected by a number of biotic and abiotic factors affecting the plants. The occurrence and activity of soil microorganisms are affected by a variety of environmental factors faced by the plant. In response to stress acclimation, plants undergo a number of metabolic and physiological changes that result in changed root exudation. Changes in the root exudation

due to metabolic changes in the plants also result in the change in microbial flora. Different sugars, amino acids, and organic acids exuded by the plant serve as nutritional supplements to the root zone bacteria. Plant roots release an enormous amount of root exudates that may represent up to 10–20% of the photosynthethates, leading to a significant stimulation of the microbial density and activity. Plant root exudates are a complex mixture of chemicals and organic compounds secreted into the complex environment drive underground interactions [63]. Microbes entering the rhizospheric environment utilize root exudates' major components as nutrients and minor components, non-nutrients, as signals to guide their movement toward the root surface and elicit changes in gene expression appropriate to the complex beneficial associations and maintain themselves in a competitive manner on the root system [64]. The exact composition of the exudates is determined by many factors, including species and nutritional status of the plant, soil structure, micronutrient status, and plant developmental stage. Colonizing microbes stimulate exudation from plant root and promote the bacterial populations from 3- to 100-fold [65]. Depending on the exact nature of the compound in the root exudates, they may play a role in activation of microbial genes responsible for recognition and initiation of symbiotic association, act as an antimicrobial plant defense agent, activate or disrupt key microbial genes responsible for biofilm formation, or simply act as an easy source of moisture, nutrients, and energy. Mucilage addition to the soil itself increased the microbial C up to 23% and the number of cultivable bacteria by 450%, which affect both the metabolic and genetic structures of the bacterial community [66].

Utilization of various sources by the microbial community and modification of rhizobacterial populations by engineered bacterium for the utilization of opines was achieved by Oger *et al*. [67] and Savka and Farrand [68] based on the concept given by Nautiyal and Dion [69]. Nautiyal and Dion [69] were the first to report catabolization of opines by the pseudomonads, as carbon and nitrogen sources. Effect of nutritional biasing on rhizobacterial colonization was studied by using mannopine (MOP) as a novel substrate (US Patent No. 5,610,044; Figure 6.2). These findings attributed to the fact that opines are excellent substrates for various soil microorganisms outside the genus *Agrobacterium* and underline how opine exudation brought rhizosphere perturbation [69, 70].

Auxin, a hormone responsible for root architecture, is produced by the root zone bacteria by using tryptophan as a precursor from the root exudates of a plant. Auxin plays an intermediary role between the action of a stressor and the realization of response phenotype. Several mechanisms have been proposed to explain stress-induced changes in auxin metabolism and/or receptiveness; however, evidence for stress-induced changes in auxin transport and catabolism is predominantly found in the literature [71].

These root zone bacteria also exudate osmolytes such as glutamate, proline, glycine betaine, and trehalose in response to stresses, which along with other PGP attributes can possibly act synergistically with plant-produced glycine

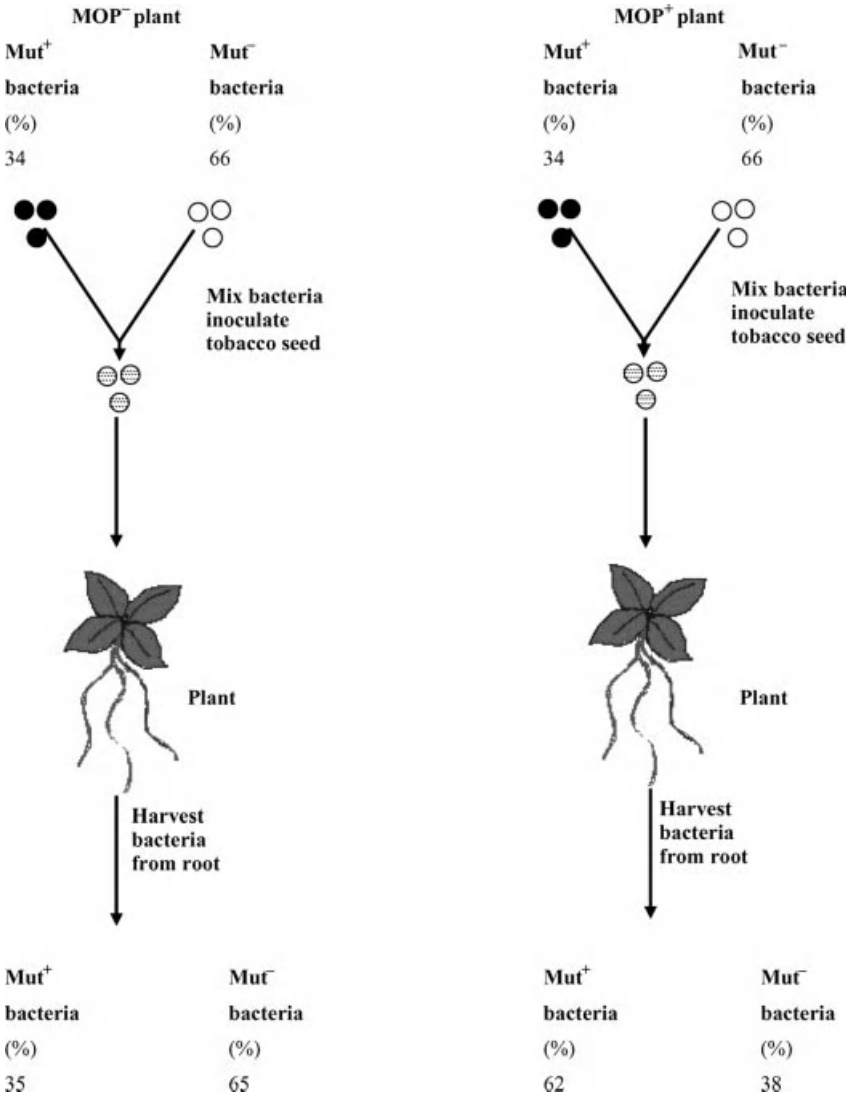

Figure 6.2 Competition between mannopine-utilizing bacteria and its non-utilizing mutant showing effect of nutritional biasing on rhizobacterial colonization. (From author's US Patent No. 5,610,044.)

betaine in response to the stress and stimulate the plant growth even under stressed conditions by abiotic stress-tolerant PGPRs [72, 73]. The long-term goal of improving plant–microbe interactions for salinity-affected fields and crop productivity can be met with a mechanism of osmoadaptation. Proline plays a major role in osmoadaptation through increase in osmotic stress that shifts the dominant osmolyte from glutamate to proline in *Azospirillum* sp.

Under abiotic stress conditions, increased proline biosynthesis was observed for various plant species inoculated with different PGPRs [74, 75]. *P. fluorescens* can survive under dry conditions and hyperosmolarity; the gene *algU* is a crucial determinant of its adaptation. Recent studies by Sandhya *et al.* [76] and Heidari *et al.* [77] report the potential of PGPR strains in alleviating drought stress effects in maize and basil.

Salinity decreased P accumulation in plants because of ionic imbalance effects, which affect both the sorption and solubilization of the insoluble phosphate, and plants face P-deficiency symptoms. Phosphate solubilizing bacteria, able to solubilize insoluble phosphate under abiotically stressed conditions, offer a scope for amelioration of abiotic stresses such as alkalinity of soil [76, 78]. These P solubilizing strains solubilize insoluble phosphates and improve plant growth even under water-deficit conditions [79]. Production of microbial metabolites during P solubilization results in a decrease in soil pH, which probably plays an important role in the solubilization and enhances the plant growth significantly, and this plant growth promoting capability can be used for the rapid revegetation of barren alkaline land. The soil microbes in the rhizosphere of plants growing on trace metal-contaminated soils play an important role in phytoremediation also.

6.4.4
PGPR-Mediated Induction of Defense Mechanism

When plants are invaded by microorganisms or damaged by mechanical injuries, major physiological changes are induced and plant defense enzymes are generally activated. In spite of major advances in our understanding of the plant defense response, little information is available on PGPR-mediated induced enzymes [80]. Nautiyal *et al.* [81] has compared the changes in antioxidant potential and phenolics in vegetable systems even after minimal processing induced by either PGPR-treated fresh or processed vegetables and fruits. *B. lentimorbus* B-30488 (B-30488) induces antioxidant potential in functional food and the PGPR strain B-30488 has the potential to induce antioxidant enzyme activities of polyphenol oxidase (PPO), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD). The antioxidant potential of the hydroalcoholic extracts in *in vitro*1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide scavenging, photochemiluminescence, percent inhibition of lipid peroxidation, and prevention of oxidation of reduced glutathione (GSH) in B-30488-treated fresh or processed vegetables and fruits demonstrates the usefulness of bioinoculant for vegetables as well as fruits. Liang *et al.* [82] demonstrated the effects of two PGPR strains, *Pseudomonas* strains 13 and 63-28, which significantly induced plant defense enzymes both locally and systemically. PGPR consortia enhanced combinations of plant defense mechanisms such as peroxidase enzymes, lignification, superoxide dismutase, or phenolic compounds more than those treated with individual PGPR strains [83].

Co-inoculation of lettuce (*Lactuca sativa* L.) with PGPR *Pseudomonas mendocina* and arbuscular mycorrhizal fungi (*Glomus intraradices* or *G. mosseae*) augmented an antioxidant catalase under severe drought conditions, suggesting that they can be used in inoculants to alleviate the oxidative damage elicited by drought [84].

6.4.5 Modulation of Plant Genes through Bacterial Intervention

There are several PGPR inoculants that seem to promote growth through one or more mechanisms, suppression of plant disease (biocontrol agents), improved nutrient acquisition (biofertilizers), or phytohormone production (biostimulants). Use of *Rhizobium* sp. and its co-inoculation with *Pseudomonas* improves growth and nodulation because of the production of phytohormones such as auxins, gibberellins, and cytokinins by the inoculant and nutrient uptake [85, 86].

Inoculation of plants by a root zone bacterium primes the physiological status of a plant through ISR and accumulation of signaling molecules against different abiotic and biotic stresses [87]. Accumulation of salicylic acid and/or jasmonic acid induces pathogenesis-related (PR) proteins and triggers the systemic acquired response and activation of a family of defense-related genes encoding proteins for resistance against a variety of pathogens. The plant–PGPR interaction plays an important role in mitigating the adverse effects of ethylene overproduction and increases the plant biomass [87]. Overproduction of ethylene due to adverse environmental conditions results in the inhibition of plant growth or death. A PGPR containing ACC deaminase activity can hydrolyze ACC, the immediate precursor of ethylene, to α-ketobutyrate and ammonia, and in this way promote plant growth. ACC deaminase producing PGPR may assist plant growth by alleviating deleterious effects of overproduced ethylene [88–90]. Application of *Achromobacter piechaudii* on tomato seedlings exposed to high salt was found to reduce the ethylene content by its ACC deaminase activity and increased the growth of tomato seedlings by as much as 66% in the presence of high salt. Induced systemic tolerance (IST) to salt stress was also noted in a new study with *Arabidopsis* [91]. Bacterial volatiles also play a regulatory role in stress adaptation by regulating the expression of HKT1, a gene responsible for inward movement of Na^+ in *Arabidopsis thaliana* [92]. *Bacillus* sp. TW4-mediated ethylene metabolism in pepper has also been reported to relieve the osmotic stress [44].

Changes in gene expression in *A. thaliana*, inoculated with *Paenibacillus polymyxa* followed by the provision of abiotic and biotic stress, support the induction of ERD15, a drought-responsive gene involved in plant response to biotic and abiotic stresses [93]. Verhagen *et al.* [94] determined changes in gene expression of *Arabidopsis* plants grown in the presence of strain WCS417, using a microarray representing about 8000 genes. Substantial changes were found in the expression of genes involved in cell rescue and defense, metabolism, transcription, cellular communication, and ethylene signaling. Wang *et al.* [95] further substantiated the work

by using 22K gene chip and performed the microarray analysis using a biocontrol agent *P. fluorescens*. Srivastava *et al*. [96] have reported the gene activation in *A. thaliana* leaves by an abiotic stress-tolerant phosphate solubilizing strain of *P. putida*. Plant growth promoting *P. putida* strain was able to enhance plant growth in *A. thaliana* by inducing ISR, hormone biosynthesis, increased nutrient uptake and amino acid biosynthesis, mitosis and meiotic division, and ethylene signaling-related genes. Barriuso *et al*. [97] demonstrated the overexpression of PR1 gene by the inoculation of ISR inducing *Arthrobacter oxydans* strain in a defense response. Defense genes involved in pathogen response were reported to be overexpressed by the priming of bacterial strain BS107 onto pepper roots by Yang *et al*. [62], proving the ISR mechanism.

6.5
PGPR-Based Practical Approaches to Stress Tolerance

6.5.1
Development and Commercialization of PGPRs: Approaches and Limitations

The large-scale application of PGPRs to crops as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers and pesticides, which often pollute the environment. The growing cost of pesticides, particularly in less affluent regions of the world, and consumer demand for pesticide-free food have led to a search for substitutes for chemical inputs. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent [98]. Biological control is thus being considered as an alternative or a supplemental way of decreasing the use of chemicals in agriculture, but their inconsistent performance under field conditions has hindered widespread commercialization. To overcome these problems, it is therefore important to understand the ultimate fate of bacteria released in a stressful environment, such as natural soil, and to fully comprehend the mechanisms involved in plant protection. Commercialization of PGPRs mainly proceeded with *Bacillus* spp. rather than pseudomonads as earlier attempts to commercialize products containing fluorescent pseudomonad strains of PGPRs generally failed due to lack of long-term viability of these asporogenous bacteria. *Bacillus* offers ecological advantages over these because it produces endospores that are tolerant to extreme environmental conditions such as heat and desiccation. Survival of introduced rhizobacteria in any given microbial community is an important factor in determining the degree of plant stimulation because competition for limited resources is crucial, and bacteria are also susceptible to environmental stressors; the most prominent beneficial effects of inoculation with a potential PGPR are to be expected in poor soils when the development of the indigenous microbial community is inhibited [99, 100]. The use of PGPR offers an attractive way to replace chemical fertilizers, pesticides, and supplements.

In general, though PGPRs perform well and promote plant growth under normal conditions, they are highly sensitive to the fluctuations in environmental conditions and are inconsistent in their performance. The inconsistent performance is due to poor shelf life, lack of multidisciplinary approach, technology constraints and optimization of fermentation technology, and mass production of PGPR strains. The consistency of PGPRs could be enhanced through several means with and without going in for genetic engineering approaches.

6.5.2 Implications of Bacterial Genes for Transgenic Development

As per estimates recorded in 2002, transgenic crops are cultivated worldwide on about 148 million acres (587 million hectares) of land by about 5.5 million farmers. Transgenic plants have many beneficial traits such as insect resistance, herbicide tolerance, delayed fruit ripening, improved oil quality, weed control, and so on. Herbicide-resistant crops (HRCs) and insect-resistant crops (Bt crops) accounted, respectively, for 59 and 15% of the total global area of all transgenic crops in 2000 [101]. For both herbicide- and insect-resistant crops, bacterial genes glyphosate oxidase, from *Ochrobactrum anthropi*, that converts glyphosate to glyoxylate and aminomethylphosphonic acid and cry toxins from *Bacillus thuringiensis* that form crystal protein have been used successfully for transgenic development. Isopentenyl transferase from *Agrobacterium tumefaciens* was another example found to reduce tobacco hornworm [102].

Trehalose synthesis under stressed conditions in bacteria is one of the mechanisms of stress adaptation. Trehalose is produced by the action of trehalose phosphate synthase and trehalose phosphate phosphatase that degrades trehalose-6-phosphate into trehalose and both of these genes in combination have been used for transgenic development in rice against abiotic stress adaptation [103]. Other than trehalose, glutamate dehydrogenases from an algae and *gutD* from *E. coli* have been overexpressed and used for transgenic development [104]. The choline oxidase gene from *Arthrobacter* sp. was also used to produce transgenic rice with high levels of glycine betaine giving tolerance against water-deficit stress. The *E. coli betA* encodes CDH while *betB* encodes BADH. Transgenic tobacco plants producing both CDH and BADH were developed by crossing *betA*- and *betB*-producing plants and selecting for double transgenic lines. Transgenic tobacco expressing both *betA* and *betB* produced a considerable amount of betaine (66 ± 18 nmol/g of fresh weight) and was able to grow in the presence of 200 mM NaCl [105]. Bacterial chaperones have been shown as a promising means for transgenic development against abiotic stress tolerance in plants [106]. *popA* gene from the phytopathogenic bacterium *Ralstonia solanacearum* and *entC* and *pmsB* bacterial genes were used to develop transgenic tobacco plants resistant to the virulent fungus *Phytophthora parasitica* and *Oidium lycopersicum* [107, 108].

6.6 Conclusions

Any strategy to address food insecurity in the arid or semi-arid regions, which are highly prone to abiotic stresses, must tackle the climatic conditions and conditions prevailing in the soil. While control of climatic factors is beyond direct or immediate human interference, local soil edaphic factors such as salinity, alkalinity, and drought are easier to manipulate to overcome stress temporarily and enhance crop productivity. PGPRs play an important role in such short-term soil manipulations, subsequently leading to improved soil properties on repeated use by production of organic acids, hormones, and so on, increased root biomass and rhizosphere effect, and induced stress tolerance in plants, to name a few. The molecular genetic basis of stress tolerance in PGPRs is being identified and harnessed to develop stress-tolerant plants and finally lead to translation of abiotic stress tolerance to the farmer's field. Thus, to meet human needs, productivity in extreme environments, PGPRs are surely a major target to be developed for the improvement of tolerance to abiotic stresses, including extremes in water availability, temperature, and soil contamination by salts and heavy metals. PGPR are bioresources having novel and potential tool for providing substantial benefits to the agriculture exposed to stresses. Application of PGPRs is gaining worldwide importance and acceptance and appears to be the trend for the future.

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7
Are Viruses Always Villains? The Roles Plant Viruses May Play in Improving Plant Responses to Stress

Stephen J. Wylie and Michael G.K. Jones

Abstract

Viruses have traditionally been seen as pathogens of plants and animals. Recent research has shown that most viruses induce no pathology in natural plant hosts and in some cases they may be of benefit in reducing damage from both biotic and abiotic stressors. New technologies are revealing that viruses are far more abundant and diverse than previously known, and unexpected roles as symbionts and as sources of genetic raw material for evolution are informing a new appreciation of the roles plant viruses play in nature.

7.1
Introduction

Approximately 2000 virus species are recognized by the International Committee on the Taxonomy of Viruses (ICTV), and many are the stuff of nightmares. The influenza virus, for example, appears year after year, and scientists are locked into an annual battle to develop new vaccines against new strains. We are constantly reminded that this common human virus has the potential to mutate and quickly kill millions in devastating pandemics, as it did in 1918 [1]. Ebola virus causes death in 68% of human infections from multiple organ dysfunction syndrome [2]. Rinderpest devastated human civilizations for millennia by cutting swathes through their cattle and buffalo herds where they were domesticated. It is likely that lethal viruses such as rinderpest were responsible for some of the 10 great plagues of Egypt described in the Hebrew Bible [3].

In domesticated crop plants, viral epidemics have probably occurred since plants were first domesticated 10 000 years ago. They still occur today, and are particularly serious in places where subsistence farming is widely practiced, such as in parts of sub-Saharan Africa. For example, the important subsistence species cassava (*Manihot esculenta*) is an essential source of dietary carbohydrate. The plant is ideally suited to the region as it is able to yield a crop even on very marginal soils and under drought conditions. It is susceptible to a number of begomoviruses (family

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Geminiviridae) collectively known as cassava mosaic viruses. Cassava mosaic disease (CMD) was recorded for the first time about 100 years ago but it remained benign until the 1990s when a recombinant hybrid of two begomoviruses was associated with a severe and rapidly spreading epidemic throughout East and Central Africa [4]. The epidemic affected most of Uganda causing losses of US$ 60 million each year between 1992 and 1997 [5]. Farmers abandoned growing cassava in large parts of the country, and in eastern districts widespread food shortages led to famine-related deaths [6].

7.2
Viruses Are Abundant and Diverse

Today we are beginning to recognize viruses as the most abundant life forms on Earth, and for holding the greatest genetic diversity. It is this great diversity that makes viruses such a successful group. Although viruses are the simplest of life's many forms, their genomes are highly plastic, enabling them to ride the evolutionary wave where it takes them. They colonize life on every continent and some viruses even parasitize other viruses [7]. As new technologies have become available to study viral abundance and diversity, some astonishing figures have emerged. In seawater, for example, viruses are enormously abundant. Estimates range from 3 million virus particles per milliliter in the deep ocean to 100 million virus particles per milliliter in coastal waters. Assuming the volume of the oceans is 1.3×10^{21} liters and the average abundance of viruses is 3×10^{9} per liter, then ocean waters contain $\sim 4 \times 10^{30}$ viruses. Because a marine virus is about 100 nm long, if they were stretched end on end they would span approximately 10 million light years [8]. The roles these viruses play in marine ecosystems are largely unknown but are likely to be highly significant.

In plant virology, the picture is less clear, but early indications are that there too viruses are considerably more abundant in plants than previously recognized. Approximately 900 plant viruses are recognized by the ICTV, in addition to a large number that have been described but are not assigned taxa. In a metagenomic study of randomly selected rainforest plants in Costa Rica, it was found that thousands of previously unknown viruses inhabit them [9]. Interestingly, none of the infected plants showed visible symptoms commonly associated with infection by viruses. In a similar metagenomic study of natural tallgrass prairie in Oklahoma, 25% of plants randomly sampled contained virus sequences, but only 2.3% had any visible symptoms of disease [10]. So far these types of studies in plants are preliminary, but they promise to reveal a diversity of plant viruses that is well beyond our current understanding.

7.3
Wild Versus Domesticated

Most of the plant viruses currently described are pathogens of cultivated plants, yet in natural ecosystems viruses appear to be abundant but induce few or no

damaging symptoms of virus infection. What could be the reason for these apparently divergent reactions? Do symptoms occur more widely in wild plants than are observed? Is the prevalence of symptom-inducing viruses an artifact inadvertently created by plant pathologists whose skills lie in detecting disease and disease-causing viruses in domesticated plants? Whatever the case, the question remains, why are domesticated plants often so severely affected by pathogenic viruses, when their wild cousins are not?

Fields of crops differ in crucial ways from surrounding wild plant communities. Edible domesticated plants have been bred for greatly enhanced food value, and are thereby favored by arthropod vectors of plant viruses. Modern crops are usually genetically uniform and are planted in dense stands, enabling ease of harvest. This means that viruses that successfully invade one individual are able to rapidly spread through the crop. The wild progenitors of most crop species evolved in lands far distant from where they are grown today. This means that crops have only recently become exposed to viruses that evolved in the areas they currently grow. Recent associations between a plant and a virus it has never "seen" before are much more likely to induce severe symptoms than an ancient association [11]. None of these factors apply to wild plants living in natural systems. Wild plants have multiple defense mechanisms against the organisms that feed on them. There is wide genetic variation between individuals and plants may grow widely apart from one another. Possibly the most important factor is that the viral associations they have may be of a long-standing nature.

7.4
New Encounters

Judging from the wide diversity of plant viruses currently known, plants and viruses have undoubtedly coevolved over millennia. The place where a virus or its host evolves is its center of biodiversity. There, its members have the greatest genetic diversity. An example of this was reported from wild leguminous plants indigenous to a small region in the southwestern tip of Australia. Endemic *Hardenbergia comptoniana* plants from there were often infected with a potyvirus named *Hardenbergia mosaic virus* (HarMV). There is a considerable variation in morphology of *H. comptoniana* plants, indicating wide genetic diversity in the host, and plants responded to infection by the virus in widely different ways – from asymptomatic to severe reactions (in a small number of cases). When over 30 isolates of the virus were collected from across its natural range, the viral coat protein genetic diversity was very high, up to 24%. Such high variation is typical of an organism that evolved in an isolated area or host over an extended period of time [12]. When this virus spread naturally from its natural host to a recently introduced leguminous crop plant, narrow-leafed lupin (*Lupinus angustifolius*), the new host reacted with severe deformation and flower abortion. In some cases, the lupin plants died [13].

It is probable that scenarios resembling this one are the initial events that eventually lead to viral epidemics. The trigger for epidemics may be the physical location of a crop species close to original vegetation and/or the introduction of a new vector that is capable of transmitting certain types of viruses. An important example is the spectacular emergence of the begomoviruses over the past 20 years or so. This group of viruses and their aggressive partner, the whitefly *Bemisia tabaci*, have piggybacked on transportation and agricultural production systems to explode from their centers of origin to became serious threats to primary production in tropical and subtropical areas and in glasshouses the world over [14]. The role of humans in this process is obvious, and as the climate warms, new opportunities for viruses and their vectors will undoubtedly arise.

Not every domesticated plant/virus association results in deformation or death. There are a number of "latent" or "symptomless" viruses known that do not induce symptoms in their hosts.

7.5
Roles for Viruses in Adaptation and Evolution

If most virus relationships with wild plants generally do not induce disease, do they have an ecological role? Do virus infections benefit plants in any way? In some cases, they do. Some plants infected with a mild strain of a virus are immune to much more damaging strains of the same virus. Indeed, in regions where citrus tristeza virus is a severe pathogen, growers deliberately infected trees with a mild strain of the virus to protect their crops from serious damage [15].

Another means by which virus infection may benefit a host plant is through the process of "spillover," a competitive strategy resembling biological warfare. This is recorded in wild oats (*Avena fatua*) infected by the generalist virus (one with a wide host range) *Barley yellow dwarf virus* (BYDV). Wild oats display mild symptoms when infected with BYDV, and but other grasses growing in the same plant communities become severely stunted after infection. Wild oats gain a competitive advantage when they act as a reservoir for BYDV, which is spread by vectors to nearby susceptible grasses, thereby limiting competition. BYDV spillover from *A. fatua* decreased the abundance of two other host grass species through pathogen-mediated competition [16].

Under laboratory conditions, some plants infected with viruses are able to withstand longer periods without water than uninfected plants. Xu *et al.* [17] showed that *Nicotiana benthamiana* plants infected with tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), brome mosaic virus (BMV), or tobacco rattle virus (TRV) survived longer under drought conditions than uninfected plants. The same was true for rice infected with BMV, for *Nicotiana tabacum* (tobacco) plants infected with TMV, and for beet, cucumber, pepper, watermelon, squash, tomato, *Chenopodium amaranticolor*, and *Solanum habrochaites* plants infected with CMV. In addition, beet infected with CMV survived cold treatments that killed uninfected plants.

The precise mechanisms of this phenomenon are not known. A widespread protective mechanism against drought and frost in plants is the accumulation of certain organic metabolites – the osmoprotectants and antioxidants [18–20]. Virus infection also stimulates accumulation of some of these compounds. A profile of the metabolites in BMV-infected rice and CMV-infected beet showed that levels of plant osmoprotectants were higher in virus-infected plants than in uninfected plants [17]. Virus presence can stimulate other stress-related responses. In *Arabidopsis*, cauliflower mosaic virus infection induced lipid transfer protein expression, which is also regulated by environmental stimuli, for example, high and low temperatures, drought, abscisic acid, and osmotic stress [21]. Similarly, heat shock proteins and defense-like proteins are induced in some plants infected by viruses [22], conceivably priming them to withstand biotic and abiotic stresses.

A mutualistic association among a virus (*Curvularia* thermal tolerance virus), a fungal endophyte (*Curvularia protuberata*), and a tropical panic grass (*Dichanthelium lanuginosum*) is essential for survival of all three species under the extreme conditions in which they grow. The plant can tolerate soil temperatures of up to 65 °C in geothermal areas of Yellowstone National Park. When the plant and fungus are separated, neither is able to grow at temperatures above 38 °C. When the fungus was "cured" of the virus and reintroduced to the plant, heat tolerance was not conferred, but heat tolerance was restored after the virus was reintroduced. The virus-infected fungus confers heat tolerance not only to its native panic grass host but also to an unrelated host plant, which suggested that the underlying mechanism involves pathways that are conserved between plants [23].

White clover plants are often infected by the persistent virus *White clover cryptic virus* (WCCV). When white clover plants are grown in high nitrogen-containing soils, the plant uses the coat protein gene of WCCV to suppress nodulation [24].

Perhaps the most profound way in which viruses might benefit their hosts is as a source of genetic material. Horizontal gene transfer during infection can occur from host to virus or from virus to host, and both have been observed. In the host to virus direction, viral acquisition of a number of plant genes including a heat shock protein in *Beet yellows virus* has happened over evolutionary history [25]. In the virus to host direction, retrovirus (RNA viruses with reverse transcriptase activity) elements are common in the genomes of many nonplant organisms, including humans where human endogenous retroviral (HERV) elements account for 42.2% of the genome [26]. Some are of benefit; for example, at least 50% of the human-specific HERV-K long-terminal repeats act *in vivo* as active promoters for host DNA transcription, contributing to the expression of nearby genes, whereas others are detrimental, being implicated in disease [27].

In plants, instead of retrovirus sequences their genomes contain elements of reverse-transcribing DNA viruses (pararetroviruses) such as *Banana streak virus*, *Tobacco vein clearing virus*, and *Petunia vein clearing virus* [28], and surprisingly, some non-reverse-transcribing RNA viral elements [29]. Most plant pararetroviral elements are inactive during normal plant development but some are activated by biotic and abiotic stresses such as wounding, pathogen attack, or tissue culture [30–32]. Genetic material from RNA viruses is derived from plus-sense,

minus-sense, and double-stranded RNA viruses. Some RNA viral sequences integrated in plant genomes are obviously of ancient origin as they are conserved across genera, whereas others are more recent, retained only in a limited number of species. These sequences serve as molecular tools for deciphering evolutionary relationships among plants. There is as yet little information about whether integrated viral components are utilized by plants. The *Arabidopsis thaliana* gene ILR2 has high sequence identity with the coat protein gene of *Rosellinia necatrix partitivirus 2* (RnPV2) and is thought to be derived from it. ILR2 regulates indole-3-acetic acid (IAA)–amino acid conjugate sensitivity and is functional in root development [29].

7.6 Conclusions

The examples given show that in at least some cases viruses play positive roles in plant adaptation and evolution. The widely held dogma, held even by some plant pathologists, that viruses are always villains is certainly incorrect. It is likely that many more persistent and mutualistic relationships between viruses and plants, and other creatures [33–36], will be uncovered as new technologies make it easier to detect and describe viruses and their effects on hosts. Similarly, as more plant genome sequences become available, deeper roles for viruses in plant evolution may be revealed. Study into all aspects of plant/virus associations is ramping up as high-throughput sequencing technologies are utilized in this exciting field. Questions to be asked in greater depth than were previously possible include the roles viruses play in plant ecology and evolution, the importance of mutualistic associations under a climate predicted to become warmer and drier, and the role of host biodiversity in disease emergence.

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8
Risk Assessment of Abiotic Stress Tolerant GM Crops

Paul Howles and Joe Smith

Abstract

Genetically modified (GM) crops engineered with herbicide tolerance and/or insect resistance have been grown on a commercial scale throughout the world for well over a decade. A new generation of GM crops, containing a greater variety of traits, including tolerance to abiotic stresses, is currently in field trials and/or approaching commercialization. Whereas engineering of herbicide tolerance or insect resistance can be achieved by insertion of single genes with clearly defined roles and highly specific mechanisms, tolerance to abiotic stresses involves the interaction of numerous genes and regulatory pathways. This chapter outlines the likely issues for consideration in risk assessment for the commercial release of a GM plant with a novel abiotic stress tolerance trait. To inform the discussion, the chapter begins with an overview of current understanding of plant responses to abiotic stress, and how this knowledge is being used to engineer various types of stress tolerance into GM plants.

8.1
Introduction

In 2010, the global area planted with biotech crops containing engineered herbicide tolerance and insect resistance was in excess of 145 million hectares [1]. However, it is likely that the future course of genetically modified (GM) crop commercialization will be characterized by plants containing a greater variety of engineered traits. These traits, sometimes referred to as second-generation GM crop traits, include abiotic and biotic stress tolerance, as well as increased nutritional value and modification to improve the use of the plant material as biofuels [2].

The impetus for the development of these plants can be put down to a number of factors. First, in recent years the needs and associated environmental consequences of the increasing world population have become a growing source of concern. In the year 1800 the world population was under 1 billion, while it had exceeded 6 billion in 2000, with estimates that it may exceed 9 billion by 2050 [3]. Human

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civilization has always impacted upon the environment, but industrialization and population growth have led to its degradation at an unprecedented rate. Interlinked with this is the concern that agriculture will no longer be able to feed the increasing population in many parts of the world. With such a background, maintaining if not increasing crop yields has become a focal point of attention among both politicians and agricultural scientists.

Second, from the perspective of the biotech companies, although herbicide-tolerant and insect-resistant GM crops have proven a financial success, diversification of the portfolio of available products by any company is sound strategy and may potentially reap significant commercial gains [4]. Finally, although attempts at the generation of plants with increased stress tolerances through traditional breeding and mutagenesis have met with some success, they have not produced plants that cope with any of these stresses in a profoundly remarkable manner [5]. Further, at least in the case of breeding with wild relatives, undesirable genes are often transferred along with those expressing the desired trait, necessitating multiple backcrosses to remove them (even then presuming they are not linked). For these reasons, use of other technologies, such as genetic modification, has become more attractive to tackle these stresses [6].

8.2 Abiotic Stress

Abiotic stress can be defined as any nonliving factor that produces a harmful impact upon a plant (or animal). These factors are extremes in the surrounding environment, the most important being drought, excess water (floods), salinity, the presence of toxic chemicals such as heavy metals, nutrient deficiencies, excessive cold or hot temperatures, UV irradiation, fire, and high winds.

Although all these factors can substantially reduce yield and/or restrict the geographical range of a plant, drought is the most significant. The availability of only a limited amount of water, or even none at all, negatively impacts the growth of crops worldwide [7]. Moreover, agriculture accounts for over 80% of freshwater used by humans, but most of this water is lost, either through direct evaporation into the atmosphere or through transpiration by plants themselves [8]. However, the simultaneous occurrence of more than one abiotic stress is usually more detrimental to plant growth than exposure to a single stress. As abiotic stress combination can rarely be analyzed as a sum of the individual stresses, it has been suggested that such combinations should be viewed as new stresses [9].

Natural mechanisms by which plants deal with abiotic stresses are often classified as either avoidance or tolerance [10]. Avoidance mechanisms refer to those that affect the morphology and/or physiology of the plant, the most common being root structure or depth, the length of the life cycle, and the number and structure of stomata. Tolerance mechanisms are those that result from changes in the levels and/or activity of molecules within cells, but that are not associated with large-scale morphological/physiological effects. Such molecules

include osmoprotectants, membrane ion transporters, dehydrin proteins, and reactive oxygen species.

Morphological and physiological avoidance responses to many abiotic stresses, especially heavy metals, nutrient deficiency, and UV irradiation, are similar [11]. These frequently include inhibition of cell elongation, redifferentiation of some cells, and the localized stimulation of cell division (leading, for example, to the formation of lateral roots). Responses to drought include reducing the surface area exposed to evaporation (losing leaves), while both salinity and drought are characterized by closing of stomata and reduction in growth [12, 13]. On the other hand, tolerance to an abiotic stress is manifested by responses such as reestablishment of cellular homeostasis, detoxification, and repair of damaged tissues [14].

Ultimately, the response of a plant to abiotic stress is due to changes in the temporal and spatial expression of genes. However, it is the tolerance mechanisms (Figure 8.1), involving switches in gene expression that are easier to characterize (especially by modern technologies such as genomics), that provide greatest scope for biotechnological manipulation [10, 15].

8.3
Abiotic Stress Traits are Mediated by Multiple Genes

As stated above, the engineering of herbicide tolerance and insect resistance has involved the insertion of single genes with clearly defined roles and mechanisms, which directly produce the desired trait. However, tolerance to abiotic stresses involves the interaction of numerous genes and their associated regulatory pathways. Exposure of *Arabidopsis* to stresses such as drought and cold affects the expression of hundreds, if not thousands, of genes [16]. These complex changes in gene expression provide difficulties for researchers in unraveling these traits. Although the expression of a gene may be experimentally linked to tolerance to a given abiotic stress, the role that this gene plays in producing that trait may not be clear.

Furthermore, even if the expression of multiple genes can be associated with each other, and with tolerance to a given abiotic stress, it may be technically difficult to generate a GM plant with all these genes. Obviously, it is easier to insert single genes into transformation vectors and characterize the resulting GM plants. Generating a GM plant containing a number of genes (either by transformation with a single vector containing all the genes or by crossing individual GM plants) is labor intensive. As a result, researchers have largely concentrated on the insertion of single genes for abiotic stress tolerance, but it is expected that multigene GM plants will follow.

A large number of genes have been implicated in abiotic stress responses, and shown to enhance tolerance to these stresses when their expression is purposely manipulated in GM plants [17]. Protein products of such genes can be classified into a number of groups. The most important of these groups are the transcriptional regulators, post-translational modifiers of proteins, proteins associated with

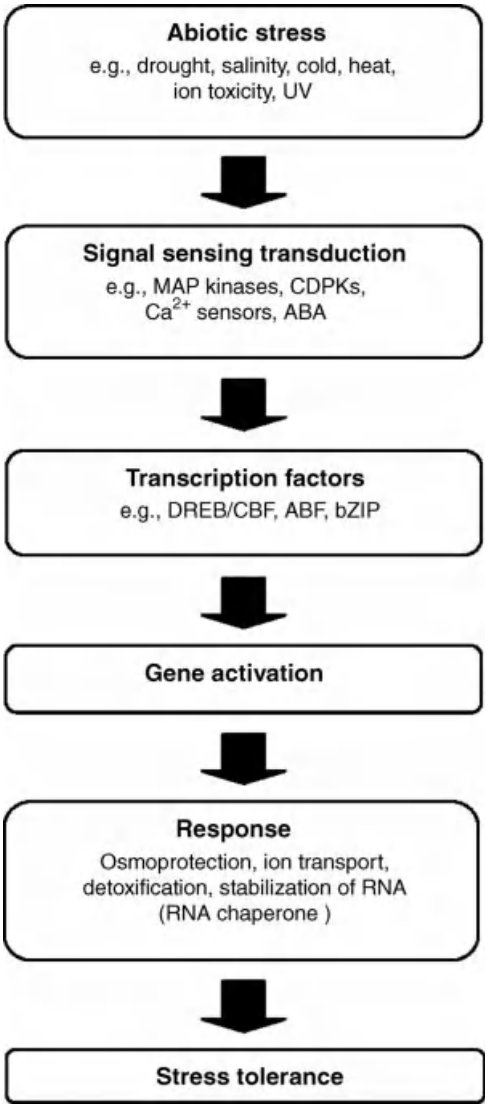

Figure 8.1 Abiotic stress responses. Abiotic stresses trigger signal pathways that often involve transcription factor activation of gene expression. The protein products of these latter genes act to mitigate the effects of the stress, with roles including repairing membranes, extruding ions, and/or promoting the translation of other proteins. Abbreviations: MAP, mitogen-activated protein; CDPK, calcium-dependent protein kinase; ABA, abscisic acid; DREB, dehydration response element binding protein; CBF, C repeat binding factor; ABF, abscisic acid responsive element binding factor; bZIP, basic leucine zipper transcription factor. (Modified from Ref. [10].)

ABA (abscisic acid) metabolism, phospholipases, and proteins known to protect the conformation of other proteins, the so-called molecular chaperones [18]. Although the engineering of constitutive or inducible expression of genes is the common strategy to confer stress tolerance, this trait can sometimes be generated by downregulation [19].

The genes that code for the cold shock proteins (CSPs) from bacteria, which function as RNA chaperones, are promising candidates for the production of both drought and freezing tolerance [7, 20]. Other genes likely to prove significant in the engineering of abiotic stress tolerance are those for which the protein products regulate the expression of multiple (downstream) genes [14, 17]. These genes code for components of signal transduction pathways, such as transcription factors. Here the strategy is to manipulate signal transduction pathways, by either the activation or deactivation of key components, to transmit the signal in a manner that evokes a more effective response. Nevertheless, selecting worthwhile candidate genes for the engineering of abiotic stress tolerance is demanding. Often the lack of a clear understanding of the link between a gene and the expressed trait means that the use of some single genes may be based upon naïve experimental design and expected outcomes [21]. Also, plants possessing single genes are less likely to show the desired phenotype when assayed under various field conditions [18].

8.4 Pleiotropy and Abiotic Stress Responses

Due to the regulatory role of many genes implicated in abiotic stress tolerances, it is not surprising that expression of such genes has often been associated with pleiotropic effects. The overlap (sharing of components) between signal transduction pathways could occur at the stage of receptors, signaling pathways, and/or transcription factors. Some of these pathways are also linked to hormones such as ABA and ethylene, these hormones being essential components of both abiotic and biotic stress responses as well as developmental processes. In the case of ABA, while some abiotic stress response pathways are dependent upon this hormone and others act without reference to it, there is likely interaction of these response pathways in downstream steps [22]. As the ethylene signaling pathway is linked to a range of biochemical processes, manipulation of a component of this pathway for one purpose is frequently accompanied by other unintended effects [23].

The mitogen-activated protein kinases (MAPKs) are regulatory proteins, presumptively acting in "upstream" roles, which are known to be involved in abiotic stress responses. In general, these proteins form a sequential cascade, transducing a signal from the plasma membrane to the cytoplasm or nucleus. Some MAPK pathways in plants have been linked to multiple abiotic stresses [24, 25]. In *Arabidopsis*, a MAPK cascade was found to be induced by both cold and salinity, and engineered overexpression of one of the pathway genes gave tolerance to both of these stresses [26]. Overexpression of this gene was marked by changes in

expression of over 150 genes, including upregulation of genes coding for transcription factors such as DREB1C (described below).

In "downstream" positions, a number of transcription factors have been linked to multiple abiotic stress responses. Perhaps the most well-known example is the DREB/CBF transcription factors that bind to the DRE/CRT (dehydration responsive element/C-element) sequences in the promoters of genes, inducing the expression of these genes [27, 28]. In *Arabidopsis*, while the *DREB1A*, *DREB1B*, and *DREB1C* genes appear specifically induced by only cold, both the *DREB2A* and *DREB2B* genes are responsive to drought and high salinity [28]. However, overexpression of the *DREB1A* and *DREB1B* genes in *Arabidopsis* induced tolerance to a range of abiotic stresses. Homologues of the *Arabidopsis DREB2* genes from other plant species are likewise generally responsive to multiple abiotic stresses in wild-type plants. The *DREB2A* gene from maize, for example, was found to be induced by cold, heat, drought, and salinity [29].

Other transcription factors that are responsive to a range of abiotic stresses include members of the calcium-dependent protein kinase (CDPK) and ABA-responsive element binding protein (AREB) families [30–32]. For example, overexpression of the maize ABP9 protein (a member of the AREB family) in *Arabidopsis* resulted in enhanced tolerance to drought, salinity, cold, and oxidative stress [32]. At least in the case of *Arabidopsis*, members of the AREB family of proteins likely form both homo- and heterodimers in the nucleus and possess overlapping functions [33].

A pleiotropic effect may be unrelated to abiotic stress tolerance. In particular, there often appears to be crosstalk between abiotic and biotic stress response pathways [16]. Many examples illustrate this phenomenon. Overexpression in tobacco of a rice gene coding for a ubiquitin E3 ligase produced plants with enhanced resistance to infection by both bacterial and viral pathogens, and increased drought tolerance [34]. Further, the modified plants had defects in their growth and development. Similarly, overexpression of a tobacco transcription factor in that plant resulted in both tolerance to salt and resistance to a bacterial pathogen [35], while individual overexpression of two fungal chitinases in tobacco induced tolerance to salt and heavy metals, as well as increased resistance to fungal and bacterial pathogens [36]. Examples from natural populations also illustrate pleiotropic effects. One of these is the observed correlation in some plants between tolerance to heavy metals and ability to resist attack by pathogens, a phenomenon that likely involves at least partially overlapping signaling pathways [37].

8.5 General Concepts of Risk Analysis

The trialing and commercialization of GM crops has been accompanied by the development of stringent regulatory requirements, aimed to protect the health and safety of people and protect the environment. A number of documents have played a significant role in the development of the logic and rationale for risk analysis. The

most important of these are the 1983 report from the US Academy of Sciences National Research Council (the "Red Book") and the 1986 OECD booklet entitled *Recombinant DNA Safety Considerations* (the "Blue Book") [38, 39]. The latter was the first intergovernmental document that addressed the risks posed by GMOs. It proposed that potential risks should be considered before the release of any GMO, and that all risk assessments should be guided by existing data on human and environmental effects of organisms, as well as proceeding on a case-by-case basis [40]. Later, Annex III of the 2000 Cartagena Protocol on Biosafety (a supplement to the Convention on Biological Diversity) provided a limited outline on how risk assessments should be conducted. This includes emphasizing that any assessment should be conducted in a scientifically sound and transparent manner.

Although the procedure of risk analysis differs between countries, it is essentially based upon risk assessment, a risk management plan, and risk communication. Risk assessment involves the identification of risks (hazards), and the subsequent characterization of the likelihood and consequences of each. In essence, it involves postulating a series of "what if" scenarios, whereby plausible pathways to potential harm are generated [41, 42]. The assessment of individual risks could be presented in either qualitative or quantitative terms, but usually in the case of GMOs they are described in qualitative language. The qualitative risk analysis approach used in Australia by the Office of the Gene Technology Regulator (OGTR) is described in the *Risk Analysis Framework* [43]. This includes use of a risk matrix, with likelihood and consequence axes, to estimate the level of risk, the extremes being negligible and high.

The conclusions from the risk assessment are used to formulate a risk management plan, which is designed to mitigate any identified risks. Risk communication refers to consultations with relevant government organizations, scientists, and the members of the public. This process occurs throughout risk analysis.

8.6
Risk Assessment and Abiotic Stress Tolerance

Risk assessments for small-scale field trials of GM crops with introduced genes associated with abiotic stress tolerance traits have been conducted in a number of countries, including the United States and Australia. In the United States, the Animal and Plant Health Inspection Service (APHIS) has published preliminary environmental and plant pest risk assessments for the nonregulated status of Monsanto's drought-tolerant maize (MON87460) and environmental assessments (2007, 2009) for ArborGen's cold-tolerant/altered lignin *Eucalyptus* [44–47].

In Australia, the OGTR has prepared risk assessments and risk management plans for field trials of a number of GM crops with abiotic stress tolerances including tolerance to drought, heat, and waterlogged soils (see Table 8.1 and http://www.ogtr.gov.au). Risk assessments for these trials were conducted within a context that included limits in time and space as well as controls to minimize the likelihood of any adverse effects. The same approach was used for the assessment of field trials

Table 8.1 Dealings involving an intentional release (DIR) into the environment, issued by the Australian Office of the Gene Technology Regulator, which involve plants with abiotic stress tolerance traits.

Application number	Crop	Trait
DIR067/2006	Cotton	Waterlogging tolerance
DIR070/2006	Sugarcane	Enhanced water use efficiency
DIR071/2006	Wheat	Drought tolerance
DIR077/2007	Wheat and barley	Drought and boron tolerances
DIR080/2007	Wheat	Drought tolerance
DIR081/2007	Cotton	Water use efficiency
DIR083/2007	Cotton	Waterlogging tolerance
DIR095	Sugarcane	Drought tolerance and nitrogen use efficiency
DIR100	Wheat	Heat tolerance and water use efficiency
DIR102	Wheat	Drought, salinity, and cold tolerances

All these DIRs have been for small-scale field trials (limited and controlled releases).

of GM crops with herbicide tolerance and/or insect resistance. The consideration of appropriate limits and controls in the risk assessment of proposed field trials minimizes the likelihood of an adverse effect, and these limits and controls are imposed as license conditions to maintain this context and the validity of the assessment.

The commercial release of any GM plant implies the large-scale and largely unregulated growth of the plant in the environment. The context for such an assessment would differ from the context for a field trial, as there would be few or no limits and controls. The risk assessment for the release of such a plant will entail considering many of the same issues considered in risk assessments for commercial release of GM plants with herbicide tolerance and/or insect resistance. However, additional issues arise that are peculiar to abiotic stress traits, in particular the choice of comparator and potential wider pleiotropic effects. Further, the complex molecular character of responses to abiotic stress (in contrast to the relatively simple mechanisms of herbicide tolerance and insect resistance traits) implies that any risk assessment may have to proceed without knowledge of the exact mechanism underlying the trait. It has been suggested that an in-depth knowledge of the mechanism is really unnecessary for any risk assessment of these crops, the defining trait *per se* being the important starting point for assessment [48]. Nevertheless, if the mechanism of tolerance is clearly understood, incorporating it into risk assessment will be beneficial, especially in relation to potential pleiotropic effects, and will add weight to the conclusions.

As a background to the risk assessment of any GM plant, it must be appreciated that not only may the expression of the introduced gene(s) induce an adverse effect, but such an effect may also result from the act of insertion into the host genome. The transformation of genes into plants often results in integration occurring preferentially in certain chromosomes, and in certain regions of these chromosomes [49, 50]. However, unless a gene is specifically introduced

into an already engineered and characterized site [51], it is not possible to predict the exact site of insertion. In this context, an insertion could disrupt the sequence of an endogenous gene, leading to the complete absence of the final gene product (whether that be RNA (e.g., rRNA, microRNA) or protein) or the expression of a nonfunctional gene product. Alternatively, the insertion could result in either overexpression or underexpression of an adjacent gene. The subsequent alteration in the level of the derived protein, or its catalytic product(s), may be the cause of an adverse effect.

8.6.1 Choice of Comparator

The standard comparator for the risk assessment of GM plants is the conventional non-GM parent plant. For herbicide tolerance and insect resistance traits, the non-GM variety provides an adequate comparator as the engineered traits are found neither in any variety of the same species, nor usually in any related species. Moreover, such GM plants are usually engineered to be put back into the environments they normally inhabit, but with a trait that will enhance their production.

However, the selection of a comparator for GM plants with abiotic stress tolerance presents two issues. First, abiotic stress tolerance traits are sometimes found in other varieties of the same species. These latter plants often possess other traits that make them unsuitable for agriculture, but at the same time, their expression of the engineered trait may make them useful comparators. In such cases, it may be prudent to assess the GM plant against both the unmodified parent plant and those related varieties that express the trait. For example, a number of comparator lines may make it easier to ascertain if the GM trait is accompanied by increased weediness. Also, any pleiotropic effects observed in the GM plant may occur in unmodified relatives, and the consequences of these effects may have been the subject of published studies.

Second, a plant may be engineered with a trait that enables it to thrive in a particular environment where the unmodified plant is incapable of surviving beyond germination. In this case, information on the weediness of the unmodified plant in that environment will not be available or obtainable by future research, and the weediness of the GM plant will have to be assessed without direct reference to this comparator. An example is a plant engineered to withstand cold. Such a plant will be able to grow in cooler climates, especially the winters, where the unmodified parent plant cannot persist. The latter plant will hence be unable to provide comparative data from the new environment for risk assessment [52].

8.6.2 Production of an Allergenic or Toxic Substance

The insertion into a plant of one of more genes for a particular abiotic stress tolerance trait could be accompanied by an increase in the level of an endogenous, or the production of a novel, allergenic, or toxic compound. Such a trait

would almost certainly represent a distinct risk to human and/or animal health, and it is not likely that a plant possessing it would be selected for commercialization.

Consideration of the origin and known properties of the probable inserted genes makes such a scenario unlikely. Most of the genes that have been proposed as useful for abiotic stress tolerance are derived from plants, or microorganisms, with which humans have had extensive contact [17]. Any presently known, or future discovered, negative effect of the protein product of such a gene on human health would immediately disqualify the gene from ever being transformed into a plant. However, as the potential risk of any compound to human health is proportional to the amount consumed (and hence to the quantity in the food), the increased expression of any introduced gene in a GM plant may warrant consideration in any risk assessment. Also, there is the possibility that a protein, nonallergenic in its natural host, will be allergenic when expressed in a novel context, or sensitize endogenous proteins of the host [53].

The above noted regulatory nature of many candidate genes for abiotic stress tolerance engineering and the likelihood that such genes may have pleiotropic effects are notable differences to the genes used to generate herbicide tolerance and insect resistance. It is thus expected that the metabolic profile of abiotic stress-tolerant crops will be perturbated substantially more than that seen with the currently commercialized GM crops [52]. This likelihood means that in the risk assessments of abiotic stress-tolerant GM plants, greater consideration should be given to data arising from compositional analysis. Nevertheless, as discussed in more detail below, the experience from traditional breeding is that novel toxicants and allergenic compounds are rarely a problem in new crop varieties.

8.6.3
Invasiveness and Weediness

The engineering of abiotic stress tolerance into a plant may be aimed at increasing the growth and productivity of that plant in its established range, and/or extending the range of the plant into regions where environmental variables have previously restricted its survival or productivity. Even if this is not the case, compared to GM crops containing herbicide tolerance and/or insect resistance genes, those engineered to have tolerance to abiotic stresses are predicted to have greater fitness, consequently increasing the chance that they will colonize and establish in new regions [2, 54]. Many crop species are prevented from establishing successful breeding populations outside cultivated areas as a consequence of abiotic (and biotic) conditions. If one or more of these restrictive conditions are overcome by a GM crop, then it may effectively "escape" its previous environmental shackles. Whether this escape is desired or not, its consequences for the environment must be assessed.

Expansion into new regions will likely bring any plant into contact with plants with which it has previously been geographically separated. These latter plants may be either sexually compatible relatives or those with which breeding is impossible.

Hybridization between the GM crop plant and a wild relative could lead to the abiotic stress tolerance trait being transferred to the wild plant, possibly increasing its invasiveness or persistence. A further possibility to consider is that a compatible species may be endangered. Crossbreeding between the two species, possibly followed by natural backcrossing to the GM crop, may lead to extinction of the endangered species [55].

Another issue that may deserve attention is pest and disease burden. In a new environment, a GM plant could face either an increase or a decrease in pressure from pests and diseases. In the case of greater pressure from these biotic factors, the plants may become a greater reservoir for their multiplication, while if there is less pressure, the plants may be more prone to develop into weeds.

An analysis of gene expression in natural populations of sunflower provides an example of how there may be links between stress responses in a plant and weediness [56]. In this study, comparing separate wild (nonweedy) and weedy populations of sunflower, it was demonstrated that genes associated with abiotic and biotic stress were significantly overrepresented among those genes showing variation between the two types of populations. Most of these genes were downregulated in the weedy populations. This possibly reflects a cost in maintaining active abiotic and biotic stress response pathways, with weeds downregulating these pathways to concentrate on the increased rates of growth necessary to compete in agricultural fields. If this scenario is related to plants engineered with abiotic stress tolerance, expression of such traits may sometimes act as a brake on the development of weediness.

8.6.4 Pleiotropic Effects

The relatively simple biochemical basis to engineered herbicide tolerance and insect resistance has meant that associated pleiotropic effects have been limited [23]. However, as noted above, many of the genes associated with abiotic stress code for regulatory proteins, such as transcription factors, most of which have established pleiotropic properties. Although signaling pathways are often depicted as being linear, most are part of complex networks (Figure 8.2). Further, control of these pathways is nearly always exerted at a number of points, and usually entails feedback loops, where the product of a downstream reaction affects the expression of an upstream step [57]. Consultation of the published literature will enable all known roles of a gene, or at least its protein product, to be established, and from such data plausible scenarios constructed for how the gene may influence other traits. However, pleiotropic effects from the insertion of a gene into the genome of a plant will often be difficult to predict.

Increased toxicity, allergenicity, and weediness of a GM plant may all be due to pleiotropic effects of the introduced gene. In regards to weediness, the frequent occurrence in plants engineered for one abiotic tolerance of pleiotropic effects related to other abiotic and/or biotic stresses would likely be important in any risk

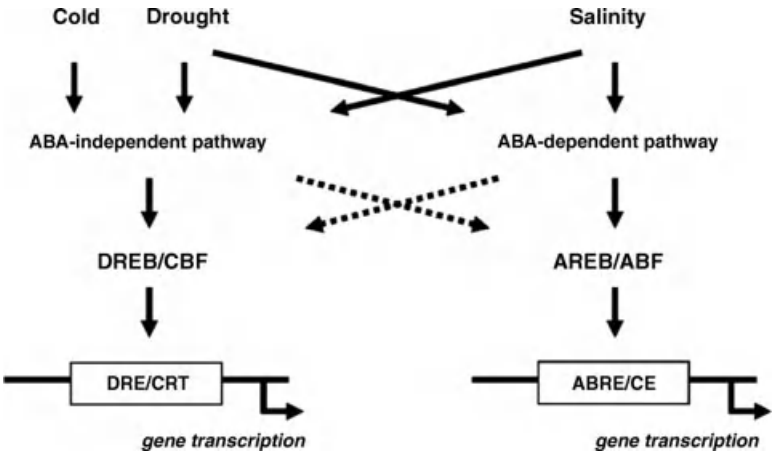

Figure 8.2 Outline of the signaling pathways leading from the abiotic stresses of drought, cold, and salinity to the activation of response genes. These pathways can be either dependent on or independent of ABA, and there may be overlap of these pathways in downstream steps. Involvement of the same pathway in response to multiple stresses may result in pleiotropic effects. Exposure and tolerance to one stress may be accompanied by tolerance to one or more other stresses. Two major groups of transcription factors, DREB/CBF and AREB/ABF, are illustrated but other groups of such factors are also induced by abiotic stresses. These factors bind to specific *cis*-elements in the promoters of "stress-inducible" target genes, thus activating their expression. The protein products of the latter genes participate in the induction of the abiotic stress tolerance phenotype. For abbreviations, see Figure 8.1. (Modified from Refs [22, 28].)

assessment. However, other pleiotropic effects of the genetic modification, such as changes in the levels of organic metabolites and inorganic ions (possibly affecting the nutritional value), may occur in a GM plant.

Laboratory and then field trials are the best method to collect information pertaining to the presence and significance of any pleiotropic effects in a GM plant. Expression of two cold shock proteins, *E. coli* CSPA and *B. subtilis* CSPB, in *Arabidopsis*, rice, and maize conferred the desired cold, heat, and drought tolerance (indicating the genetic interrelatedness of these traits). No pleiotropic effects were noted in relation to plant size or development [7]. In a more theoretical study, the *Arabidopsis* ABF3 gene was overexpressed in that plant [58]. Overexpression of this gene conferred tolerance to drought and other abiotic stresses. Microarray analysis suggested that this was due only to modification of pathways that normally respond to these stresses. It was concluded that beyond modifying the abilities of plants to resist other abiotic stresses, unintended pleiotropic effects in engineering drought tolerance through this specific genetic modification may be minimal.

Published risk assessments for small-scale field trials of engineered abiotic stress-tolerant plants have briefly considered the issue of gene pleiotropy. Assessments produced by the OGTR for such field trails note that the insertion of any

gene can potentially have pleiotropic effects, giving rise to novel traits (see www.ogtr.gov.au). APHIS' environmental risk assessment for field trials of modified *Eucalyptus* (2009) makes particular reference to the use of a stress-inducible promoter (*rd29A*) to drive the production of the CBF protein [45]. This promoter will likely alleviate negative effects on agronomic traits, which have been observed when CBF genes are driven by a constitutive promoter. As part of the environmental risk assessments of maize modified for drought tolerance, APHIS used data from the applicant regarding abiotic and biotic stress responses of the modified plant compared to the control [46, 47].

It should be appreciated that any unintended pleiotropic effect may not be detrimental to the survival of a plant or its use as a food commodity, but in fact advantageous. This especially applies to biotic responses. If a GM plant engineered for an abiotic stress is found to be more resistant to infection by a pathogen, then it may well prove a boon for the use and commercialization of that plant.

Finally, although rarely mentioned in the risk assessments of GM plants, the epistatic interactions of genes are worth considering. Here, two or more genes interact with each other to control the expression of a single phenotype. The introduction of a genetic modification to a plant may therefore lead to the obscuring of a trait that is beneficial to the survival and reproduction of the plant.

8.6.5
Gene Transfer to Another Organism

Genes can be transferred between organisms by either so-called "horizontal" and "vertical" means. The transfer of a gene to a recipient organism, whether an endogenous gene or a genetic modification introduced into the donor organism by genetic engineering and transformation, is not in itself an adverse outcome. The risk is that the transfer of a gene could confer upon the recipient organism an undesirable trait, such as toxicity, allergenicity, or weediness.

Horizontal gene transfer is the transfer of genes to other organisms without a parent–offspring relationship. The major mechanism involving plants as the donor organism is the transduction of genetic material by viruses [59]. A number of conditions have to be met for genetic material to be transferred from a plant to another organism by horizontal gene transfer, and for this material to become part of the genetic makeup of the descendents of that recipient organism. These include contact between the plant (or material derived from it) and the organism, transfer of the genetic material to the organism, and survival and reproduction of the recipient organism. Moreover, for any genetic material to be maintained in a recipient organism and its descendants, there must be a selective advantage. Each of these steps is in itself unlikely, and as such the successful completion of all an extremely rare event.

Some putative examples of DNA transfer from plants to bacteria are known, and the feasibility of the process has been demonstrated in experimental studies [60, 61]. However, there is no evidence to conclude that the process is widespread, and many examples reflect extremely ancient events. Viruses are capable of taking up

host DNA sequences, but only some, most particularly retroviruses and DNA viruses, are surmised to commonly transfer DNA to host eukaryotic genomes [62]. It should also be noted that if any host DNA is transferred to a viral genome, it most often represents sequences of viral origin that have previously been incorporated into the plant genome. It is likely that there has been transfer of DNA between plants and fungi, but such transfer is again proposed to be rare, and even then it may largely involve plants that are not angiosperms [63, 64]. Animals may eat and digest a GM plant, but the transfer of DNA to the germ line cells has not been demonstrated.

In summary, the risk of horizontal gene transfer from any GM plant to a recipient organism is negligible. Other than the case of using viral sequences in a construct to be inserted in a plant (which potentially may be prone to recombination with infecting plant viruses), there is no reason to expect that the sequences in a construct itself are more subject to horizontal gene transfer than the endogenous DNA. The basic assessment of these risks will not be changed by the use of DNA sequences designed to produce abiotic stress tolerance.

Vertical gene transfer in plants occurs by hybridization with a sexually compatible plant, either of the same or of different genera. Hybridization is a common and well-documented phenomenon in the plant kingdom [65]. The risk with an abiotic stress-tolerant plant is that the plant could hybridize with another plant and either transfer an undesirable trait to the recipient or induce such a trait in the recipient. Hybridization may be more likely with plants with abiotic stress tolerance, if as noted above these traits themselves may increase the fitness of the plants, leading to an extension of their range of habitats and the chance that they will cross with sexually compatible plants.

8.7
Abiotic Stress Tolerance Engineered by Traditional Breeding and Mutagenesis

Traditional breeding and mutagenesis have both been used to generate crops with tolerance to various abiotic stresses. Examples from traditional breeding include drought-tolerant varieties of barley, chickpea, pearl millet, rice, and tomatoes [66–69]. The generation of some of these plants has involved hybridization with appropriate wild relatives, previous research having established that these possess useful traits. Mutagenesis, through exposure of seed to either radiation or chemical treatment, has likewise produced a number of successful varieties of crop species. These include drought-resistant and cold-tolerant varieties of wheat, and varieties of rice with various agronomic and nutritional traits [70–72].

Traditional plant breeding has nearly always incorporated backcrossing and observation of the plants over a number of generations prior to commercialization. Plants containing the desired properties are selected, whereas those failing to have these properties, or exhibiting unwanted features, are discarded. As standard practice, breeders have compared their newly generated varieties against the parent(s) and other related varieties on the market. This approach has meant that the safety

of these novel plants for human consumption is largely based on trial and error, but its utility has been rarely questioned [73, 74]. It has resulted in the release of numerous new plant varieties, with virtually no undesirable toxic and allergenic properties for the consumer [75]. Such plants present a useful context for any risk assessment of a plant with a GM abiotic stress tolerance trait.

A GM plant usually contains only one or a small number of insertions of a known gene, the genomic positions of these insertions usually being defined. Hence, it is anticipated that GM plants with engineered abiotic stress tolerance will rarely have properties (such as weediness or compounds of concern to human health) that are not predictable on the basis of the known functions of inserted gene(s). Nonetheless, it remains appropriate that risk assessment proceeds on a case-by-case basis.

8.8 Conclusions

Herbicide-tolerant and insect-resistant GM crops are widely grown. In 2010, crops containing one or both of these traits were grown in over 25 countries. Significant proportions of canola, cotton, maize, and soybean grown worldwide (over 75% for soybean) are GM crops with these traits [1]. GM plants with other introduced traits, such as abiotic stress tolerance, are nearing commercial release. Regulatory decision making for approval of these crops will continue to use the same risk assessment framework established for the current GM crops. However, it is expected that the potential weediness of plants with abiotic stress tolerance traits will receive greater attention, reflecting both the intrinsic nature of these traits and the increased possibility of associated pleiotropic effects.

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9
Biofertilizers: Potential for Crop Improvement under Stressed Conditions

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Abstract

Biofertilizers are low-cost, renewable source of plant nutrients that supplement chemical fertilizers. Besides promoting increased absorption of nutrients, they also help plant to withstand various biotic and abiotic stresses. They do not directly supply any nutrients to crops what chemical and organic fertilizers do and are cultures of special bacteria and fungi. The installation cost of a biofertilizer unit is very low and production technology is also very simple. Moreover, the long-term use of biofertilizers is economical, eco-friendly, more efficient, productive, and accessible to marginal and small farmers compared to chemical fertilizers. Although there are plenty of evidences of crop improvement under stressed conditions using biofertilizers, a few factors often limit the potential of biofertilizers in fields, which include poor shelf life, lack of suitable carrier materials, susceptibility to high temperature, and problems in transportation and storage. Thus, more field application-based research and outreach activities are required to get optimum results from biofertilizer application and its promotion.

9.1
Introduction

Indiscriminate application of synthetic fertilizers has led to the pollution and contamination of soil and water basins and destroyed microorganisms and friendly insects, making the crops more prone to diseases and leading to reduced soil fertility. Therefore, in order to make agriculture sustainable, it is necessary to implement a balanced and responsible agricultural practice. Thus, an approach is adopted to introduce into the soil potential microorganisms, a practice known as inoculation. The inoculants are also known as biofertilizers. They are low-cost, renewable source of plant nutrients that supplement chemical fertilizers. Besides promoting increased absorption of nutrients, they also help plant to withstand various biotic and abiotic stresses. In this chapter, the discussion is restricted to a review of the main group of biofertilizers and their role in crop improvement under stressed conditions.

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9.2 What Is Biofertilizer?

Biofertilizer is a ready-to-use live formulation of such beneficial microorganisms that on application to seed, root, or soil mobilize the availability of nutrients by their biological activity. However, many other definitions and interpretations of the term biofertilizer exist [1]. Important functions of such microbes are as follows:

- Convert ambient nitrogen into forms that the plants can use (nitrate and ammonia).
- Solubilize the insoluble forms of phosphates such as tricalcium, iron, and aluminum phosphates in available forms.
- Decay organic residues with nutrients and CO_2 release.
- Produce hormones and antimetabolites that promote plant growth.
- Defend plants against pathogens by outcompeting pathogens for food.
- Increase soil porosity by gluing soil particles together that aid in water filtration. If pore spaces are too small, they cannot break the surface tension of a water droplet, and water will run off, instead of saturating the soil, where it can be taken up by plant roots.

9.3 How It Differs from Chemical and Organic Fertilizers

Both chemical and organic fertilizers are widely used in agriculture and their advantages and disadvantages are well documented (Table 9.1). Biofertilizers differ from chemical and organic fertilizers in the sense that they do not directly supply any nutrients to crops and are cultures of special bacteria and fungi. The production technology for biofertilizers is relatively simple and installation cost is very low compared to chemical fertilizer plants [2]. Besides, the long-term use of biofertilizers is economical, eco-friendly, more efficient, productive, and accessible to marginal and small farmers compared to chemical fertilizers [3].

9.4 Type of Biofertilizers

Based on functional differences, biofertilizers can be classified as follows:

1) Nitrogen-fixing biofertilizer, for example, *Rhizobium* for legume crops, *Azotobacter*/*Azospirillum* for nonlegume crops, *Acetobacter* for sugarcane only, and blue-green algae (BGA) and *Azolla* for lowland paddy.
2) Phosphorus solubilizing biofertilizer, for example, *Bacillus*, *Pseudomonas*, and *Aspergillus*.
3) Phosphorus mobilizing biofertilizer, for example, mycorrhizae.
4) Plant growth promoting biofertilizer, for example, *Bacillus* sp. and *Pseudomonas* sp.

Table 9.1 Advantages and disadvantages of using chemical and organic biofertilizers [2].

Chemical fertilizers	Organic fertilizers
Advantages 1. Nutrients are soluble and immediately available to the plants; therefore, the effect is usually direct and fast. 2. The price is lower and more competitive than organic fertilizers, which makes them more acceptable and often applied by users. 3. They are quite high in nutrient content; only relatively small amounts are required for crop growth.	*Advantages* 1. The nutrient supply is more balanced, which helps to keep plants healthy. 2. They enhance soil biological activity, which improves nutrient mobilization from organic and chemical sources and decomposition of toxic substances. 3. They enhance the colonization of mycorrhizae, which improves P supply. 4. They enhance root growth due to better soil structure. 5. They increase the organic matter content of the soil, therefore improving the exchange capacity of nutrients, increasing soil water retention, promoting soil aggregates, and buffering the soil against acidity, alkalinity, salinity, pesticides, and toxic heavy metals. 6. They release nutrients slowly and contribute to the residual pool of organic N and P in the soil, reducing N leaching loss and P fixation; they can also supply micronutrients. 7. They supply food and encourage the growth of beneficial microorganisms and earthworms. 8. They help to suppress certain plant diseases, soil-borne diseases, and parasites.

(*continued*)

Table 9.1 (*Continued*)

Chemical fertilizers	Organic fertilizers
Disadvantages 1. Overapplication can result in negative effects such as leaching, pollution of water resources, destruction of microorganisms and friendly insects, crop susceptibility to disease attack, acidification or alkalization of the soil, or reduction in soil fertility – thus causing irreparable damage to the overall system. 2. Oversupply of N leads to softening of plant tissue resulting in plants that are more sensitive to diseases and pests. 3. They reduce the colonization of plant roots with mycorrhizae and inhibit symbiotic N fixation by rhizobia due to high N fertilization. 4. They enhance the decomposition of soil OM, which leads to degradation of soil structure. 5. Nutrients are easily lost from soils through fixation, leaching, or gas emission and can lead to reduced fertilizer efficiency.	*Disadvantages* 1. They are comparatively low in nutrient content, so larger volume is needed to provide enough nutrients for crop growth. 2. The nutrient release rate is too slow to meet crop requirements in a short time; hence, some nutrient deficiency may occur. 3. The major plant nutrients may not exist in organic fertilizers in sufficient quantity to sustain maximum crop growth. 4. The nutrient composition of compost is highly variable; the cost is high compared to chemical fertilizers. 5. Long-term or heavy application to agricultural soils may result in salt, nutrient, or heavy metal accumulation and may adversely affect plant growth, soil organisms, water quality, and animal and human health.

Table 9.2 Important microorganisms constituting biofertilizer and their contribution in nutrient fixation and mobilization.

Microorganisms	Nutrient fixed (kg/ha/year)
Actinorhizae (*Frankia* sp.)	150 kg N_2
Algae	25 kg N_2
Azolla	900 kg N_2
Azospirillum	10–20 kg N_2
Rhizobium	50–300 kg N_2
Azotobacter	0.026–20 kg N_2
Mycorrhizae	Solubilize food phosphorus (60%)
Phosphate solubilizing bacteria and fungi	Solubilize about 50–60% of the fixed phosphorus in the soil

Some examples of important microorganisms constituting biofertilizers and their contribution to nutrient fixation and mobilization are illustrated in Table 9.2 [4].

9.5 Description and Function of Important Microorganisms Used as Biofertilizers

9.5.1 Rhizobia

Rhizobia have the ability to fix atmospheric nitrogen (diazotroph). They make a symbiotic association with legumes and some nonlegumes such as *Parasponia*. *Rhizobium* bacteria enter into the roots through root hairs. They release certain stimulatory root exudates and form nodules. Inside the root, rhizobia invade expanded cells of cortex, and then differentiate into nitrogen-fixing "bacteroids." However, N_2-fixing capability of rhizobia varies greatly depending on the host plant species. Therefore, selection of best strains must take rhizobia host compatibility for selection of biofertilizers. The plant roots supply essential minerals and newly synthesized substances to the bacteria. Because of their N_2-fixing ability, legumes are less reliant on inorganic N fertilizers than many other nonlegume crops such as cereals and pasture grasses. N fixation by legumes can also maintain soil fertility and can be of benefit to the following crop. *Rhizobium* inoculation is a well-known agronomic practice to ensure adequate N supply for legumes in place of N fertilizer. Heavier application of inoculums mixed into peat granules trickled into soil as the seeds are planted is an alternative technique to encourage nodulation [2].

9.5.2 *Azotobacter* and *Azospirillum*

Azotobacter is a heterotrophic free-living nitrogen-fixing bacterium present in alkaline and neutral soils. It also increases germination and vigor in young plants leading to improved crop stands.

Apart from its ability to fix atmospheric nitrogen in soils, it can also synthesize growth promoting substances, namely, auxins, gibberellins, and also to some extent the vitamins. Many strains of *Azotobacter* sp. can also produce antifungal compounds to fight against many plant pathogens. Response of *Azotobacter* has been seen in rice, maize, cotton, sugarcane, pearl millet, vegetables, and some plantation crops.

Azospirillum is a free-living or nonsymbiotic bacterium (does not form nodules but makes association by living in the rhizosphere) and does not need a specific host plant. *Azospirillum* species establish an association with many plants, particularly with C_4 plants such as maize, sorghum, sugarcane, and so on. It is the most common organism and can form associative symbiosis on a large variety of plants.

9.5.3
Blue-Green Algae or Cyanobacteria

Among the N_2-fixing microorganisms, only blue-green algae are able to generate their own photosynthate from CO_2 and water [5]. This trophic independence makes BGA especially attractive as a biofertilizer. The blue-green algae are small organisms and can be seen under the microscope as a single cell or large accumulation of cells (colonies) or strings of cells (trichomes). Some accumulations may be so large that they are easily seen with the naked eye. Blue-green algae are also known by different names, such as cyanophytes, cyanobacteria, and most recently cyanoprokaryotes. They have a similar external appearance to that of algae and their requirements for light, nutrients, and carbon dioxide are also similar. Certain types of blue-green algae have tiny gas vesicles in their cells, which regulate them to float to the water surface or sink to the bottom in response to the changing of light and nutrient availability.

The blue-green alga (*Anabaena azollae*) forms a symbiotic relationship with *Azolla* (aquatic fern) and fixes atmospheric nitrogen. BGA is associated with the *Azolla* occurring in a ventral pore in the dorsal lobe of each vegetative leaf. The endophyte fixes atmospheric nitrogen and resides inside the tissue of the water fern. Individually, BGA and *Azolla* can also be used in paddy fields. BGA are capable of performing photosynthetic activity as well as fix the atmospheric nitrogen in the flooded rice ecosystem. *Azolla* is a fast growing water fern and can double its weight within a week. *Azolla* is rich organic manure also. It mineralizes the soil nitrogen rapidly, which is made available to the crop in a very short period. Nitrogen release from *Azolla* is slow but steady, without leaching losses. It also serves as a protein-rich feed to fish and poultry. They use energy derived from photosynthesis to fix nitrogen, and hence are called autotrophs. They are free-living organisms. In addition to fixing atmospheric nitrogen, BGA also synthesize and liberate some growth promoting substances, namely, auxin and amino compounds, that stimulate the growth of rice plants.

9.6 Phosphate Solubilizing Bacteria

Under acidic or calcareous soil conditions, large amounts of phosphorus are fixed in the soil but are unavailable to the plants. Inorganic forms of P are solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the P ions, that is, phosphate, releasing P into solution [6]. Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s [7, 8]. Release of P by PSB from insoluble and fixed/adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation [9].

9.7 Plant Growth Promoting Rhizobacteria

Plant growth promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria that, when grown in association with a host plant, result in stimulation of host growth. PGPR modes include fixing N_2, increasing the availability of nutrients in the rhizosphere, positively influencing root growth and morphology, and promoting other beneficial plant–microbe symbioses. Some researchers have indicated that PGPR will often have multiple modes of action. The PGPR can induce mycorrhizal formation by stimulating hyphal growth out of the roots and also through provoking sporulation [10, 11]. Ratti *et al.* [12] found that a combination of the arbuscular mycorrhizal fungus *Glomus aggregatum* and the PGPR *Bacillus polymyxa* and *Azospirillum brasilense* maximized biomass and P content of the aromatic grass palmarosa (*Cymbopogon martinii*) when grown with an insoluble inorganic phosphate.

9.8 Mycorrhiza

Mycorrhizae form mutualistic symbiotic relationships with plant roots of more than 80% of land plants including many important crops and forest tree species [13–15]. They possess special structures known as vesicles and arbuscules. The plant roots transmit substances (some supplied by exudation) to the fungi, and the fungi aid in transmitting nutrients and water to the plant roots. The fungal hyphae may extend the root lengths 100-fold. The hyphae reach into additional and wetter soil areas and help plants absorb many nutrients, particularly the less available mineral nutrients such as phosphorus, zinc, molybdenum, and copper. There are seven types of mycorrhizae: arbutoid mycorrhiza, ectomycorrhiza, endomycorrhiza

or arbuscular mycorrhiza, ectendomycorrhiza, ericoid mycorrhiza, monotropoid mycorrhiza, and orchid mycorrhiza [14, 16–19]. The two dominant types of mycorrhizae are ectomycorrhizae (ECM) and arbuscular mycorrhizae (AM) that can improve water and nutrient uptake and provide protection from pathogens but only a few families of plants are able to form functional associations with both AM and ECM fungi [20, 21]. Because they provide a protective cover, mycorrhizae increase seedling tolerance to drought, high temperatures, infection by disease fungi, and even extreme soil acidity. Plants that have coarse or limited root system benefit the most [2].

9.9 Inoculation of Biofertilizers

There are many ways to apply bacterial and fungal biofertilizers including seed coating, root dipping, seedling inoculation, scattering by hand, and in-furrow application with or without some carrier for the microorganisms, for example, peat, fly ash, clay materials, composts, or stickers [22].

Seed inoculation uses a specific strain of microbe that can grow in association with plant roots; soil conditions have to be favorable for the inoculants to perform well. Selected strains of N_2-fixing *Rhizobium* bacteria have proven to be effective as seed inoculants for legumes. The seed treatment can be done with any of two or more bacteria without antagonistic effect. In the case of seed treatment with *Rhizobium*, *Azotobacter*, or *Azospirillum* along with PSB, first the seeds must be coated with *Rhizobium*, *Azotobacter*, or *Azospirillum*. When each seed has a layer of the aforementioned bacteria, then the PSB inoculant has to be treated on the outer layer of the seeds. This method will provide maximum numbers of population of each bacterium to generate better results.

In soil inoculation, microbes are added directly to the soil where they have to compete with microbes already living in the soil that are already adapted to local conditions and greatly outnumber the inoculums. Inoculants of mixed cultures of beneficial microorganisms have considerable potential for controlling the soil microbiological equilibrium and providing a more favorable environment for plant growth and protection. Although inoculations with PSBs have not been very effective, joint inoculation of PSBs with mycorrhizae and N_2-fixing bacteria has been successful [2].

9.9.1 Carrier Materials for Biofertilizers

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy handling, long-term storage, and high effectiveness of biofertilizers. Among various types of biofertilizers, bacterial inoculant is one major group that includes

rhizobia, nitrogen-fixing rhizobacteria, plant growth promoting rhizobacteria, phosphate solubilizing bacteria, and so on. Various types of materials are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10–40 μm. Peat is the most frequently used carrier material for seed inoculation. Peat-based rhizobial inoculant is already used in many countries and a lot of information is available on the properties and effect of the inoculant. The properties of a good carrier material [23] for seed inoculation are as follows:

1) nontoxic to inoculant bacterial strain;
2) good moisture absorption capacity;
3) easy to process and free of lump-forming materials;
4) easy to sterilize by autoclaving or gamma irradiation;
5) available in adequate amounts;
6) inexpensive;
7) good adhesion to seeds and good pH buffering capacity;
8) nontoxic to plants.

For soil inoculation, carrier materials with granular form (0.5–1.5 mm) such as peat, perlite, charcoal, and soil aggregates are generally used. Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered.

1) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying conditions until placed into soil.
2) Survival of the inoculant bacteria during the storage period.
3) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micropore to the inoculant bacteria will be desirable. In this sense, materials with microporous structure, such as soil aggregate and charcoal, will be good carriers for soil inoculants.

Selection of carrier material is also a crucial step for formulation and application of fungal biofertilizers. Like a bacterial biofertilizer, the success of a carrier-based fungal biofertilizer depends on whether (a) it is economically viable to produce, (b) it does not alter the viability and function of the inoculum, and (c) it is easy to carry and enhance dispersal during application. The inoculum formulation may comprise one or more AM fungi and other organisms that together enhance the ability of the inoculum to form mycorrhizal associations with the target plant. The formulations are available in the form of powder, tablets/pellets or granules, gel beads, and balls [5, 22].

Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for long storage period. Gamma irradiation is the most suitable way of carrier sterilization, because the sterilization process makes almost no

change in physical and chemical properties of the material. In brief, carrier material is packed in thin-walled polyethylene bag, and then gamma irradiated at 50 kGy (5 Mrad). Another way of carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 °C. It should be noted that during autoclaving, some materials change their properties and produce toxic substance to some bacterial strains. http://www.fnca.mext.go.jp/bf/bfm/pdf/3_Carriers_for_Biofertilizer0331final.pdf.

9.10
Potential Role of Various Biofertilizers in Crop Production and Improvement

9.10.1
Bacterial Biofertilizers

Biofertilizers or microbial inoculants as an economic input play a significant role in increasing crop productivity; fertilizer (chemical and organic) doses can be lowered and more nutrients can be harvested from the soil. Under temperate conditions, inoculation of *Rhizobium* improved number of pods per plant, number of seeds per pod, and 1000-seed weight (g), and thereby yield over the control. The number of pods per plant, number of seeds per pod, and 1000-seed weight (g) recorded were 25.5, 17.1, and 4.7% more over the control, respectively, which was statistically significant [24]. In rice under lowland conditions, the application of BGA and *Azospirillum* proved significantly beneficial in improving leaf area index (LAI) and all yield attributing aspects. Grain yield and harvest index also exhibit a discernable increase with use of biofertilizers [25]. Afzal [26] found that seed and straw yield of green gram increased significantly up to single inoculation with *Rhizobium* under $20\,kg\,N + 45\,kg\,P_2O_5\,ha^{-1}$ fertility level. Field trials carried out in different locations have demonstrated that under certain environmental and soil conditions inoculation with azotobacteria has beneficial effects on plant yields. The effect of *Azotobacter chroococcum* on vegetative growth and yields of maize has been studied by numerous authors [27–32], as well as the effect of inoculation with this bacterium on wheat [33–38]. The inoculation of faba bean with *Rhizobium* constantly resulted in severe increments in the fresh and dry weights of shoot, root, nodules, number of nodules, nodule dry weight, grain yield, and N_2 fixation [39]. Inoculation with a compatible strain of rhizobia was found to enhance nodulation, dry weight of nodules, nitrogen fixation, and yield of alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*), cluster bean (*Cyamopsis tetragonolobus*), field pea (*Pisum sativum*), and common bean (*Phaseolus vulgaris*) grown in dry land. It was concluded that the productivity of leguminous crops in dry land could be improved by *Rhizobium* inoculation [40]. *Acetobacterium* species *Asaia bogorensis* [41] has been demonstrated to enhance growth of pineapple plants, probably through N_2 fixation activity or by producing phytohormones [42, 43].

Phosphate rock minerals are often too insoluble because of their fixation in acidic and alkaline soils to provide sufficient P for crop uptake. Use of PSBs can increase crop yields up to 70% [44]. Combined inoculation of arbuscular mycorrhiza and PSB gives better uptake of both native P from the soil and P coming from the phosphatic rock [45, 46]. Higher crop yields result from solubilization of fixed soil P and applied phosphates by PSB [47]. Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation [48, 49]. *Pseudomonas* sp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, and nutrient availability and uptake in soybean crop [50]. Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* [51], while co-inoculation of PSB and PGPR reduced P application by 50% without affecting corn yield [52]. Inoculation with PSB increased sugarcane yield by 12.6% [53]. Sole application of bacteria increased the biological yield, while the application of the same bacteria along with mycorrhizae achieved the maximum grain weight [54]. Single and dual inoculation along with P fertilizer was 30–40% better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20% against sole P fertilization [55]. Mycorrhiza along with *Pseudomonas putida* increased leaf chlorophyll content in barley [54]. Rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake. Seed yield of green gram was enhanced by 24% following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* [47]. Growth and phosphorus content in two alpine *Carex* species increased by inoculation with *Pseudomonas fortinii* [56]. Integration of half dose of NP fertilizer with biofertilizer gives crop yield as with full rate of fertilizer, and through reduced use of fertilizers the production cost is minimized and the net return maximized [57].

Several associations between plants and beneficial bacteria also show a protective response under restrictive environmental conditions. Wheat and faba beans subjected to saline stress showed greater growth when inoculated with *Azospirillum*, compared to noninoculated plants [58, 59]. This favorable effect may be attributable directly to bacteria or indirectly to the effect on plant physiology. The production of microbial metabolites such as polysaccharides modifies the soil structure, and has a positive effect on plants grown under water stress. Growth parameters of sunflower plants under water stress inoculated with an exopolysaccharide (EPS)-producing *Rhizobium* sp. were greater than those in uninoculated plants [60]. Promotion effect in wheat plants was also observed after inoculation with an EPS-producing *Pantoea agglomerans* isolate [61]. In wheat plants inoculated with *Paenibacillus polymyxa*, the aggregation of rhizospheric soil depended on a bacterial polysaccharide that enlarged the amount of soil adhering to roots [62, 63]. Bacteria can also stimulate the plant to turn on particular metabolic activity like increasing its exudates, and consequently, improve rhizospheric soil qualities [64]. In the same way, after inoculation of *Arabidopsis* with *P. polymyxa*, the water stress gene ERD15 is switched on [65]. Inoculated plants show improved response against pathogenic colonization and drought stress in comparison to control plants. Hence, it seems that inoculation induces protection against biotic agents, and also against abiotic ones.

9.10.2 Fungal Biofertilizers

The fungi that are probably most abundant in agricultural soils and widespread from arctic to tropics are AM fungi. They account for 5–50% of the biomass of soil microbes [66]. AM fungi belong to nine genera: *Acaulospora*, *Archaeospora*, *Entrophospora*, *Gerdemannia*, *Geosiphon*, *Gigaspora*, *Glomus*, *Paraglomus*, and *Scutellospora* [17]. They play an important role in plant growth, health, and productivity [67, 68]. AM fungi help plants to absorb nutrients, especially the less available mineral nutrients such as copper, molybdenum, phosphorus, and zinc [69]. They increase seedling tolerance to drought, high temperatures, toxic heavy metals, high or low pH, and even extreme soil acidity [2, 70, 71]. AM fungi can also affect plant growth indirectly by improving the soil structure, providing antagonistic effects against pathogens and altered water relationships [13]. AM fungi can reduce the severity of soil-borne pathogens and enhance resistance in roots against root rot disease [2, 72–74]. This results because of competition for colonization sites or nutrients in the same root tissues and production of fungistatic compounds [68, 75]. AM fungi have been shown to have benefits to host plants including increasing herbivore tolerance, pollination, soil stability, and heavy metal tolerance. The use of AM fungi as biofertilizers is not new; they have been produced for use in agriculture, horticulture, landscape restoration, and soil remediation for almost two decades [76]. It was not until the past two decades that the hypothesis of a better adaptation of mycorrhized plants to unfavorable environmental conditions was confirmed by further experiments: inoculation of AM fungi resulted in the reduction of harmful impacts of heavy metals [77], of high salt concentrations in soil [78], and of stress induced by herbicides [79], cold [80], and repotting [81]. Mycorrhized plants exhibit improved growth, in particular under dryness-induced stress conditions [82]. Other fungal biofertilizers that have been used to improve plant growth are *Penicillium* species. They are phosphate solubilizing microorganisms that improve phosphorus absorption in plants and stimulate plant growth [83, 84]. Application of *Penicillium bilaiae* can increase dry matter, phosphorus uptake, and seed yield in canola (*Brassica napus*) [85, 86]. *Penicillium radicum* and *P. italicum* are also phosphate solubilizing taxa [83, 87, 88]. Several species of *Aspergillus* have been reported to be involved in the solubilization of inorganic phosphates such as *A. flavus*, *A. niger*, and *A. terreus* [89]. These fungi are able to solubilize inorganic phosphate through the production of acids, for example, citric acid, gluconic acid, glycolic acid, oxalic acid, and succinic acid [90]. *Aspergillus fumigatus* isolated from compost has been reported to be potassium releasing fungus [91]. *Trichoderma* species can not only reduce the occurrence of disease and inhibit pathogen growth when used as mycofungicides, but also increase the growth and yield of plants [92–95], survival of seedlings, plant height, leaf area, and dry weight [96], improve mineral uptake, release minerals from soil and organic matter, enhance plant hormone production, induce systematic resistance mechanisms, and induce root systems in hydroponics [97]. For these reasons, *Trichoderma* species are known as plant growth promoting fungi [98–100].

9.11 Conclusions

Application of microorganisms as biofertilizers has shown great potential as an effective means in the development and growth of crop plants under stressed environmental conditions. However, regardless of application methods, the number of viable cells reaching the field soil from commercial biofertilizers is smaller than the existing number in soil or rhizosphere microorganisms; hence, the added microorganisms are unlikely to have a beneficial impact on the plant unless multiplication occurs. Moreover, the population of introduced microorganisms will decline and be eliminated in a very short time, often days or weeks. The formulation of inoculums, method of application, and storage of the product are all critical to the success of a biological product. Poor shelf life, lack of suitable carrier materials, susceptibility to high temperature, and problems in transportation and storage are main constraints of biofertilizers that still need to be solved in order to obtain effective inoculation. Thus, more and more field-based research is required to remove existing bottlenecks and to get optimum benefit from biofertilizers. Technical training of manufacturers on standard production processes and quality control, organization of regular technical training to the extension workers and farmers to adopt and popularize the technology should also be encouraged.

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Part III
Species-Specific Case Studies

Section IIIA: Graminoids

Improving Crop Productivity in Sustainable Agriculture, First Edition.
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10
Rice: Genetic Engineering Approaches for Abiotic Stress Tolerance – Retrospects and Prospects

Salvinder Singh, M.K. Modi, Sarvajeet Singh Gill, and Narendra Tuteja

Abstract

Abiotic stresses, especially salinity and drought, are the primary causes of crop loss worldwide. The moisture availability is essential for plant growth and agricultural production. Soil moisture stress causes significant water deficits that lead to low production and ultimately result in significant economic losses worldwide. A major limitation of crop production is imposed by a suite of stresses, resulting in 30–60% yield losses globally each year. Minimizing these losses is a major area of concern for all countries. Engineered abiotic stress resistance is an important target for increasing agricultural productivity. Therefore, it is desirable to develop multi-stress-tolerant varieties. Plants are nonmobile organisms and therefore need to adapt to environmental stresses mostly by modulating their growth and development in addition to physiological and biochemical changes. The plant life style of continuous development also requires proper maintenance of many important functions in the stress environment. Plants may acclimate to the limited stress conditions by integrating stress information with developmental programs. Plant adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Consequently, engineering genes that protect and maintain the function and structure of cellular components can enhance tolerance to stress. Rice (*Oryza sativa*) is a staple food for more than half the world and a model for studies of monocotyledonous species. The rice is also frequently impacted by several abiotic stressors, the most important of which are drought, salinity, and cold. Exposure to environmental conditions outside of acceptable tolerance ranges can negatively affect rice growth and production. In this chapter, plant, particularly rice, responses to abiotic stress are presented, with particular attention to the genes and pathways related to environmental stress tolerance. It is apparent that, while progress has been made in identifying genes involved in stress adaptation, many questions remain. Understanding the mechanisms of stress response in rice is important for all research designed to develop new rice varieties with improved tolerance.

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10.1 Introduction

Plant growth and productivity can be adversely affected by abiotic stress. Plants are exposed to a number of potentially adverse environmental conditions such as water deficit, high salinity, extreme temperature, and submergence. In response, plants have evolved delicate mechanisms, from the molecular to the physiological level, to adapt to stressful environments.

Rice is the staple food for more than half of the world's population. Evolved in a semiaquatic, low-radiation habitat, rice exhibits distinct tolerance and susceptibilities to abiotic stresses among domesticated cereal crops [1]. Rice thrives in waterlogged soil and can tolerate submergence at levels that would kill other crops, and is moderately tolerant of salinity and soil acidity but is highly sensitive to drought and cold [1]. Cultivated over a broad region between 45° north and south latitudes, rice plants are faced with low temperature in temperate regions, submergence in tropical regions, water deficit in humid tropics, and other stressors [2].

Arabidopsis is a good model in plant molecular biology and genetics research, and the majority of studies examining the impacts of abiotic stress have employed this plant [3–7]. The signaling pathways and regulatory network in *Arabidopsis* have been well characterized and well reviewed. Although progresses were also made on rice, reviews were less focused on this most important crop because of little functional genes characterized. In recent years, multiple genes contributing to abiotic stress responses in rice were identified by using genetics, reverse genetics, and molecular biology method. Here, we present a summary to display those progresses so as to better understand genetics and molecular mechanisms of crops' response to abiotic stress.

10.2 Single Action Genes

10.2.1 Osmoprotectants

Severe osmotic stress causes detrimental changes in cellular components. In stress-tolerant transgenic plants, many genes involved in the synthesis of osmoprotectants – organic compounds such as amino acids (e.g., proline), quaternary and other amines (e.g., glycine betaine and polyamines), and a variety of sugars and sugar alcohols (e.g., mannitol, trehalose, and galactinol) that accumulate during osmotic adjustment – have been used to date [8]. Many crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress-tolerant organisms. It is believed that osmoregulation would be the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity, and high temperature.

Therefore, a widely adopted strategy has been to engineer certain osmolytes or overexpress such osmolytes in plants, as a potential route to breed stress-tolerant crops, particularly rice.

Various strategies are being pursued to genetically engineer osmoprotection in plants. The first step involved in obtaining stress-tolerant transgenic plants has been to engineer genes that encode enzymes for the synthesis of selected osmolytes [9]. This has resulted in a profusion of reports involving osmoprotectants such as glycine betaine [10–19] and proline [20–23]. Iskandar *et al.* [24] demonstrated that the expression of genes encoding proline biosynthesis was associated with both sucrose accumulation and water deficit, but amino acid analysis indicated that proline was negatively correlated with sucrose concentration, and while total amino acid concentrations increased about sevenfold under water deficit, the relatively low concentration of proline suggested that it had no osmoprotectant role in sugarcane culms. The results show that while there was a change in stress-related gene expression associated with sucrose accumulation, different mechanisms respond to the stress induced by water deficit, because different genes had altered expression under water deficit [24]. Similarly, Dobra *et al.* [25] reported the possibility of improving tolerance to heat and drought (alone and in combination) by elevation of the osmoprotectant proline content; stress responses were compared in tobacco plants constitutively overexpressing a gene for the proline biosynthetic enzyme Δ(2)-pyrroline-5-carboxylate synthetase (P5CSF129A; EC 2.7.2.11/1.2.1.41) and in the corresponding wild-type (WT) plants. A number of "sugar alcohols" (mannitol, trehalose, myo-inositol, and sorbitol) have been targeted for the engineering of compatible solute overproduction, thereby protecting the membrane and protein complexes during stress [26–37]. *Arabidopsis* plants transformed with the mannose-6-phosphate reductase (M6PR) gene from celery were dramatically more salt tolerant (at 100 mM NaCl) as exhibited by reduced salt injury, less inhibition of vegetative growth, and increased seed production relative to the wild-type plants [38]. In this study, transcriptome analysis reveals that the M6PR transgene activated the downstream abscisic acid (ABA) pathway by upregulation of ABA receptor genes (PYL4, PYL5, and PYL6) and downregulation of protein phosphatase 2C genes (ABI1 and ABI2). In the M6PR transgenic lines, there were also increases in transcripts related to redox and cell wall strengthening pathways. These data indicate that mannitol-enhanced stress tolerance is due at least in part to increased expression of a variety of stress-inducible genes [39]. Ahn *et al.* [40] recently reported that the methylation of myo-inositol yields *O*-methyl inositol (D-ononitol) catalyzed by myo-inositol methyltransferase (IMT) when plants are subjected to abiotic stress. D-Ononitol can serve as an osmoprotectant that prevents water loss in plants. The authors isolated the IMT cDNA from *Glycine max* and found by RT-PCR analysis that GmIMT transcripts are induced by drought and salinity stress treatments in the leaves of soybean seedlings. They confirmed the protein product of GmIMT and its substrate using a recombinant system in *Escherichia coli*. Transgenic *Arabidopsis* plants overexpressing GmIMT displayed improved tolerance to dehydration stress treatment and to a lesser extent high salinity stress treatment.

Tonoplast intrinsic protein (TIP) is a subfamily of the aquaporin (AQP), also known as major intrinsic protein (MIP) family, and regulates water movement across vacuolar membranes. Some reports have implied that TIP genes are associated with plant tolerance to some abiotic stresses that cause water loss, such as drought and high salinity. Wang *et al*. [41] isolated TIP from cDNA library screening of *Glycine soja*, and named it as GsTIP2;1. The expression patterns of GsTIP2;1 in *G. soja* under low-temperature, salt, and dehydration stresses were different in leaves and roots. Though GsTIP2;1 is a stress-induced gene, overexpression of GsTIP2;1 in *Arabidopsis thaliana* depressed tolerance to salt and dehydration stresses, but did not affect seedling growth under cold or favorable conditions. Higher dehydration speed was detected in *Arabidopsis* plants overexpressing GsTIP2;1, implying that GsTIP2;1 might mediate stress sensitivity by enhancing water loss in the plant. Such a result is not identical to previous reports, providing some new information about the relationship between TIP and plant abiotic stress tolerance [41].

Similarly, transgenics engineered for the overexpression of polyamines have also been developed [42–47]. Studies on the identification/isolation/cloning of genes that are associated with improved flooding stress tolerance have also focused on enzymes of the glycolytic and alcohol fermentation pathways indicating that respiratory pathway is affected in a major way in response to anaerobic stress. Research on genetically altering the levels of *pdc* and *adh* in tobacco and rice has been extensively carried out to elucidate their role in submergence tolerance. Transgenic rice over- and underexpressing pyruvate decarboxylase 1 (*pdc1*) gene has also been developed, which showed a positive correlation of higher PDC activities with survival after submergence [48].

The results of transgenic modifications of biosynthetic and metabolic pathways in most of the above-mentioned cases indicate that higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species (ROS) and by their chaperone-like activities in maintaining protein structures and functions [49–52]. However, pleiotropic effects (e.g., necrosis and growth retardation) have been observed due to disturbance in endogenous pathways of primary metabolism. Also, there are also some reports showing a negative effect of osmotic stress on yield potential [53]. Genetic manipulations of compatible solutes do not always lead to a significant accumulation of the compound (except in some cases of proline overproduction [54]), thereby suggesting that the function of compatible solutes is not restricted to osmotic adjustment, and that osmoprotection may not always confer drought tolerance. A recent review [55] shows that virtually none of the studies that tested the effect of osmotic adjustment on yield under water stress showed any benefit at all, since some benefit of osmotic adjustment might be in the ability of plants to maintain root growth under severe stress [56]. Another recent study with chickpea has also shown that osmotic adjustment provided no beneficial effect on yield under drought stress [57]. Besides, the results of simulation modeling also suggest that changes in a given metabolic process [58, 59] may end up with little benefit for actual yield under stress [60]. For agricultural practices, oversynthesis of compatible solutes should

not account for the primary metabolic costs, and hence to minimize the pleiotropic effects, overproduction of compatible solutes should be stress inducible and/or tissue specific [61].

10.2.2 Late Embryogenesis Abundant Proteins

Proteins that may protect macromolecules and membranes include Late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, chaperones, and mRNA binding proteins. LEA proteins represent another category of high molecular weight proteins that are abundant during late embryogenesis and accumulate during seed desiccation and in response to water stress [62]. Among the several groups of LEA proteins, those belonging to group 3 are predicted to play a role in sequestering ions that are concentrated during cellular dehydration. These proteins have 11-mer amino acid motifs with the consensus sequence TAQAAKEKAGE repeated as many as 13 times [63]. The group 1 LEA proteins are predicted to have enhanced water binding capacity, while the group 5 LEA proteins are thought to sequester ions during water loss. Constitutive overexpression of the HVA1, a group 3 LEA protein from barley, conferred tolerance to soil water deficit and salt stress in transgenic rice plants [64]. Constitutive or stress-induced expression of the HVA1 gene resulted in the improvement of growth characteristics and stress tolerance in terms of cell integrity in wheat and rice under salt and water stress conditions [65, 66]. More than 100 group 1 LEA genes, also termed Em genes, have been identified from plants, bacteria, and animals [67]. The wide distribution indicates the functional importance of these genes. Shih *et al.* [67] characterized a novel Em-like gene, OsLEA1a of rice (*Oryza sativa*). The encoded OsLEA1a protein has an N-terminal sequence similar to that of other plant Em proteins but lacks a 20-mer motif that is the most significant feature of typical Em proteins. The location of the sole intron indicates that the second exon of OsLEA1a is the mutated product of a typical Em gene. Transcriptome analysis revealed OsLEA1a mainly expressed in embryos, with no or only a few transcripts in osmotic stress-treated vegetative tissues. Structural analysis revealed that the OsLEA1a protein adopts high amounts of disordered conformations in solution and undergoes desiccation-induced conformational changes [67].

Hanin *et al.* [68] reported the accumulation of dehydrins (DHNs) or group 2 LEA proteins typically in maturing seeds or in vegetative tissues following salinity, dehydration, cold, and freezing stresses. In yet another study, a LEA gene, CarLEA4 (GenBank Accession No. GU247511), was isolated from chickpea based on a cDNA library constructed with chickpea seedling leaves treated with polyethylene glycol (PEG) [68]. CarLEA4 contained two exons and one intron within genomic DNA sequence and encoded a putative polypeptide of 152 amino acids. CarLEA4 had a conserved pfam domain and showed high similarity to the group 4 LEA proteins in secondary structure. It was localized in the nucleus. The transcripts of CarLEA4 were detected in many chickpea organs, including seedling leaves, stems, roots, flowers, young pods, and young seeds. CarLEA4 was

inhibited by leaf age and showed expression changes in expression during seed development, pod development, and germination. Furthermore, the expression of CarLEA4 was strongly induced by drought, salt, heat, cold, ABA, IAA, GA(3), and MeJA. The authors suggest that CarLEA4 encodes a protein of LEA group 4 and may be involved in various plant developmental processes and abiotic stress responses [68]. A LEA protein gene OsLEA3-1 was identified and overexpressed in rice to test the drought resistance of transgenic lines under the field conditions. OsLEA3-1 is induced by drought, salt, and abscisic acid, but not by cold stress [69]. The author used three expression constructs consisting of the full-length cDNA driven by the drought-inducible promoter of OsLEA3-1 (OsLEA3-H), the CaMV 35S promoter (OsLEA3-S), and the rice Actin1 promoter (OsLEA3-A) and transformed them into the drought-sensitive japonica rice Zhonghua 11. Drought resistance prescreening of T(1) families at anthesis stage revealed that the overexpressing families with OsLEA3-S and OsLEA3-H constructs had significantly higher relative yield (yield under drought stress treatment/yield under normal growth conditions) than the wild type under drought stress conditions, although a yield penalty existed in T(1) families under normal growth conditions. Nine homozygous families, exhibiting overexpression of a single copy of the transgene and relatively low yield penalty in the T(1) generation, were tested in the field for drought resistance in the T(2) and T(3) generations and in the PVC pipes for drought tolerance in the T(2) generation. Except for two families (transformed with OsLEA3-A), all the other families (transformed with OsLEA3-S and OsLEA3-H constructs) had higher grain yield than the wild type under drought stress in both the field and the PVC pipes conditions. No significant yield penalty was detected for these T(2)and T(3) families. These results indicate that transgenic rice with significantly enhanced drought resistance and without yield penalty can be generated by overexpressing OsLEA3-1 gene with appropriate promoters and following a bipartite (stress and nonstress) in-field screening protocol [69].

10.2.3 Detoxifying Genes

The higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species and by their chaperone-like activities in maintaining protein structures and functions [49–52]. In most of the aerobic organisms, there is a need to effectively eliminate reactive oxygen species generated as a result of environmental stresses. Depending on the nature of the ROS, some are highly toxic and need to be rapidly detoxified. In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge the ROS. These antioxidant systems include various enzymes and nonenzymatic metabolites that may also play a significant role in ROS signaling in plants [70]. A number of transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy. These include transgenic plants overexpressing enzymes involved in

oxidative protection, such as glutathione peroxidase (GPX), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase [71, 72]. Kim *et al*. [73] demonstrated proteomic analysis of ROS-related proteins in rice roots. The authors applied the PEG fractionation technique combined with two-dimensional gel electrophoresis, which rendered more well-separated protein spots. Out of the 295 chosen proteins, 93 were identified by MALDI-TOF mass spectrometry. The proteins were classified as relating to metabolism (38.7%), ROS-related proteins (22.5%), protein processing/degradation (8.6%), stress/defense (7.5%), energy (6.5%), and signal transduction (5.4%). The high percentage of ROS-related proteins found in rice root indicated the role of ROS in rice root growth. Treatment with ROS quenching chemicals such as reduced glutathione (GSH), diphenyleneiodonium (DPI), and ascorbate inhibited root growth dose dependently. Forty-nine proteins identified were either up- or downregulated by GSH treatment, of which 14 were ROS-related proteins, such noticeably modulated ones as glutathione S-transferase (GST), superoxide dismutase, and L-ascorbate peroxidase. The protein levels of four GSTs (NS4, NS8, NS56, and NS57), three APXs (NS46, NS49, and NS50), and MnSOD (NS45) were strongly reduced by GSH treatment but slightly reduced by ascorbate and DPI. Ascorbate and DPI strongly inhibited expression levels of a catalase A (NP23) and an APX (NS65) but did not affect APX (NS46, NS49, and NS50) protein levels. Northern analysis demonstrated that changes in transcript levels of five genes – GST (NS4), GST (NS43), MnSOD (NS45), APX (NS50), and APX (NS46/49) – in response to ROS quenching chemicals were coherent with patterns shown in two-dimensional electrophoresis analyses. The authors concluded that these proteins may play an important role in maintaining cellular redox homeostasis during rice root growth [73].

Similarly, transgenic tobacco plants overexpressing SOD in the chloroplast, mitochondria, and cytosol have been generated [74, 75] and these have been shown to enhance tolerance to oxidative stress induced by methyl viologen (MV) in leaf disk assays. Overexpression of chloroplast Cu/ZnSOD showed a dramatic improvement in the photosynthetic performance under chilling stress conditions in transgenic tobacco [76] and potato plants [77]. While transgenic alfalfa (*Medicago sativa*) plants cv. RA3 overexpressing MnSOD in chloroplasts showed lower membrane injury [78], the tobacco transgenic plants overproducing alfalfa aldose reductase gene (MsALR) showed lower concentrations of reactive aldehydes and increased tolerance against oxidative agents and drought stress [79]. Tobacco transgenic plants overexpressing MnSOD showed enhanced tolerance to oxidative stress only in the presence of other antioxidant enzymes and substrates [80], thereby showing that the genotype and the isozyme composition also have a profound effect on the relative tolerance of the transgenic plants to abiotic stress [81]. Oxidative stress-related genes such as *ApGPX2* and *AcGPX2* (GPX-like proteins), *ALR* (aldose/aldehyde reductase), *Apx1* (ascorbate peroxidase), *APX2* (ascorbate peroxidase), *Apx3* (ascorbate peroxidase), *Apx3* (ascorbate peroxidase), *Apx* (ascorbate peroxidase), *AO* (ascorbate oxidase), *AtMDAR1* (monodehydroascorbate reductase; ascorbate regeneration), *DHAR* (regeneration of ascorbate), *Gly1;gly2* (glutathione-based detoxification of methylglyoxal), *GmTP55* (antiquitin-like protein), *GST* (glutathione

S-transferase overexpression), *GST/GPX* (glutathione S-transferase with glutathione peroxidase), *GPX* (glutathione peroxidase), *katE* (*E. coli* catalase), *ndhCKJ* (NADPH dehydrogenase), *NtPox* (glutathione peroxidase), *Nt107* (glutathione S-transferase), *parB* (glutathione S-transferase), *SOD* (Cu-, Mn-, Fe-, ZnSOD), *SOD* (Cu/Zn superoxide dismutase), *SOD* (Fe superoxide dismutase), *SOD* (Mn superoxide dismutase), vtc1, vtc2, npq1, and cad2 (reactive oxygen metabolism mutants), and vtc-1 (ascorbate-deficient mutant) were transferred in many crops.

10.2.4 Multifunctional Genes for Lipid Biosynthesis

Transgenic approaches also aim to improve photosynthesis under abiotic stress conditions through changes in the lipid biochemistry of the membranes [82]. Adaptation of living cells to chilling temperatures is a function of alteration in the membrane lipid composition by increased fatty acid unsaturation. The accumulation of toxic compounds generated by the interaction between reactive oxygen species and polyunsaturated fatty acids of membrane lipids can significantly damage plant cells [83]. Three rice stress-induced aldo-keto reductase (AKR) genes have been studied. The transcription level of OsAKR1 was greatly induced by abscisic acid and various stress treatments; the other two AKR genes tested were moderately stress inducible. The OsAKR1 recombinant protein exhibited a high nicotinamide adenine dinucleotide phosphate-dependent catalytic activity to reduce toxic aldehydes including glycolysis-derived methylglyoxal (MG) and lipid peroxidation-originated malondialdehyde (MDA). The function of this enzyme in MG detoxification was demonstrated *in vivo* in *E. coli* and in transgenic plants overproducing the OsAKR1 protein. Heterologous synthesis of the OsAKR1 enzyme in transgenic tobacco plants resulted in increased tolerance against oxidative stress generated by methyl viologen and improved resistance to high temperature. In these plants, lower levels of MDA were detected following both MV and heat treatment due to the activity of the OsAKR1 enzyme. The transgenic tobacco plants also exhibited higher AKR activity and accumulated less MG in their leaves than the wild-type plants, both in the presence and in the absence of heat stress. These results support the positive role of OsAKR1 in abiotic stress-related reactive aldehyde detoxification pathways and its use for improvement of stress tolerance in plants [83]. Genetically engineered tobacco plants overexpressing chloroplast glycerol-3-phosphate acyltransferase (GPAT) gene (involved in phosphatidylglycerol fatty acid desaturation) from squash (*Cucurbita maxima*) and *A. thaliana* [84] showed an increase in the number of unsaturated fatty acids and a corresponding decrease in the chilling sensitivity. Besides, transgenic tobacco plants with silenced expression of chloroplast x3-fatty acid desaturase (Fad7, which synthesizes trienoic fatty acids) were able to acclimate to high temperature as compared to the wild-type plants [85].

10.2.5
Heat Shock Protein Genes

Plants respond to heat stress by enhancing the expression of genes encoding heat shock protein (HSP) genes through activation of heat shock factors (HSFs) that interact with heat shock elements (HSEs) present in the promoter of HSP genes. Plant HSFs have been divided into three conserved classes, namely, A, B, and C [86]. The authors showed the expression profile through microarray analysis and quantitative real-time PCR showed that eight OsHsfs express at a higher level during seed development, while six HSFs are upregulated in all the abiotic stresses studied. The expression of OsHsfA2a gene in particular was greatly stimulated by heat stress in both root and shoot tissues and also during panicle and seed development. OsHsfA3 was found more responsive to cold and drought stress, while OsHsfA7 and OsHsfA9 showed developing seed-specific expression. This study also revealed that spliced variants generally accumulated at a higher level in all the tissues examined. Different hormones/elicitors such as ABA, brassinosteroids, and salicylic acid also alter OsHsf gene expression. Calcium in combination with heat stress further elevated the level of HSF transcripts. Expression analysis by both microarray and real-time RT-PCR revealed a unique stable constitutive expression of OsHsfA1 across all the tissues and stresses. A detailed *in silico* analysis involving identification of unidentified domains has been done by the MEME motif tool in their full-length proteins as well as in DNA binding domains. Analysis of 1 kb putative promoter region revealed presence of tissue-specific, abiotic stress and hormone-related *cis*-acting elements, correlating with expression under stress conditions [86]. Mittal *et al.* [87] demonstrated that the binding of Hsfs to HSEs also leads to transcriptional regulation of heat shock genes. Genome-wide, 953 rice genes contain perfect-type, 695 genes contain gap-type, and 1584 genes contain step-type HSE sequences in their 1 kb promoter region [87]. The rice genome contains 13 class A, 8 class B, and 4 class C Hsfs (OsHsfs) and has OsHsf26 (which is of variant type) genes. Chemical cross-linking analysis of *in vitro* synthesized OsHsf polypeptides showed formation of homotrimers of OsHsfA2c, OsHsfA9, and OsHsfB4b proteins. Binding analysis of polypeptides with oligonucleotide probes containing perfect-, gap-, and step-type HSE sequences showed that OsHsfA2c, OsHsfA9, and OsHsfB4b differentially recognize various model HSEs as a function of varying reaction temperatures. These differential patterns pertaining to binding with HSEs and protein–protein interactions may have a bearing on the cellular functioning of OsHsfs under a range of different physiological and environmental conditions [87].

Genetic engineering for increased thermotolerance by enhancing heat shock protein synthesis in plants has been achieved in a number of plant species [88–90]. There have been a few reports on positive correlations between the levels of heat shock proteins and stress tolerance [91, 92]. Although the precise mechanism by which these heat shock proteins confer stress tolerance is not known, a recent study demonstrated that *in vivo* function of thermoprotection of small heat shock

proteins is achieved via their assembly into functional stress granules (HSGs [93]). Genes encoding for molecular chaperones, for example, *APG6* (chloroplast structure), *atDjA2* and *atDjA3* (J-domain molecular chaperone family), *AtMTP3* (metal tolerance protein), *Atsbp1* (selenium binding protein), *atRZ-1a* (RNA chaperone protein), *BiP* (endoplasmic reticulum binding protein), *CaHSP26* (chloroplast-localized small heat shock protein), *hs* (heat shock transcription factor), *Hsp101* (heat shock protein), *Hsp17.7* (heat shock protein), *Hsp70* (heat-inducible antisense HSP70), *LeHSP100/ClpB* (chloroplast HSP), *mHSP22* (mitochondrial small HSP), *P5CR* (inducible heat shock promoter (IHSP)), *pBE2113/hiC6* (overexpressed HIC6 cryoprotective protein), *S1pt::ECS* (glutamylcysteine synthetase), *TLHS1* (overexpressed class I cytosolic small HSP), and *wx* (control amylose synthesis), were used for transformation in crop plants.

10.2.6
Regulatory Genes

Many genes that respond to multiple stresses such as dehydration and low temperature at the transcriptional level are also induced by ABA [94], which protects the cell from dehydration [95, 96]. In order to restore the cellular function and make plants more tolerant to stress, transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels [97]. To overcome such constraints, enhancing tolerance toward multiple stresses by a gene encoding a stress-inducible transcription factor (TF) that regulates a number of other genes is a promising approach [98, 99]. Therefore, a second category of genes of recent preference for crop genetic engineering includes those that switch on transcription factors regulating the expression of several genes related to abiotic stresses.

10.2.7
Transcription Factors

An attractive target category for manipulation and gene regulation is the small group of transcription factors that have been identified to bind to promoter regulatory elements in genes that are regulated by abiotic stresses [100, 101]. During the past decade, many transcription factors, belonging to different families, have been shown to act as positive or negative regulators of stress-responsive genes, thus playing an extremely important role in stress signaling [102]. The transcription factors also activate cascades of genes that act together in enhancing tolerance toward multiple stresses. Dozens of transcription factors are involved in the plant response to drought stress [8, 103]. Most of these fall into several large transcription factor families, such as AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc finger, and WRKY. Individual members of the same family often respond differently to various stress stimuli. On the other hand, some stress-responsive genes may share the same transcription factors, as indicated by the significant overlap of the gene expression profiles that are induced in response to different stresses [54, 104]. Transcriptional

activation of stress-induced genes has been possible in transgenic plants overexpressing one or more transcription factors that recognize promoter regulatory elements of these genes. Two families, bZIP and MYB, are involved in ABA signaling and its gene activation. Many ABA-inducible genes share the (C/T) ACGTGGC consensus, *cis*-acting ABA-responsive element (ABRE) in their promoter regions [105]. Introduction of transcription factors in the ABA signaling pathway can also be a mechanism of genetic improvement of plant stress tolerance. Constitutive expression of ABF3 or ABF4 demonstrated enhanced drought tolerance in *Arabidopsis*, with altered expression of ABA/stress-responsive genes, for example, rd29B, rab18, ABI1, and ABI2 [106]. Several ABA-associated phenotypes, such as ABA hypersensitivity and sugar hypersensitivity, were observed in such plants. Moreover, salt hypersensitivity was observed in ABF3- and ABF4-overexpressing plants at the germination and young seedling stages, indicating the possible participation of ABF3 and ABF4 in response to salinity at these particular developmental stages. Improved osmotic stress tolerance in 35S:At-MYC2/AtMYB2 transgenic plants as judged by an electrolyte leakage test was reported by Abebe *et al*. [29]. Transgenic *Arabidopsis* plants constitutively overexpressing a cold-inducible transcription factor (CBF1; CRT/DRE binding protein) showed tolerance to freezing without any negative effect on the development and growth characteristics [107]. Overexpression of *Arabidopsis* CBF1 (CRT/DRE binding protein) has been shown to activate cor homologous genes at nonacclimating temperatures [108]. The CBF1 cDNA when introduced into tomato (*Lycopersicon esculentum*) under the control of a CaMV35S promoter improved tolerance to chilling, drought, and salt stresses but exhibited dwarf phenotype and reduction in fruit set and seed number [109]. Another transcriptional regulator, Alfin1, when overexpressed in transgenic alfalfa (*M. sativa* L.) plants regulated endogenous MsPRP2 (NaCl-inducible gene) mRNA levels, resulting in salinity tolerance, comparable to a few available salt-tolerant plants [110]. Lee *et al*. [111] produced thermotolerant *Arabidopsis* plants by derepressing the activity of ATHSF1, a heat shock transcription factor leading to the constitutive expression of heat shock proteins at normal temperature.

Several stress-induced cor genes such as rd29A, cor15A, kin1, and cor6.6 are triggered in response to cold treatment, ABA, and water-deficit stresses [112]. There have been numerous efforts in enhancing tolerance toward multiple stresses such as cold, drought, and salt stresses in crops other than the model plants such as *Arabidopsis*, tobacco, and alfalfa. DREBs (dehydration-responsive element binding factors) are important plant transcription factors that regulate the expression of many stress-inducible genes mostly in an ABA-independent manner and play a critical role in improving the abiotic stress tolerance of plants by interacting with a DRE/CRT *cis*-element present in the promoter region of various abiotic stress-responsive genes [113]. An increased tolerance to freezing and drought in *Arabidopsis* was also achieved by overexpressing CBF4, a close CBF/DREB1 homologue whose expression is rapidly induced during drought stress and by ABA treatment, but not by cold [114]. Similarly, a *cis*-acting element, dehydration-responsive element (DRE) identified in *A. thaliana*, is also involved in ABA-independent gene expression under drought, low-temperature, and high salt stress conditions in

many dehydration-responsive genes such as rd29A that are responsible for dehydration- and cold-induced gene expression [115–117]. Several cDNAs encoding the DRE binding proteins, DREB1A and DREB2A, have been isolated from *A. thaliana* and shown to specifically bind and activate the transcription of genes containing DRE sequences [118]. DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression. The transcriptional activation of stress-induced genes has been possible in transgenic plants overexpressing one or more transcription factors that recognize regulatory elements of these genes. In *Arabidopsis*, the transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes [100]. DREB1A cDNA under the control of CaMV 35S promoter in transgenic plants elicits strong constitutive expression of the stress-inducible genes and brings about increased tolerance to freezing, salt, and drought stresses [118]. Strong tolerance to freezing stress was observed in transgenic *Arabidopsis* plants that overexpress CBF1 (DREB1B) cDNA under the control of the CaMV 35S promoter [107]. Subsequently, the overexpression of DREB1A has been shown to improve the drought and low-temperature stress tolerance in tobacco, wheat, and groundnut [119–123]. The use of stress-inducible rd29A promoter minimized the negative effects on plant growth in these crop species. However, overexpression of DREB2 in transgenic plants did not improve stress tolerance, suggesting involvement of post-translational activation of DREB2 proteins [118]. Recently, an active form of DREB2 was shown to transactivate target stress-inducible genes and improve drought tolerance in transgenic *Arabidopsis* [124]. The DREB2 protein is expressed under normal growth conditions and activated by osmotic stress through post-translational modification in the early stages of the osmotic stress response.

Water-deficit stress is detrimental for rice growth, development, and yield. Transcriptome analysis of 1-week-old rice (*O. sativa* L. var. IR64) seedlings under water-deficit stress conditions using Affymetrix 57K GeneChip® has revealed 1563 and 1746 genes to be up- and downregulated, respectively [125]. In an effort to amalgamate data across laboratories, the authors identified 5611 differentially expressing genes under varying extrinsic water-deficit stress conditions in six vegetative and one reproductive stage of development in rice. Transcription factors involved in ABA-dependent and ABA-independent pathways have been found to be upregulated during water-deficit stress. Members of zinc-finger TFs, namely, C_2H_2, C_2C_2, C_3H, LIM, PHD, WRKY, ZF-HD, and ZIM, along with TF families such as GeBP, jumonji, MBF1, and ULT, express differentially under water-deficit conditions. NAC (NAM, ATAF, and CUC) TF family emerges to be a potential key regulator of multiple abiotic stresses. Among the 12 TF genes that are co-upregulated under water-deficit, salt, and cold stress conditions, 5 belong to the NAC TF family. They identified water-deficit stress-responsive genes encoding key enzymes involved in biosynthesis of osmoprotectants such as polyols and sugars; amino acid and quaternary ammonium compounds; cell wall loosening and structural components; cholesterol and very long chain fatty acids; and cytokinin and secondary metabolites. Comparison of genes responsive to water-deficit stress conditions with genes preferentially expressed during panicle and seed development revealed a significant

overlap of transcriptome alteration and pathways [125]. Another ABA-independent, stress-responsive, and senescence-activated gene expression involves ERD gene, the promoter analysis of which further identified two different novel *cis*-acting elements involved in dehydration stress induction and in dark-induced senescence [126]. Similarly, transgenic plants developed by expressing a drought-responsive AP2-type TF, SHN1-3 or WXP1, induced several wax-related genes, resulting in enhanced cuticular wax accumulation and increased drought tolerance [127, 128]. Thus, clearly, the overexpression of some drought-responsive transcription factors can lead to the expression of downstream genes and the enhancement of abiotic stress tolerance in plants [129].

The regulatory genes/factors reported so far play a significant role not only in drought and salinity stresses, but also in submergence tolerance. More recently, an ethylene response factor (ERF)-like gene Sub1A, one of the cluster of three genes at the Sub1 locus, has been identified in rice and the overexpression of Sub1A-1 in a submergence-intolerant variety conferred enhanced submergence tolerance to the plants [130], thus confirming the role of this gene in submergence tolerance in rice.

10.2.8
Other Transcription Factors

Other kinds of transcription factors also play vital roles in transcriptional regulation during stress conditions, although little information is available about their upstream regulators or direct targets. It has been demonstrated that Sub1A-1, an ethylene response factor, is the major genetic determinant for submergence tolerance in rice [130]. Sub1A-1 finely modulates acclimation responses to sudden and total inundation to maintain the capacity of regrowth when water subsides. For example, Sub1A-1 inhibits leaf elongation by suppressing the expression of expansin-encoding genes and restrains carbohydrate consumption by reducing the expression of α-amylose genes and sucrose synthase genes [131].

Although multiple *cis*-elements and *trans*-factors for transcriptional regulation under abiotic stress have been identified and characterized, a clear explanation of the complex transcription network and the huge number of abiotic stress-responsive genes remains elusive. For example, it was found that only 12% of the cold-responsive genes in *Arabidopsis* are regulated by CBF/DREB [132]. In addition, some of the CBF/DREB regulating genes have no DRE/CRT element in their promoters. It is very likely that these genes are controlled by CBF/DREB regulating transcriptional factors (e.g., RAP2.1) [132]. Chen *et al.* [133] used transcription factor microarray analysis to demonstrate that at least 30 transcription factors in *Arabidopsis* were induced by abiotic stress. A similar situation exists in rice. Microarray analysis revealed that transcription factor genes are rich within the group of the earliest salt-responsive genes [134]. Considering the spatial and temporal expression pattern of the abiotic stress-responsive genes, it is highly likely that many transcription factors and regulatory pathways related to abiotic stress remain to be identified [102].

10.2.9 Signal Transduction Genes

Genes involved in stress signal sensing and a cascade of stress signaling in *A. thaliana* have been of recent research interest [101, 135]. Components of the same signal transduction pathway may also be shared by various stress factors such as drought, salt, and cold [135]. Although there are multiple pathways of signal transduction systems operating at the cellular level for gene regulation, ABA is a known component acting in one of the signal transduction pathways, while others act independently of ABA. The early response genes have been known to encode transcription factors that activate downstream delayed response genes [7]. Although specific branches and components exist [136], the signaling pathways for salt, drought, and cold stresses all interact with ABA, and even converge at multiple steps [137]. Abiotic stress signaling in plants involves receptor-coupled phosphorelay, phosphoinositol-induced Ca^{2+} changes, mitogen-activated protein kinase (MAPK) cascade, and transcriptional activation of stress-responsive genes. A number of signaling components are associated with the plant response to high-temperature, freezing, drought, and anaerobic stresses [138].

One of the merits for the manipulation of signaling factors is that they can control a broad range of downstream events that can result in superior tolerance for multiple aspects [139]. Alteration of these signal transduction components is an approach to reduce the sensitivity of cells to stress conditions or induce a low level of constitutive expression of stress genes [140]. Overexpression of functionally conserved At-DBF2 (homologue of yeast DBF2 kinase) showed striking multiple stress tolerance in *Arabidopsis* plants [141]. Pardo *et al.* [142] also achieved salt stress-tolerant transgenic plants by overexpressing calcineurin (a Ca^{2+}/calmodulin-dependent protein phosphatase), a protein phosphatase known to be involved in salt stress signal transduction in yeast. Transgenic tobacco plants produced by altering stress signaling through functional reconstitution of activated yeast calcineurin opened up new routes not only for study of stress signaling, but also for engineering transgenic crops with enhanced stress tolerance [140]. Overexpression of an osmotic stress-activated protein kinase, SRK2C, resulted in a higher drought tolerance in *A. thaliana*, which coincided with the upregulation of stress-responsive genes [143]. Rao *et al.* [144] reported that mitogen-activated protein kinase kinase kinases (MAPKKKs) are important components of MAPK cascades, which are universal signal transduction modules and play important role in plant growth and development. In the sequenced *Arabidopsis* genome, 80 MAPKKKs were identified and are currently being analyzed for their role in different stresses. In rice, economically important monocot cereal crop, only five MAPKKKs were identified so far. The authors revealed computational analysis of sequenced rice genome and identified 75 MAPKKKs. EST hits and full-length cDNA sequences (from KOME or GenBank database) of 75 MAPKKKs supported their existence. Phylogenetic analyses of MAPKKKs from rice and *Arabidopsis* have classified them into three subgroups, which include Raf, ZIK, and MEKK. Conserved motifs in the deduced amino acid sequences of rice MAPKKKs strongly

supported their identity as members of Raf, ZIK, and MEKK subfamilies. Further expression analysis of the MAPKKKs in MPSS database revealed that their transcripts were differentially regulated in various stress- and tissue-specific libraries [144, 145]. Similarly, a truncated tobacco MAPKKK, NPK1, activated an oxidative signal cascade resulting in cold, heat, salinity, and drought tolerance in transgenic plants [146, 147]. However, suppression of signaling factors could also effectively enhance tolerance to abiotic stress [92].

10.2.10
Functional Proteins

Plant stress tolerance depends on the correct regulation of physiological mechanisms. This is achieved by multiple functional proteins participating in developmental, biosynthetic, and metabolic pathways. Based on changes at the transcript and/or activity level, functional proteins protect cells from stress by the removal of toxic elements, restoration of cellular homeostasis, and eventual recovery of normal growth patterns. In this chapter, we focus on enzymes associated with ROS scavenging and sodium transporters.

10.2.11
ROS Scavenging System

ROS, including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-), are generated during aerobic metabolism and under abiotic stress conditions. They are capable of unrestricted oxidation of various cellular components and can lead to membrane lipid peroxidation, protein oxidation, and enzyme inhibition [148, 149]. Plant cells remove excess ROS produced under stress conditions by enzymatic and nonenzymatic mechanisms.

ROS scavenging enzymes include superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, and catalase [150]. APX and GPX are the most studied scavenging enzymes in rice [151–153]. They belong to the plant peroxidase superfamily and catalyze the conversion of H_2O_2 to H_2O. Because ROS also function as second messengers, their generation and removal are tightly regulated in different cellular components. There are eight APX enzymes in rice: two cytosolic (OsAPX1 and OsAPX2), two peroxisomal (OsAPX3 and OsAPX4), one mitochondrial (OsAPX6), and three chloroplastic isoforms (OsAPX5, OsAPX7, and OsAPX8) [153]. OsGPX1 and other plant GPX enzymes, on the other hand, are cytosolic [150, 151]. Under salt stress, OsAPX2, OsAPX7, and OsAPX8 show altered transcript levels [153], but only OsAPX8 is induced in roots [154]. NaCl, ABA, and H_2O_2 can enhance the expression of OsAPX8 in rice roots, while the NaCl-induced expression of OsAPX8 is mediated through the accumulation of ABA but not H_2O_2 [154]. Even isoforms with the same subcellular location may have distinct functions. Expression analysis reveals that OsAPX2 is upregulated by salt [153]. *Arabidopsis* plants expressing OsAPX2 exhibit higher ROS scavenging activity and salt tolerance than those expressing OsAPX1 [155]. Kim *et al.* [73] applied PEG fractionation technique

combined with two-dimensional gel electrophoresis in rice root proteomic studies. Out of the 295 chosen proteins, 93 were identified by MALDI-TOF mass spectrometry. The proteins were classified as relating to metabolism (38.7%), ROS-related proteins (22.5%), protein processing/degradation (8.6%), stress/defense (7.5%), energy (6.5%), and signal transduction (5.4%). The high percentage of ROS-related proteins found in rice root indicates the role of ROS in rice root growth. Treatment with ROS quenching chemicals such as reduced glutathione, diphenyleneiodonium, and ascorbate inhibited root growth dose dependently. Forty-nine proteins identified were either up- or downregulated by GSH treatment, of which 14 were ROS-related proteins, such noticeably modulated ones as glutathione S-transferase, superoxide dismutase, and L-ascorbate peroxidase. The protein levels of four GSTs (NS4, NS8, NS56, and NS57), three APXs (NS46, NS49, and NS50), and MnSOD (NS45) were strongly reduced by GSH treatment but slightly reduced by ascorbate and DPI. Ascorbate and DPI strongly inhibited expression levels of a catalase A (NP23) and an APX (NS65) but did not affect APX (NS46, NS49, and NS50) protein levels. Northern analysis demonstrated that changes in transcript levels of five genes – GST (NS4), GST (NS43), Mn-SOD (NS45), APX (NS50), and APX (NS46/49) – in response to ROS quenching chemicals were coherent with patterns shown in two-dimensional electrophoresis analyses. The authors suggested that these proteins may play an important role in maintaining cellular redox homeostasis during rice root growth [73].

Nonenzymatic antioxidants include the major cellular redox buffers such as ascorbate and glutathione, as well as carotenoids and tocopherol [149, 150]. Alleviation of oxidative injury by the use of antioxidants can enhance plant resistance to abiotic stress. Guo *et al*. [156] found that feeding rice roots with L-ascorbic acid and its immediate precursor protected plants against oxidative damages, suggesting that manipulation of ascorbic acid biosynthesis could be a strategy for improving stress tolerance. During the antioxidation process, ascorbate itself is oxidized to dehydroascorbate; dehydroascorbate reductase (DHAR) re-reduces the oxidized ascorbate. A high ratio of reduced to oxidized ascorbate is important for ROS scavenging efficiency. Ushimaru *et al*. [157] reported that overexpression of rice DHAR1 in *Arabidopsis* increases ascorbate levels, which leads to increased salt tolerance.

10.2.12
Sodium Transporters

Sodium is a micronutrient in plant cells. Under high salinity, excessive accumulation of Na^+ in cytosol disrupts enzymatic and photosynthetic functions and causes ion toxicity. Both Na^+ efflux and vacuolar sequestration contribute to a lower cytosolic Na^+ concentration. Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across the membranes in order to maintain ion homeostasis, as well as cytoplasmic pH and cell turgor [158]. In *Arabidopsis*, extruding Na^+ out of cell is mediated by the plasma membrane Na^+/H^+ antiporter SOS1 [159], whose activity is regulated by

the SOS3–SOS2 complex in roots and by the CBL10–SOS2 complex in shoots [160, 161]. Biochemical and genetic analyses have demonstrated that OsSOS1 is a functional homologue of SOS1. Plasma membrane preparations from yeast expressing OsSOS1 show greater capacity for Na^+/H^+ exchange, and OsSOS1 confers salt tolerance to the yeast mutant AXT3K (Δena1–4 Δnha1 Δnhx1) and the *Arabidopsis* sos1 mutant [162]. Na^+ in cytosol can also be sequestered into vacuoles by the Na^+/H^+ antiporter OsNHX1 located in the tonoplast [163, 164]. The overexpression of OsNHX1 improves salt tolerance in transgenic rice plants, without adversely affecting Na^+ and K^+ levels or plant growth [163, 165].

A high K^+/Na^+ ratio is essential for normal cellular functions. There is growing evidence that supports the idea that the capacity of plants to maintain a high K^+/Na^+ ratio correlates with salt tolerance [166]. Identification of a major quantitative trait locus (QTL) for shoot K^+ content and salt tolerance revealed that SKC1 encodes a HKT-type Na^+-selective transporter, OsHKT8 or OsHKT1;5 according to the new nomenclature [167–169]. SKC1 is preferentially expressed in parenchyma cells surrounding xylem vessels and upregulated by salinity in roots. SKC1 functions to recirculate Na^+ back to the roots by unloading Na^+ from xylem sap, thereby maintaining shoot K^+ homeostasis and enhancing salt tolerance [169, 170]. In *Arabidopsis*, SOS1 [171] and AtHKT1;1 (or AtHKT1) [172–174] also reduce shoot Na^+ concentration through the vascular system, although the mechanisms are different from SKC1 [175].

High-affinity Na^+ uptake has been reported in K^+-starved seedlings of wheat [176], rice [177], and barley [178]. However, there is no direct evidence that a channel/transporter functions in Na^+-selective uptake in plant roots. Studies using oshkt2;1 null mutants demonstrated that OsHKT2;1 (or OsHKT1) takes up Na^+ under K^+-starvation conditions [179]. Due to a dramatically reduced influx of Na^+ in roots, oshkt2;1 mutants accumulate less Na^+ in roots and shoots. They exhibit growth inhibition only under K^+-starvation and low-Na^+ conditions [179]. Unlike *Arabidopsis* containing one HKT gene, seven HKT genes have been identified in rice cv. Nipponbare [170, 177]. Given this large number of genes, rice may be a better model for understanding the role of HKTs in regulating Na^+ transport [180]. Jacobs *et al.* [181] transformed rice with the *Physcomitrella patens* Na^+ pumping ATPase (PpENA1). When grown in solutions containing 50 mm NaCl, plants constitutively expressing the PpENA1 gene are more salt tolerant and produce greater biomass than controls. Transgenics and controls accumulate similar amounts of Na^+ in leaf and root tissues under stress, which indicates that the observed tolerance is not because of Na^+ exclusion. Moreover, inductively coupled plasma analysis reveals that the concentration of other ions in the transformants and the controls is similar. The transgenic lines are developmentally normal and fertile, and the transgene expression levels remain stable in subsequent generations. The results of this study demonstrate the utility of PpENA1 as a potential tool for engineering salinity tolerance in important crop species [181].

10.3 Choice of Promoters

An important aspect of transgenic technology is the regulated expression of transgenes. Tissue specificity of transgene expression is also an important consideration while deciding on the choice of the promoter so as to increase the level of expression of the gene. Thus, the strength of the promoter and the possibility of using stress-inducible, developmental stage, or tissue-specific promoters have also proved to be critical for tailoring plant response to these stresses [182]. Some gene products are needed in large amounts, such as LEA3, thereby necessitating the need for a very strong promoter. With other gene products, such as enzymes for polyamine biosynthesis, it may be better to use an inducible promoter of moderate strength. The promoters that have been most commonly used in the production of abiotic stress-tolerant plants so far include the CaMV 35S, ubiquitin 1, and actin promoters. These promoters being constitutive in nature, by and large express the downstream transgenes in all organs and at all the stages. However, constitutive overproduction of molecules such as trehalose [183] or polyamines [184] causes abnormalities in plants grown under normal conditions. Also, the production of the above-mentioned molecules can be metabolically expensive. In these cases, the use of a stress-inducible promoter may be more desirable. In plants, various types of abiotic stresses induce a large number of well-characterized and useful promoters. Not only an ideal inducible promoter should be devoid of any basal level of gene expression in the absence of inducing agents, but the expression should also be reversible and dose dependent. The transcriptional regulatory regions of the drought-induced and cold-induced genes have been analyzed to identify several *cis*-acting and *trans*-acting elements involved in the gene expression that is induced by abiotic stress [185]. Most of the stress promoters contain an array of stress-specific *cis*-acting elements that are recognized by the requisite transcription factors; for example, the transcriptional regulation of hsp genes is mediated by the core "heat shock element" located in the promoter region of these genes, 5′ of the TATA box. All the plant hsp genes sequenced so far have been shown to contain partly overlapping multiple HSEs proximal to TATA motif. Apart from these hsp promoters, *rd29* and *adh* gene promoters induced by osmotic stress and anaerobic stress, respectively, have also been studied. The *Arabidopsis* rd29A and rd29B are stress-responsive genes, but are differentially induced under abiotic stress conditions. The rd29A promoter includes both DRE and ABRE elements, where dehydration, high salinity, and low temperatures induce the gene, while the rd29B promoter includes only ABREs and the induction is ABA dependent. Overexpression of DREB1A transcription factors under the control of stress-inducible promoter from rd29A showed a better phenotypic growth of the transgenic plants compared to those obtained using the constitutive CaMV 35S promoter [186]. A stress-inducible expression of *Arabidopsis* CBF1 in transgenic tomato was achieved using the ABRC1 promoter from barley HAV22 [187]. Gene expression is induced by the binding of DREB1A, which in itself is induced by cold and water stresses, to a *cis*-acting DRE element in the promoters of genes such as rd29A, rd17, cor6.6,

cor15A, erd10, and kin1, thereby initiating synthesis of gene products imparting tolerance to low temperatures and water stress in plants. The regions of respiratory alcohol dehydrogenase adh1 gene promoter in maize and rice that are required for anaerobic induction include a string of bases called anoxia response element (ARE) with the consensus sequence of its core element as TGGTTT. Besides, other stress-responsive *cis*-acting promoter sequences such as low-temperature-responsive elements (LTRDs) with a consensus sequence of A/GCCGAC have been identified in genes such as Cor6.6, Cor15, and Cor78. These basic findings on stress promoters have led to a major shift in the paradigm for genetically engineering stress-tolerant crops [188].

10.4 Physiological Evaluation of Stress Effect

A large number of studies have evaluated different transgenic constructs in different plant species, and under different stresses such as drought, salinity, and cold. The expression of the genes inserted and altered levels of metabolites have been reported in great detail. However, less detail is given with regard to the methods used to evaluate the stress response. Although the transgenic construct is usually reported to have increased the tolerance to drought in most of the instances, it is then referred to as such in other papers. This lack of details applies mostly to drought stress; the protocols used for salt stress are usually better described [18, 26], although the levels of salt stress used in some studies are far beyond what is found in a natural environment. It is understood that most of these studies are intended to assess the gene expression, often in model plants, under a particular stress, and extreme situations of stress are often used to ensure the gene expression. However, these studies may bring about some misleading conclusions from an agronomic or a physiological perspective, where the assessment of stress tolerance of transgenics needs to be done with respect to its crosstalk with other stress-related genes/mechanisms and where the effects of stress need to be observed over longer periods/conditions. This is particularly important, in order to closely mimic the life span of most crops under cycles of stress, rather than short exposure to very severe stresses, although we agree that short exposures to stress are certainly adequate if the purpose is to assess gene expression only. Therefore, in the following discussion, we focus on the agronomic/physiological perspective and do not mean to challenge the quality of the work done to assess gene expression.

Two major issues that typically need to be addressed in stress response evaluation of transgenics include (1) means of stress imposition, details about the stress, and growth conditions (including the intensity, timing, and quickness of imposition, etc.) and (2) "hard" data on the response of tested materials to support conclusions (comparison within the same species). Besides, precise details about the protocols used to evaluate the performance of plants to any given stress are very essential to assess the performance of materials.

10.5 Means of Stress Impositions, Growth Conditions, and Evaluations

Stress conditions used to evaluate the transgenic material in most of the reports so far are usually too severe [21, 35, 189] as plants are very unlikely to undergo such stresses under field conditions. Also, the means of evaluation are often significantly different from natural conditions. For example, Pellegrineschi *et al.* [120] compared the performance of initial events of DREB1A transgenic wheat to the wild parent by withholding water to 2-week-old seedlings grown in 5 cm × 5 cm pots, and then rewatering until maturity when they were evaluated. Untransformed plants were nearly dead within 10–15 days of stress imposition, likely because of a different pattern of water use, whereas transgenic plants survived in these small pots and "passed" the evaluation successfully; such conditions would obviously not occur in the field. Besides the type of systems used to assess plant performance, one would expect the evaluation to be made, at least, on the basis of biomass accumulated during the stress.

While the use of PEG in hydroponics can be useful to test certain response of plants under a given osmotic potential as reported by Pilon-Smits *et al.* [34, 190], it offers conditions relatively different from those present in the soil where the water reservoir is by definition finite. There is improvement in growth by increased water uptake under the water potential applied, due to osmolyte production by the transgenic plant. This is quite possible in such a system because the water reservoir is unlimited in hydroponics, and because the water potential is constant. Under soil conditions, however, the volume of soil surrounding the root where water can be extracted is limited, and the water potential of that soil quickly declines upon water uptake by roots, reaching soil water potential where even the enhanced osmolyte production of the transgenics would be unable to extract any significant additional amount of water. A more realistic test of the ability to take up water using osmotic potential-enhanced transgenics would be to compare their capacity to extract water from a soil system rather than a hydroponic system. A recent study by Sivamani *et al.* [65] reported an increased water use efficiency (WUE) in the transgenic wheat. Unfortunately, there was no control over the soil evaporation that probably accounts for most of the water loss and explained the very low values of WUE observed. Besides, investigating drought responses by using fresh weight [91] and other indirect estimates of performance such as growth rate, stem elongation [32, 191], or survival [142] is likely to give inconsistent results. While applying a drought stress, it is important to know the stages of drought stress that the plants are exposed to, for which a detailed description of growth conditions, plant size, container size, water availability, and transpiration is needed. It is also crucial to report the dry weight of tested plants, possibly before and after the stress period.

Similarly, often the stress imposed has been modified from 2 days to 2 weeks and even 4 weeks using the same experimental conditions [191], without indicating the water holding capacity of the potting mixture used as well as the plant density. This obviously leads to different types of stresses, where the plants exposed to water stress for 2 days may well have remained in stage I when water is abundant

(see below), while plants exposed to stress for 4 weeks may have spent most of the time under stage III where roots may have exhausted all the available water. Also, there are cases where a given quantity of water is applied to the plants on alternate days from 2 to 10 weeks [65], thereby disregarding the fact that the water requirements increase dramatically during the period, and probably exposing their plants to an initial flooding before a severe stress.

10.6 Adequate Protocols to Apply Drought and Salinity Stress

Unlike what seems to be a common practice in transgenic evaluation, applying drought does not consist simply in withholding water. Indeed, we cannot investigate drought responses of plants without understanding the different phases that a plant undergoes under drought in natural conditions. These steps have been described earlier [192, 193]. In phase I, water is abundant and plant can take up all the water required by transpiration and stomata are fully open. During that stage, the water loss is mostly determined by the environmental conditions to which the leaves are exposed. During stage II, the roots are no longer able to supply sufficient water to the shoot and stomata progressively close to adjust the water loss to the water supply, so that leaf turgor is maintained. In stage III, roots have exhausted all the water available for transpiration. Stomata are closed and virtually all the physiological processes contributing to growth, including photosynthesis, are inhibited. This has been used to design dry-down experiments where the response of plants to drought is taken as a function of the fraction of soil moisture available to plant (fraction of transpirable soil water, FTSW), and not as a function of number of days after which the stress has been imposed. The former allows a precise comparison of stress imposed across experiments and environmental conditions, whereas referring to stress intensity on the number of days of exposure to stress, without referring to pot size, evaporative demand, and so on, can lead to erratic and irreproducible data. Based on transpiration values, it is possible to partially compensate the water loss to apply a milder stress condition, which allows plants of different sizes to be exposed to a similar drought stress. For instance, plants exposed to water stress are allowed to lose a maximum of 70 g per day. Any water loss in excess of this value is added back on a plant basis. This allows maintaining the volumetric soil moisture content, a proxy for water stress, similar in all pots. Amount of daily water loss can be adapted to increase/decrease the level of stress. This protocol has the advantage of mimicking the situation a plant would face in the field, that is, a progressive soil drying. This method has been successfully used at ICRISAT to assess the response of 14 transgenic events of groundnut with rd29A promoter-driven DREB1A under contained greenhouse conditions [122, 123].

Regarding salinity, most of the evaluations reported so far have been carried out at the seedling stage [194], although this type of evaluation has been reported to have little correspondence, if any, with how plants will later perform under salt stress [195, 196]. Besides, evaluations are made on a short-term basis by using high

concentrations of salt, way above those found even in highly saline natural environment that obviously magnifies the effect of transgenics engineered to excrete salt. Therefore, protocols that use too severe concentrations of salt should be avoided. A few other subjects of contention include the treatments that are used as salt stress, and also the hypothesis about the major determinants of salt stress tolerance.

It is often assumed that the avoidance of Na^+ accumulation and toxicity confers salt tolerance in plants. Therefore, most of the transgenic work has dealt with genes involved in Na^+ extrusion from the root or Na compartmentation in the vacuoles. However, severe stresses (over 200–300 mM) in hydroponics [18, 121, 191] that are unlikely to occur in the natural environment will necessarily highlight those transgenics that are able to excrete Na^+ and able to maintain homeostasis, even though it may be for a short while. Whether such a strategy is adequate is still an open question.

Procedures for the salinity evaluation of crops are being optimized to be carried out in soil conditions in an outdoor facility under natural conditions at ICRISAT. Here, salt stress is applied to the soil during the early stages of germination and plant development using a staggered salt application (total amount split in three applications) to avoid an osmotic shock. Besides, plants are maintained close to 80% field capacity until maturity to avoid a possible increase in salt concentration if water is not replenished regularly. The plant tolerance to stress is evaluated based on the seed yield since no correlation between the shoot biomass and seed yield under salinity has been observed [196]. It is likely that reproduction is the key physiological process affected by salinity. Therefore, transgenic research intended to improve salt tolerance should probably be focused on those processes that appear to be sensitive. A thorough investigation of these processes can only help in devising a suitable and focused transgenic approach.

10.7 Conclusions

This chapter summarizes the recent efforts to improve abiotic stress tolerance in crop plants by employing some of the stress-related genes and transcription factors that have been cloned and characterized.

1) There is a clear and urgent need to introduce stress tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding.
2) An important issue to address is how the tolerance to specific abiotic stress is assessed, and whether the achieved tolerance compares to existing tolerance. The biological cost of production of different metabolites to cope with stress and their effect on yield should be properly evaluated.
3) A well-focused approach combining the molecular, physiological, and metabolic aspects of abiotic stress tolerance is required for bridging the knowledge gaps between short- and long-term effects of the genes and their products, and

between the molecular or cellular expression of the genes and the whole plant phenotype under stress.

4) Thorough understanding of the underlying physiological processes in response to different abiotic stresses can efficiently/successfully drive the choice of a given promoter or transcription factor to be used for transformation.

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11
Rice: Genetic Engineering Approaches to Enhance Grain Iron Content

Salvinder Singh, D. Sudhakar, and M.K. Modi

Abstract

Major staple crops are often deficient in some of the nutrients required in human diet. Thus, malnutrition is a major problem, especially in developing countries, where a diversified diet is not affordable for the majority. Several strategies have been adopted to improve nutrition. However, micronutrient deficiency is still widely spread. Rice is one of the most important staple foods for a large part of the world's population. Therefore, even a small improvement in nutritional content of rice seeds can have a dramatic impact on human health. Different approaches are being exploited to produce rice enhanced in grain iron and zinc.

11.1
Introduction

Three cereals (wheat, rice, and maize) rank at the top and account for a large share of production among the 29 basic food crops [1]. Even though rice is placed after wheat, nearly one-third of world population (mainly in Asia) depends on rice as the major source of nutritional calories [2]. Rice (*Oryza sativa* L.) is the target crop for many improvement programs because it is the staple diet for nearly 2 billion people worldwide and the major food for over half of those living in Asia [3]. Humans require more than 22 mineral elements, which can all be supplied by an appropriate diet. However, the diets of populations subsisting on cereals, or inhabiting regions where soil mineral imbalances occur, often lack Fe, Zn, Ca, Mg, Cu, I, or Se, and those people suffer from micronutrient malnutrition [4].

11.2
Micronutrient Malnutrition

The global significance of micronutrient malnutrition (MNM) – also known as hidden hunger – came to the attention of the nutrition community as early as the

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mid-1980s, when protein-energy malnutrition was widely seen as the culprit of the world's nutrition problems [5]. The 1990 World Summit for Children, convened by governments and facilitated by the UN with support from UNICEF, the World Bank, WHO, FAO, UNDP, CIDA, and USAID, was a landmark event in the fight against MNM. Three of the summit's goals directly addressed the elimination or significant reduction of deficiencies in iron, vitamin A, and iodine by the year 2000, thus providing development agencies with the necessary political mandate. Activities toward achieving these goals focused on traditional public health intervention strategies, and resulted in measurable impacts; however, the year 2000 targets are still far from being met [6–9]. In 2001, the General Assembly of the UN adopted the Millennium Development Goals (MDGs) resolution that aims to eradicate or alleviate the world's greatest health and poverty issues by 2015 [10]. Fighting MNM is an integral component of three of the eight MDGs: (1) eradication of extreme poverty and hunger; (2) reduction of child mortality; and (3) improvement of maternal health. Micronutrient supply is among the main preventive and curative interventions with proven and substantial capability to contribute toward achieving these goals [11]. Widespread MNM also results in enormous negative socioeconomic impact at the individual, community, and national levels [8]. Even though insufficient intake of any essential micronutrient will result in metabolic impairment of individuals, potentially increasing morbidity and death rates, numerous national and regional surveys have identified iron, vitamin A, and iodine as most vital among almost 30 essential micronutrients for global human health [12]. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have estimated the daily requirements of the various micronutrients in the human diet. Individuals between 25 and 50 years of age require 10–15 mg Fe and 12–15 mg Zn [12]. Nutritional deficiencies (e.g., iron, zinc, vitamin A) account for almost two-thirds of the childhood deaths worldwide. Most of those affected are dependent on staple crops for their sustenance. In low socioeconomic populations in India, the prevalence of iron deficiency anemia (IDA) may be as high as 64–68% in school-aged children and infants [13, 14].

11.2.1 Approaches to Decrease Micronutrient Deficiencies and/or Malnutrition

Traditional public health interventions, such as supplementation and industrial fortification of foods, have notably reduced MNM-induced morbidity and mortality worldwide. Nonetheless, attainment of the MDGs is not on track, mainly because classical interventions require infrastructure, purchasing power, or access to markets and healthcare systems for their success, often not available to people living in remote rural areas. Food fortification programs rely on widely distributed, industrially processed food items, usually unaffordable for half of the world's poor living on less than $2 per day, and much less another 30% who live on less than $1 per day [15].

Experience with vitamin A supplementation programs revealed that coverage achieved over the past decade in 103 priority countries has stagnated at 58%, with

high year-to-year fluctuation [7]. In India, the "Nutritional Anaemia Control Programme" has been in place since 1970 with little impact, because of mismanagement, under funding, logistic problems, and poor compliance [16]. In recent years, coverage with iron-folate supplements was around 30% for pregnant women and 10% for adolescent girls [17]. While undoubtedly money spent on these interventions is money well spent, both intervention strategies depend on uninterrupted funding. But the flow of funds can falter, as exemplified by the case of Burmese refugees along the Thailand–Burma border, where an interruption of the program led to an immediate drop in micronutrient status and consequently to health deterioration [18]. The cost per life saved is low in economic terms, when compared to WHO and World Bank benchmarks [19, 20], but these interventions perpetuate precarious dependencies.

11.2.2
Importance of Iron in Human Physiology

Humans require at least 22 mineral elements for their well-being [4, 12, 21]. These can be supplied by an appropriate diet. However, it is estimated that over 60% of the world's 6 billion people are iron (Fe) deficient, over 30% are zinc (Zn) deficient, 30% are iodine (I) deficient, and 15% are selenium (Se) deficient [22]. Iron is found in hemoglobin, which transports oxygen in the blood of vertebrates. When iron levels are low, the amount of available oxygen declines, causing a common symptom of iron deficiency called anemia. Iron deficiency causes a range of health problems in humans, including poor pregnancy-related complications, brain damage in infants, chronic hypoxia, and reduced work performance [23]. This situation is attributed to crop production in areas with low mineral phytoavailability and/or consumption of (staple) crops with inherently low tissue mineral concentrations, compounded by a lack of fish or animal products in the diet [4, 21, 24–27]. The highest risk of iron deficiency occurs during rapid growth and nutritional demand, in children, adolescence, and pregnancy. Severe anemia in pregnant women is estimated to be responsible for up to 40% of the half a million deaths associated with childbirth each year. During infancy, lack of sufficient iron in the brain causes irreversible changes in mental and psychomotor development, seriously limiting the intellectual potential of the adult [28]. Abnormalities in mental performance, including apathy, irritability, impaired attentiveness, and reduced learning capacity, have been observed as the consequences of childhood anemia [29].

11.2.3
Source of Iron for Human Nutrition

Iron in food exists mainly in the form of ferriheme and ferroheme. Ferriheme is the main source of available iron and ferriheme only accounts for 5–10% of the available iron. Hemochrome is mostly absorbed by the adherent epithelial

cells of the small intestine, and then combines with iron and forms ferritin, which finally is transported to other parts of the body to be used; the rate of absorption reaches up to 25% [24, 30]. Ferriheme exists in the food in the form of a $Fe(OH)_3$ chelate complex and the chelating molecules include proteins, amino acids, and organic acids. Ferriheme is reduced to ferroheme after release from the organic molecule under the effect of stomach acid and then absorbed, with an absorption rate of only 5%. Its low absorption rate is considered as the main reason causing iron deficiency anemia, especially in the developing countries [31].

The absorption rate of iron in plant source food is lower than 10%, for example, 1% in rice, 3% in corn and black bean, 4% in lettuce, and 5% in wheat [32]. This is due to the fact that many inhibitory factors in plant-sourced food impair the absorption of iron. The known inhibitors of iron absorption are phytic acid, polyphenolic compounds, calcium, and certain peptides from partially digested proteins [33]. Phytic acid and phenolic compounds are the most potent inhibitors and iron absorption from some foods may be extremely low unless the inhibitors of absorption are effectively overcome [34]. Phytic acid is widely present in cereal grains and legume seeds where it is an important storage form of phosphorus required for efficient germination of the seedlings [35]. Phenolic compounds are particularly high in beverages such as tea, coffee, herb teas, cocoa, and red wine. Peptides formed upon digestion can both inhibit or enhance iron absorption. Studies, in which different commercial protein sources were fed in a liquid formula meal, have indicated that soy proteins, egg albumin, and casein are important inhibitors [36, 37].

11.2.4 Approaches to Decrease Micronutrient Deficiencies

The most widely recognized strategies to reduce micronutrient malnutrition are supplementation with pharmaceutical preparations, disease reduction, food fortification, and dietary diversification through consumption of a broad variety of foods, preferably from home gardens and small livestock production [30].

Nutrition improvement with food fortification [38] and medical supplements achieved in the developed world were unfortunately not successfully transferred to many developing countries even today. Success of fortification programs and of micronutrient distribution in form of tablets to the population living in remote areas, to certain cultural groups, and to the poor in growing towns is not obvious. Therefore, those who mostly need micronutrient supplementation are not reached [39]. None of the current intervention strategies have been very successful in reducing the prevalence of micronutrient deficiency in developing countries, where malnutrition is still a major health problem.

On the contrary, genetically improved food, by breeding or gene transfer, may be a powerful intervention strategy targeting the most vulnerable population.

Genetically improved plants can reach the rural areas and represent a sustainable system, with very low recurrent costs [40, 41].

11.2.5 Pharmaceutical Preparation

Iron supplementation is only useful for reaching a rapid improvement in iron status in anemic individuals, but it is expensive and usually has poor compliance because of unpleasant gastrointestinal side effects. In contrast, the chemical synthesis of vitamin A around 1930 permitted a widespread medical supplementation and food fortification in the developed countries, eliminating ocular forms of vitamin A deficiencies [42]. Many countries even adopted a periodical distribution of high-dose vitamin A capsules. High-dose vitamin A capsules allow rapid progress in reducing vitamin A deficiency in target populations, but the success obtained is highly dependent on the economic situation of the country. Indonesia, for example, made significant progresses, eradicating *xerophthalmia* with supplement distribution in a national health program [43]. Unfortunately, the recent economic decline and civil unrest interrupted these programs leading to a reappearance of xerophthalmia [42].

11.2.6 Disease Reduction

The high prevalence of bacterial and parasitic diseases in developing countries greatly contributes to malnutrition. Hookworms cause significant gastrointestinal blood losses directly proportional to the number of worms present in the gut. Furthermore, helminth infections may stimulate inflammation, with deleterious effects on protein metabolism and erythropoiesis [44]. In fact, children from schools with high prevalence of worm infection were found to have significantly worse iron deficiency and anemia than children from low-prevalence schools [45]. Similarly, anthelminthic treatment significantly reduced malnutrition and improved iron status in infected children [44]. *Plasmodium vivax* malaria is another strong predictor of moderate to severe anemia. For example, 20% of anemic women in the plains of Nepal had malarial parasitemia [46]. The relation between several infections and iron deficiency clearly indicates the need for reducing the prevalence of hookworm and malarial infections to effectively control anemia.

11.3 Food Fortification

Food fortification has been considered the best long-term strategy for micronutrient prevention, especially in developed areas [38]. In developing countries, fortification can meet several difficulties, mainly because of lack of infrastructures and discontinuity of the programs adopted. For example, vitamin A sugar fortification

program has also been effective in raising vitamin A status of the population in Guatemala [47]. Unfortunately, the government mandated program was disrupted for economical and political reasons, and this was soon followed by a decline in vitamin A status throughout the country.

Iron is the most difficult mineral to supplement because of several technical problems related to the choice of a suitable iron compound [48]. Iron compounds of relatively high iron availability, such as ferrous sulfate, often provoke unacceptable color and flavor changes to food, whereas those compounds that are organoleptically inert, such as elemental iron, are usually poorly absorbed [49]. Iron-fortified foods that have demonstrated an improved iron status in the target population include infant cereals [50], sugar [51], and fish sauce [52]. In contrast, there is little evidence that iron fortification of major staple foods, such as wheat flour or corn flour, is a useful strategy to defeat iron deficiency, mainly because of poor bioavailability of iron compounds commonly used and the high level of phytic acid in cereal foods [34]. In conclusion, food fortification can be very effective in increasing micronutrient status; however, several limitations are evident, mainly in the developing countries and when iron is the mineral of interest.

11.4 Biofortification

Biofortification is the development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology. This approach has multiple advantages. First, it capitalizes on the regular daily intake of a consistent and large amount of food staples by all family members. Because staple foods predominate in the diets of the poor, this strategy implicitly targets low-income households. Second, after the one-time investment to develop seeds that fortify themselves, recurrent costs are low, and germplasm can be shared internationally. This multiplier aspect of plant breeding across time and distance makes it cost effective. Third, once in place, the biofortified crop system is highly sustainable. Nutritionally improved varieties will continue to be grown and consumed year after year, even if government attention and international funding for micronutrient issues fade. Finally, biofortification provides a feasible means of reaching undernourished populations in relatively remote rural areas, delivering naturally fortified foods to people with limited access to commercially marketed fortified foods that are more readily available in urban areas. Biofortification and commercial fortification, therefore, are highly complementary. In fact, biofortification may have important spin-off effects for increasing farm productivity in developing countries in an environmentally beneficial way. Mineral-packed seeds sell themselves to farmers because these trace minerals are essential in helping plants resist disease and other environmental stresses. Moreover, a higher proportion of seedlings survive, initial growth is more rapid, and ultimately yields are higher.

11.4.1 Biofortification through Classical Breeding Approach

For staple crops, thousands of different genotypes of rice, wheat, maize, beans, and cassava exist, and genotype influences the nutrient content of the plant and seeds [53]. In a recent screening of germplasm, a high variation in the Fe and Zn content was reported in the edible parts of staple foods (wheat, bean, cassava, maize, rice, and yam) between cultivars of a given species. For example, the Fe content of wheat cultivars varied from 25 to 56 mg/kg and the Zn content ranged from 25 to 64 mg/kg. Moreover, the amounts of Fe and Zn in different rice and bean seeds appear to be highly correlated with each other [54, 55]. However, the speciation and bioavailability of the minerals are not known. Research groups are searching for the genes determining high Fe and Zn levels, and several candidate genes have been identified in wheat on chromosomes 6A and 6B [56].

King *et al.* [57] recently compared Fe and Zn bioavailability in humans for two varieties of the common red bean: a Fe- and Zn-rich genotype and a lower density genotype. The bioavailability of Fe and Zn from both varieties was low, about 1.5 and 13%, respectively. The 40% increase in Fe content in the high-density beans did not improve the amount of Fe absorbed. The 75% increase in Zn content was associated with a 40% increase in total absorbed Zn. The high phytate/mineral ratios of both genotypes probably lowered their mineral bioavailability. Reducing the phytate content of nutrient-dense plants might be necessary in order for these varieties to have a positive impact on human nutrition.

Using genetic screening, Mendoza *et al.* [58] have identified several genotypes of maize, barley, and rice that are low phytic acid. In these low phytic acid (*lpa*) mutants, although the total phosphorus content of the seeds is unchanged, the phytic acid phosphorus is reduced by 55–66%. One of these maize mutants, *lpa1-1*, was used to develop inbred lines for producing hybrids. Compared with normal hybrids, few differences in agronomic characteristics were found, except for a 6% yield loss. Growth studies in chickens have resulted in increased weight gain when fed with a low phytic acid corn mutant in comparison to normal hybrids [58]. The impact of low phytic acid maize consumption on Fe and Zn bioavailability has been tested in humans. Fe bioavailability was 49% greater from tortillas made with low phytic acid maize compared with wild-type maize (Fe absorption 8.2% versus 5.5%) [58]. Adams *et al.* [59] measured Zn bioavailability from low phytic acid maize versus wild-type maize. Mean Zn absorption from polenta prepared with the low phytic acid maize was 78% greater than that for wild-type maize. Other studies, however, have shown that the phytic acid content of soy and wheat flour must be reduced by $\geq$90% to achieve a meaningful twofold increase in Fe absorption [34]. These studies demonstrate that substitution of a low-phytate variety may improve the bioavailability of these two trace minerals, but the impact on Fe nutrition in particular is uncertain. Combining crossbreeding techniques for developing low phytic acid plants with selection of highly nutrient-dense seeds could be an effective method for improving mineral nutrition.

11.4.2 Biofortification through Genetic Engineering Approach

Although metabolic engineering is the most suitable approach to fortify plants with organic nutrients, a different approach is required for minerals because they are not synthesized in the plant but obtained from the immediate environment. Transgenic strategies to increase the mineral content of crop plants have concentrated mainly on iron and zinc (which are most frequently deficient in human diets) and have used two distinct approaches: (i) increasing the efficiency of uptake and transport to harvestable tissues, and (ii) increasing the amount of bioavailable mineral accumulating in the plant, that is, accessibility after digestion [4].

One consideration particular to iron is that, although Fe(III) is the most abundant form of iron in the soil, plants cannot absorb iron in this state. Two different pathways are used to convert Fe(III) into Fe(II) for absorption: strategy I (all other plants) involves the expression of Fe(III) reductases and the subsequent absorption of Fe(II), whereas strategy II (graminaceous plants, that is, grasses and cereals) involves the secretion of chelating chemicals, called phytosiderophores, that bind to Fe(III) before absorption. The overexpression of several of these transport and chelating proteins promotes metal accumulation. For example, efforts to increase iron uptake in roots by genetic modification have focused on strategy I plants, for instance, through the expression of iron transport proteins [60]. For strategy II plants, iron accumulation can be enhanced by the production of higher levels of phytosiderophores; for instance, the expression in rice of the barley NAATA and NAATB genes, encoding nicotianamine aminotransferases (involved in phytosiderophore biosynthesis), resulted in increased iron uptake [61]. There seems to be some crosstalk between the iron and zinc transport pathways because transgenic plants and mutants with overexpressed Fe(III) reductases and iron transporters also show increased zinc accumulation. This could reflect the enhanced synthesis of nicotianamine, which increases the mobilization of both metals in the vascular tissue. Thus, the overexpression of nicotianamine synthase (NAS) also leads to iron and zinc accumulation; for example, the expression of barley *HvNAS1* in tobacco (*Nicotiana tabacum*) doubled the iron and zinc concentrations in leaves [62].

The second approach to mineral biofortification is to express recombinant proteins that enable minerals to be stored in a bioavailable form by genetic transformation strategies [26, 63–79]. Transgenic tobacco plants with high iron content have been produced via *Agrobacterium*-mediated gene transfer of the soybean *ferritin* cDNA under control of a CaMV 35S promoter [80]. A positive correlation was observed between ferritin levels and iron accumulation in ferritin-transformed tobacco leaves, raising the possibility that higher iron concentration could be achieved by increasing ferritin expression [80]. In rice, the same ferritin expression, driven by a constitutive promoter, led to an increase in iron content of the vegetative parts but not in the seeds [81]. Goto *et al.* [82] reported that transgenic lettuce, constitutively expressing ferritin, grew larger and faster than the control. Moreover, they reported the endosperm-specific expression of soybean ferritin in rice seeds with maximum threefold increase in one of the transformants [80]. Expression of

the French bean ferritin under control of the glutelin promoter in the endosperm of *japonica* rice showed an increase of iron content up to twofold [66]. The overexpression of the soybean ferritin gene driven by the endosperm-specific glutelin promoter in *indica* rice grains led to an increase of both iron and zinc concentration in brown grain as well as in the polished grain [69]. The higher iron accumulation upon ferritin overexpression could imply that low iron concentrations in the seed may not result from low iron availability for transport, but rather from a lack of sequestering capacity in the seed.

An extremely high expression of soybean ferritin driven by the rice glutelin promoter was reported in rice endosperm [72, 73], with levels of the transgenic protein up to 13-fold higher than previously reported [80]. Unfortunately, the maximum iron concentration in the seeds of the new lines was only about 30% higher compared with previous transformants and about threefold higher than that in the nontransformed control seeds. The mean iron concentration in leaves of high ferritin-expressing lines decreased to less than half of the nontransformed plants, and the plants showed chlorosis after flowering, even on iron-rich medium. Accumulation of iron in the seeds of hyperexpression ferritin rice did not only depend on the expression level of exogenous ferritin but may also be limited by Fe uptake and transport [72].

11.4.3
Biofortification by Decreasing Antinutrient Contents

The amount of iron absorbed from a diet can also be increased by improving its bioavailability. This can be achieved by reducing antinutrients such as phytic acid. Phytic acid has been completely degraded in cereals used for baby food by adding commercial exogenous phytases [83] or by activating the native phytases by a combination of soaking, germinating, and fermenting [84]. Phytic acid reduction in soybean and soy foods by fermentation has been reported to result in the prevention of iron deficiency anemia in children [85]. The addition of a commercial phytase to rice porridge allowed an almost complete degradation of the endogenous phytic acid and a threefold increase in iron absorption [86].

In humans, when a microbial phytase from *Aspergillus niger* was added to a high phytate bread roll prior to consumption, iron absorption increased from 14.3 to 26.1%, suggesting that effective and complete phytate degradation occurred in the stomach [87]. This report confirmed previous results from *in vitro* studies of addition of *A. niger* phytase at pH and temperature conditions similar to those of the stomach, which resulted in a complete phytate degradation [88].

A large number of studies have shown that the *A. niger* phytase can be synthesized efficiently in transgenic plants, such as canola, tobacco, and soybean [89]. Phytase-expressing tobacco plants, showing a high transgenic phytase content in their seeds, were visually indistinguishable from nontransformed plants, indicating that the constitutive expression of the enzyme affects neither morphology nor growth rate. Similarly, germination was not affected by the presence of the enzyme. Analysis for phytase activity of seeds stored at 4 °C, as well as at room temperature, showed that no significant decrease in the activity occurred over a

period of a year, demonstrating that active phytase can be produced and stored with few losses in plant seeds [90]. However, *A. niger* phytase is inactivated at temperatures higher than 60 °C. Therefore, this phytase is not useful in transgenic cereals for human consumption, because cooking procedures typically inactivate the enzyme. One solution would be the activation of the transgenic phytase before cooking, during seed development, seed storage, or other processes. An alternative that has been explored is the use of a thermotolerant phytase from *Aspergillus fumigatus* [91] in transgenic rice seeds [66]. *A. fumigatus* phytase displays a high resistance to heat inactivation, similar to enzymes from thermophilic organisms, because of its ability to refold properly after denaturation. The isolated fungal enzyme was boiled together with rice flour and a residual 59% phytase activity was observed. However, expression of the heat-tolerant *A. fumigatus* phytase in rice endosperm resulted in a strong reduction of enzyme activity after cooking [66]. For unknown reasons, the *in planta* synthesized phytase is not as thermotolerant as the one expressed in fungi. Eventually, the different glycosylation pattern in plants may affect the heat stability. Possibly other microbial phytases that are not denaturated under high temperatures will provide higher heat stability *in planta* and will allow a phytic acid degradation upon rice digestion.

11.4.4
Biofortification by Increasing Iron Bioavailability Promoting Compounds

Breeding strategies to increase iron bioavailability by strongly reducing antinutrient content remain controversial. Besides the well-known beneficial effect on iron nutrition, antinutrients in the seeds may play an important role in plant growth and human health [92, 93]. An alternative strategy for bioavailability improvement may be to increase the levels of promoter compounds [40]. Studies have shown that cysteine is the only free amino acid with an enhancing effect on iron absorption in humans [94]. Therefore, a better iron availability is expected by increasing the amount of cysteine residues in crop plants. A group of cysteine-rich, low molecular weight polypeptides are the metallothionein proteins (MTs) [95]. MTs are small, metal-binding proteins that are present in animals, fungi, cyanobacteria, and plants. Genes encoding MT-like proteins have been identified in different plant species. Lucca *et al.* overexpressed the cysteine-rich MT gene in rice, increasing cysteine content of the soluble seed protein about sevenfold [66]. It remains to be demonstrated that the cysteine-containing peptides formed during the digestion of MT enhance iron absorption in humans in a similar way to free cysteine, glutathione, or cysteine-containing peptides of meat. High cooking temperatures can change the content of sulfhydryl groups (SH) from cysteine residues to cystine and cysteic acid, and therefore, they may hamper the positive effect of cysteine-containing peptides on iron absorption. Low cooking temperatures (30–70 °C) increase the content of reactive SH groups in undigested meat, because the unfolding of meat proteins exposes SH groups, which are otherwise hidden within the protein structure [96]. However, at boiling temperatures (90–120 °C), the SH groups oxidize to disulfide (SS) groups. This could be a major hindrance for the transgenic rice seeds overexpressing the

endogenous MT protein, because rice seeds need to be boiled at 100 °C before consumption. A recent study showed that increasing cooking temperatures of meat at 120 °C did not impair nonheme iron absorption. There is a tendency toward a higher absorption when meat cooked at 120 °C. These findings do not support a specific role for SH groups produced from cysteine residues in the meat promoting nonheme iron absorption because the cysteine content of the meat decreased with increasing cooking temperature. In addition, the observed reduction of heme iron content by higher temperatures must be considered in evaluating total iron absorbed [97].

11.5
Iron Uptake and Transport in Plants

11.5.1
The Reduction Strategy

Nongraminaceous plants use the reduction strategy, or strategy I, to obtain iron. Upon iron deficiency, protons are released to increase the solubility of iron, via H^+-ATPases of the root plasma membrane [98, 99]. Several *Arabidopsis* H^+-ATPase (AHA) family members are induced in iron-deficient roots [100, 101], suggesting possible roles for these enzymes in iron deficiency responses.

After acidification, Fe^{3+} is reduced to Fe^{2+} by a membrane-bound ferric reductase oxidase (FRO), FRO2 [102], one of the eight members of the FRO family. FRO genes are expressed differentially at the tissue level; for example, FRO2 is specific to roots, whereas FRO6 and FRO7 are specific to shoots [103, 104]. FRO proteins are also predicted and/or experimentally shown to localize to different subcellular compartments [103, 105, 106]. Therefore, each FRO family member has a specific role in different organs or subcellular compartments, signifying that reduction-based iron transport is not limited to the root plasma membrane.

Once Fe^{3+} is reduced, Fe^{2+} is transported into the root by iron-regulated transporter 1 (IRT1), a member of the zinc-regulated transporter (ZRT)- and IRT-like protein (ZIP) family [107]. Analysis of plants overexpressing IRT1 from the cauliflower mosaic virus 35S promoter shows that IRT1 is present only in iron-deficient roots, suggesting that it is controlled post-transcriptionally [60]. A recent follow-up study showed that iron-induced turnover of IRT1 requires two lysine residues located in the intracellular loop of IRT1 between transmembrane domains III and IV [108]. This is consistent with the turnover of ZRT1, a yeast zinc transporter that also belongs to the ZIP family [109]. In its variable loop region, ZRT1 has a lysine residue that is ubiquitinated to target it for protein degradation under zinc-sufficient conditions. When either IRT1 lysine residue was substituted with arginine and the variant was overexpressed, the plants accumulated higher levels of iron than did wild-type plants; in contrast, plants overexpressing wild-type IRT1 contained iron levels similar to those of wild-type plants [108]. However, the accumulation of iron was not associated with increased ferric chelate reductase activity, implying that this activity is not rate limiting, at least under the conditions tested [108].

11.5.2
The Chelation Strategy

To acquire iron, grasses use a mechanism based on chelation, known as strategy II, in which phytosiderophores (PSs), such as mugineic acids (MAs), are released to chelate Fe^{3+}. The resulting Fe(III)–PS complexes are then transported into the roots via transporters belonging to the Yellow Stripe (YS) family, named for the YS1 PS transporter of maize (*Zea mays*) [110]. Grasses can also take up Fe^{2+} in addition to Fe(III)–PS [111, 112]. Rice (*O. sativa*) plants that cannot synthesize PS, owing to a mutation in the nicotianamine aminotransferase (NAAT) gene, do not show growth defects if Fe^{2+} is supplied [111]. However, unlike strategy I plants, neither H^+-ATPase nor Fe^{3+}–chelate reductase activity is induced under iron deficiency. This probably reflects an adaptation to flooded rice paddies, where Fe^{2+} is more abundant than Fe^{3+} owing to reduced levels of oxygen [112].

11.5.3
Regulation of the Reduction Strategy

In *Arabidopsis*, Fe-regulated (Fer)-like iron deficiency-induced transcription factor (FIT), a basic helix–loop–helix (bHLH) transcription factor orthologous to the tomato (*Solanum lycopersicum*) FER protein [113], is required to regulate iron deficiency responses [111, 114, 115]. FIT is highly induced in the epidermis of iron-deficient roots, and *fit* mutants are chlorotic and seedling lethal unless watered with supplemental iron [100], showing phenotypes similar to those of *irt1* plants. FRO2 is transcriptionally regulated by FIT, and FRO2 transcripts and root ferric chelate reductase activity are both absent in *fit* mutants [100, 114]. Meanwhile, IRT1 is post-transcriptionally regulated by FIT and *fit* mutants do not accumulate IRT1 protein, despite the induction of IRT1 mRNA by iron deficiency. Because overexpressing FIT does not affect FRO2 and IRT1 expression, FIT was postulated to function as a heterodimer [100]. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) results showed that genes encoding four additional bHLH transcription factors (AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101) were induced by iron deficiency in roots and leaves [116]. Because FIT is not expressed in leaves, the partially overlapping expression pattern suggests that the four bHLH proteins also have other roles besides interacting with FIT. Biomolecular fluorescence complementation showed that AtbHLH38 and AtbHLH39 interact with FIT. FIT–AtbHLH38 or FIT–AtbHLH39 complexes activated transcription driven by the FRO2 and IRT1 promoters in yeast, suggesting that FRO2 and IRT1 are direct targets of these complexes. AtbHLH38 and AtbHLH39 are seemingly functionally redundant because their single null mutants lack phenotypes. When either AtbHLH38 or AtbHLH39 was overexpressed with FIT, the plants accumulated more iron in their shoots than did wild-type plants, consistent with the idea that FIT functions as a heterodimer. The fact that all these genes are themselves iron-regulated indicates there must be an upstream iron sensor [117].

11.5.4 Iron Signaling and Sensing in Plants

Hormones have roles in iron deficiency signaling. Physiological studies showed that ferric chelate reductase activity decreased upon ethylene inhibitor treatment but increased upon addition of ethylene precursors [118]. Similarly, treatment with ethylene inhibitors repressed expression of IRT1, FRO2, and FIT, whereas expression of these genes was enhanced when ethylene precursors were added [119, 120]. Cytokinins negatively regulated IRT1, FRO2, and FIT expression at the transcript level, independent of the iron status of the plants [121]. This repression required cytokinin receptors but not FIT, and conditions that inhibit root growth, such as osmotic stress induced by mannitol or NaCl and hormonal treatments with auxin or abscisic acid, repressed iron deficiency response genes [121]. Because cytokinins inhibit root growth, the results imply that cytokinin treatment restricted nutrient uptake via a growth-dependent pathway by transiently arresting root elongation to reduce nutrient demand. Another example of the close link between root growth and nutrient uptake is provided by phosphate uptake. Phosphate starvation responses require continuous root growth, whereas inhibiting cell cycle activity represses phosphate uptake in response to decreased phosphate demand [122]. Nitric oxide (NO) is proposed to transmit iron deficiency signals [123–125]. NO reverted the chlorotic phenotypes of maize mutants defective in iron uptake [123] and stimulated accumulation of *Arabidopsis* ferritin transcripts and protein by acting downstream of iron [125]. In tomato, NO was rapidly produced in roots as an early response to iron deficiency; this helped to facilitate iron uptake, presumably by regulating root hair growth and by enhancing expression of iron uptake-related genes, because treatment with NO enhanced FER, LeFRO1, and LeIRT1 mRNA levels [124]. Nitric oxide, plant hormones, and other molecules could also act in concert or downstream or upstream of each other. With more evidence accumulating for different molecules involved in iron signaling, a necessary task is to determine how they integrate into a bigger pathway [77].

11.5.5 Iron Transport within the Plant

For proper storage and use of iron, it must be safely translocated to different parts of the plant and compartmentalized into specific organelles in the cell. Indeed, it is essential to maintain iron homeostasis both at the intercellular and at the intracellular level.

11.5.5.1 Intercellular Iron Transport

To avoid handling toxic free cellular iron during translocation throughout the plant, chelates such as citrate and nicotianamine (NA) are used [110]. Depending on the iron–chelate complex formed, different transport systems are involved in distributing iron throughout the plant.

Fe(III)–citrate is the major form of iron present in xylem exudates, and citrate is thought to be involved in long-distance iron transport from roots to shoots [126]. Ferric reductase defective 3 (FRD3), a multidrug and toxin efflux (MATE) family member, is localized to the plasma membrane of cells in the pericycle and vasculature and functions in iron translocation from roots to shoots by loading citrate into the xylem [127]. *frd3* xylem exudates contained less citrate and iron than did exudate from wild-type plants, and *frd3* mutant phenotypes were rescued by supplementing with citrate, consistent with the role of FRD3 as a citrate transporter. Heterologous studies in *Xenopus* oocytes confirmed that FRD3 does transport citrate. After citrate loaded into the xylem chelates iron, either Fe(III) citrate complexes are taken up at different locations via as yet unidentified transporters or the complexes might be reduced by FROs and Fe^{2+} would then be transported into various cells of the plant [128].

NA is a nonproteogenic amino acid that chelates both Fe^{2+} and Fe^{3+}, in addition to other divalent metals such as copper, zinc, manganese, cobalt, and nickel. NA is synthesized and used in all plants, regardless of their iron uptake strategy, and is a precursor of MA, a PS that is only found in graminaceous plants [110]. NA is structurally similar to PSs and chelates iron for intercellular transport in the phloem. A characteristic phenotype of plants lacking NA is interveinal chlorosis in young growing leaves, as seen in the tomato chloronerva (chln) mutant, which is defective in NA synthase [129].

YS-like (YSL) family members are thought to transport metal–NA complexes. There are eight YSLs in *Arabidopsis*. YSL1 and YSL3 are suggested to be involved in mobilizing metals, including iron, from leaves for use in developing seeds [130]. *ysl 1* and *ysl3* are functionally redundant because the single mutants lack visible phenotypes, whereas *ysl1 ysl3* double mutants show severe interveinal chlorosis, lower iron content in roots, leaves, and seeds, decreased fertility, arrested pollen and embryo development, and defects in mobilizing metals from leaves during senescence [130].

YSLs represent a subfamily of the *Arabidopsis* oligopeptide transporter (OPT) family, and *AtOPT3* was also reported to be involved in supplying iron for seed development [131]. OPT3 is expressed in the vasculature, pollen, and developing embryos [131]. In *opt3-2* mutant plants, where OPT3 expression is reduced, both the yield and iron content of *opt3-2* seeds decreased [131]. Although the mutant roots exhibited constitutive iron deficiency responses, their leaves were necrotic and accumulated high levels of iron. These results imply that OPT3 is also involved in regulating iron at the whole plant level.

11.5.5.2 **Subcellular Iron Transport**

Organelles such as chloroplasts and mitochondria require iron to carry out various metabolic processes and serve as reservoirs to keep iron for later use. This is essential to regulate iron not only at the cellular level but also at the organism level. For example, defects in organellar iron homeostasis can cause a lethal phenotype, as seen in the *Arabidopsis* frataxin mutant, which is defective in mitochondrial iron homeostasis [132]. Although understanding of subcellular iron transport remains limited, studies are gradually providing insights into subcellular trafficking of iron in plants [77].

11.5.5.3 **Vacuoles**

Vacuoles are crucial compartments for iron storage and sequestration within plant cells. In particular, studies have shown that the vacuole is an essential compartment for iron storage in seeds [132, 133].

Vacuolar iron transporter 1 (VIT1) imports iron into the vacuole [134]. Heterologous expression of VIT1 in yeast complemented the high iron-sensitive phenotype of the Ca^{2+}cross-complementer 1 (*ccc1*) mutant by increasing its vacuolar iron content, indicating that VIT1 transports iron into the vacuole. In *Arabidopsis*, VIT1 is highly expressed in developing seeds, and *vit1* mutants are unable to survive when germinated in alkaline soil, where iron availability is greatly limited. Synchrotron X-ray fluorescence microtomography revealed that iron is concentrated in the provasculature of developing embryos. Most strikingly, such distribution of iron was abolished in *vit1-1* mutant seeds, even though the seed iron content was not altered. These results highlight the relevance of proper localization of vacuolar iron stores in the embryo for seedling development [134].

Two metal transporters of the natural resistance-associated macrophage protein (NRAMP) family, NRAMP3 and NRAMP4, both export iron from vacuoles. NRAMP3 and NRAMP4 genes are induced under iron deficiency and, although the single mutants lack phenotypes owing to presumed functional redundancy, germination of *nramp3 nramp4* double mutants was arrested under iron-limiting conditions. Mutant seeds contain wild-type levels of iron; however, electron microscopy showed the disappearance of iron from wild-type vacuoles during germination, whereas vacuoles of the *nramp3 nramp4* mutant remained unaltered, supporting a role for these transporters in iron mobilization from the vacuole. Taken together with the *vit1* phenotypes, these data suggest that vacuoles are an important site of iron storage and that iron remobilization during germination is crucial for seedling development when iron supply is low [133].

11.5.5.4 **Chloroplasts**

Iron is required for photosynthetic electron transport, chlorophyll biosynthesis, Fe–S cluster assembly, heme biosynthesis, and other essential metabolic processes that occur in chloroplasts [135]. It is also quantitatively significant in plastids, which contain 80% of the iron found in a leaf cell [136]. Because the photosynthetic electron transport chain produces reactive oxygen species (ROS), iron should be tightly regulated in chloroplasts to avoid oxidative damage via the Fenton reaction. In plants, the iron storage protein ferritin, which stores up to 4500 iron atoms, is found in plastids [137]. There are four FER genes in *Arabidopsis*; FER1 is proposed to be involved in senescence [138]. Age-dependent senescence was accelerated in *fer1* loss-of-function mutants owing to iron toxicity under excessive ROS accumulation. A recent study with mutants that lack seed (*fer2*) or leaf ferritins (*fer1*, *fer3*, *fer4*) showed that ferritins are essential for protection against oxidative damage but are not the major iron pool for either seedling development or proper functioning of the photosynthetic apparatus [139].

Recently, a chloroplast ferric chelate reductase, FRO7, was characterized that was required for seedling survival under iron-limiting conditions [105]. Chloroplasts

isolated from *fro7* mutants had 75% less ferric chelate reductase activity and contained 33% less iron than did wild-type chloroplasts, demonstrating that FRO7 has a role in chloroplast iron acquisition.

There exit no reports to indicate that ZIP transporters are localized to chloroplasts. However, a cyanobacterial permease-like protein, permease in chloroplasts 1 (PIC1), was reported as a chloroplast iron transporter [140]. Although PIC1 was also identified as part of the chloroplast inner envelope protein-conducting channel [141], its expression complemented the yeast *fet3 fet4* mutant defective in iron uptake; furthermore, *pic1* mutant plants show phenotypes consistent with a defect in iron transport, such as severe chlorosis, heterotrophic growth, and accumulation of ferritins. Whether PIC1 transports Fe^{2+} or Fe^{3+} and whether a reductase is associated with the process are still unknown [140].

11.5.5.5 **Mitochondria**

Mitochondria, similar to chloroplasts, are organelles with a high iron demand [136]. Iron is used as a cofactor in the respiratory electron transport chain and Fe–S clusters are assembled in mitochondria in addition to chloroplasts. In animals, mitochondrial ferritins have been identified [142, 143], and proteomics and electron microscopy suggest that mitochondrial ferritins are also present in *Arabidopsis*. As in chloroplasts, mitochondria must deal with ROS generated from the electron transport chain and must strictly maintain iron homeostasis. Although plant mitochondrial iron importer(s) have not yet been identified, three orthologues of the yeast (*Saccharomyces cerevisiae*) ABC transporter of the mitochondrion 1 (ATM1) are found in *Arabidopsis* [144]. ScATM1 exports Fe–S clusters from the mitochondrial matrix, and the yeast *atm1* mutants exhibit slow growth and respiration defects, lack cytochromes, and constitutively accumulate high levels of mitochondrial iron [144]. *AtATM1*, *AtATM2*, and *AtATM3* are localized to mitochondria. Among the three ATMs in *Arabidopsis*, *AtATM3*, also known as STARIK (STA1), is the most similar to ScATM1 and complements the yeast *atm1* phenotypes [145]. *AtATM3* is thought to export Fe–S clusters in plant, and *sta1* mutant plants are dwarf and chlorotic. *AtATM1* (STA2) partially suppressed the *Arabidopsis sta1* and the yeast *atm1* mutant phenotypes [145], whereas the function of AtATM2 remains uncharacterized [144]. FRO8 was detected in a mitochondrial proteomic study [106]. This implies that ferric chelate reductase(s) might also be involved in mitochondrial iron transport, as seen for chloroplast iron transport [105].

11.6 Conclusions

The real challenges to solve malnutrition in the developing world are reduction of poverty and diseases and the increase of infrastructure and education. All these issues are important and should continue to be addressed, as they have been in the past. In developing countries, food biofortification is increasingly recognized as an

effective approach to improve the micronutrient status of large parts of the populations. Improving nutritional quality of rice grains through genetic modification and traditional breeding holds promising possibilities for food-based solutions to micronutrient deficiencies.

Increasing iron root absorption by transgenic approaches improved the plants' ability to cope with iron-deficient conditions. However, up to now, no evidence for increased micronutrient content in the edible part was reported. A modest increase of iron content in seeds was obtained after overexpression of a zinc transporter in barley and through expression of the iron storage protein ferritin. However, iron concentrations in leaves of high ferritin overexpressing rice plants were even less than one-tenth of the nontransformants, and the plants showed chlorosis symptoms. This seems to indicate that only the combination of strategies for higher iron acquisition and transport with an increased sequestration and storage capacity will allow an important accumulation of iron in the edible part of the plant. Furthermore, to be important for human nutrition, iron availability has to be improved, possibly by crossing the rice lines with higher iron content with those allowing better iron bioavailability, that is, those with lower phytic acid content or rich in peptides, which enhance iron absorption.

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12
Pearl Millet: Genetic Improvement for Tolerance to Abiotic Stresses

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Abstract

Pearl millet (*Pennisetum glaucum*) is an important cereal grown in adverse agroclimatic conditions where other crops fail to produce economic yields. Because of the cultivation of pearl millet mainly in rainfed production systems of arid and semiarid regions, drought is a primary constraint in its cultivation. In addition, high temperatures and salinity are emerging as new challenges in pearl millet cultivation in specific production environments. This chapter reviews the research dealing with improvement in drought tolerance of pearl millet and also updates the progress made in improving high temperature and salinity tolerance. Response of pearl millet to moisture stress at various growth stages has clearly established that yield losses are maximum when moisture stress coincides with grain filling stage, which is commonly referred to as terminal water stress. Various physiological and morphological traits have been examined as alternative selection criteria to further enhance tolerance to terminal drought. Conventional approaches to improve drought tolerance in pearl millet have a very short history and attempts have met with some success. Various novel approaches have been attempted in pearl millet for enhancing yield under drought environments. These include use of adapted germplasm, genetic diversification of adapted landraces through introgression of suitable elite genetic material, and exploitation of heterosis to amalgamate drought tolerance and high yield. Molecular breeding is fast emerging as a supplement approach to enhance drought adaptation at a faster rate with greater precision. Molecular marker-based genetic linkage maps of pearl millet are available and genomic regions determining yield under drought environments have been identified preparing a road map for marker-assisted selection. Genetic differences in tolerance to salinity and high temperature at both seedling and grain filling stages have been established and screening techniques standardized. The germplasm and breeding material with a higher degree of tolerance to high temperature and salinity have been identified in order to use them in breeding programs.

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12.1 Introduction

Pearl millet (*Pennisetum glaucum* (L). R. Br.), a C_4 plant belonging to the family Poaceae, has a very high photosynthetic efficiency and dry matter production capacity. It is grown under the most adverse agroclimatic conditions where other crops fail to produce economic yields. Pearl millet is usually cultivated in regions with characteristically low and erratic rainfall, high mean temperature, high potential evaporation, and infertile and shallow soils with poor water holding capacity. In spite of this, pearl millet has a remarkable ability to respond to favorable environments because of its short developmental stages and capacity for high growth rate, thus making it an excellent crop for short growing season and under improved crop management.

Pearl millet is cultivated on about 30 m ha in more than 30 countries of 5 continents, namely, Asia, Africa, North America, South America, and Australia (Figure 12.1). The majority of crop area is in Asia (>10 m ha) and Africa (about 18 m ha). At individual country level, India has the highest area (9.3 m ha) and production (9.5 m tons) and the major pearl millet growing Indian states are Rajasthan, Maharashtra, Gujarat, Uttar Pradesh, and Haryana. In Africa, the majority of pearl millet acreage is in western Africa where it is grown in 17 countries, though Niger, Nigeria, Burkina Faso, Mali and Senegal account for nearly 90% of

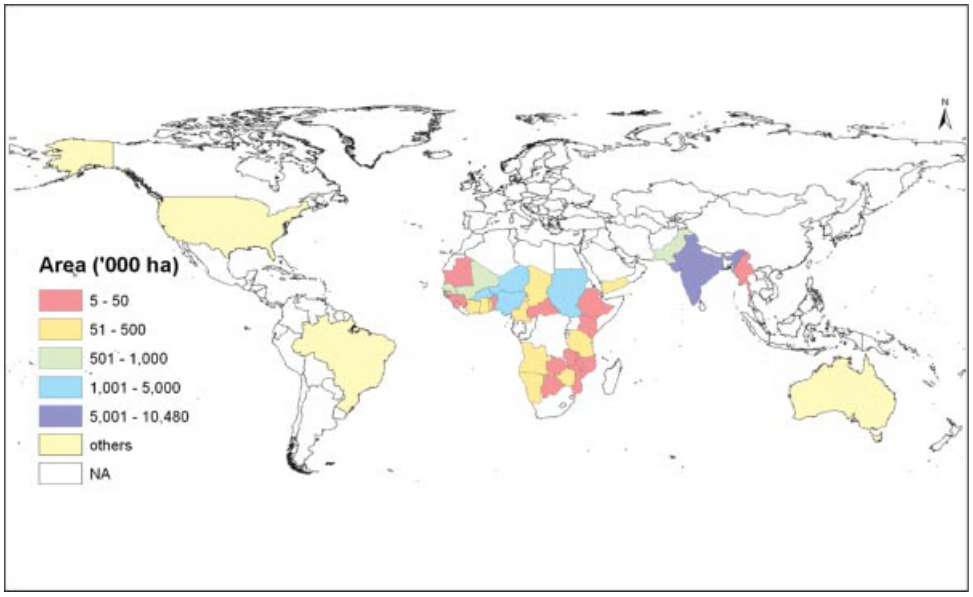

Figure 12.1 Distribution of pearl millet cultivation across world.

total cultivated area in Africa. Pearl millet cultivation is also recently expanding to some of the non-traditional areas such as Brazil (about 2 m ha), and it is being experimented as a grain and forage crop in the United States, Canada, Mexico, the West Asia and North Africa (WANA), and Central Asia.

Pearl millet is primarily grown for food for human consumption and its dry fodder forms the basis of livestock ration during the dry period of year (November–January) in India [1]. Its grains are mostly used for human consumption in the form of diverse food types such as leavened and unleavened flat breads and porridges. Several bakery products and extruded and weaning food products are also prepared. Besides, pearl millet is a highly nutritious cereal with high level of metabolizable energy and protein and more balanced amino acid profile [2]. Its grains have higher densities of iron and zinc [3], the two most important micronutrients for human. Pearl millet flour can also be substituted up to 20% for wheat flour in making leavened bread. Grains of pearl millet are also used as cattle and poultry feed. Pearl millet is also an excellent forage crop because of its lower hydrocyanic acid content than sorghum. Its green fodder is rich in protein, calcium, phosphorous, and other minerals with oxalic acid within safe limits.

Pearl millet production is confronted with relatively fewer biotic stresses compared to other crops. Among the diseases, downy mildew (*Sclerospora graminicola* (Sacc.) Schroet.) is the most important constraint in both India and Africa, especially on hybrids in India. Other diseases of relatively minor importance include smut (*Moesziomyces penicillariae*), rust (*Puccinia substriata* var. p*enicillariae*), blast (*Pyricularia grisea*), and ergot (*Claviceps fusiformis*). Insect–pests and parasitic weed Striga are significant challenges in Africa but not in India.

Because of the cultivation of pearl millet largely in rainfed production systems of semiarid and arid regions of world, crop growth is constrained by several abiotic stresses. Drought is the primary abiotic constraint and is caused by low and erratic distribution of rainfall. The mean annual rainfall in pearl millet-growing areas in India ranges from 150 to 750 mm and most of it is received during June–September [4]. In western Africa, the crop is cultivated in regions with annual rainfall ranging from 300 to 900 mm [5], the majority of which is received between May and October. The bulk of the crop in the Sahelian zone is grown with an annual rainfall of 300–600 mm and a growing season of 75–100 days. The Sudanian zone receives an annual rainfall of 600–900 mm with a growing season of 100–150 days. The coefficient of variation of annual rainfall ranges from 20% to 30% leading to variable drought conditions within and between crop seasons.

Other abiotic constraints to pearl millet production include high temperatures, both during the germination and seedling stage in the rainy season, and during the flowering period during the summer season (in parts of India). Salinity is also being increasingly recognized as a significant abiotic constraint in many pearl millet growing areas in Africa and India, but more so in the prospective areas in the WANA region and Central Asia. This chapter largely deals with drought tolerance as significant research efforts have gone into the understanding of and breeding for this trait. It also presents preliminary observations on heat and salinity tolerance.

12.2
Drought: Its Nature and Effects

A great deal of work has been done on understanding the response of pearl millet to moisture stress at various growth stages with a view to understanding its adaptation to drought stress conditions. It has been conclusively established that effects of water stress depend on the developmental stage during which the crop is subjected to stress. Consequently, pearl millet research has concentrated on exploring the effects of drought at specific growth stages.

12.2.1
Seedling Phase

Severe moisture stress during emergence and the early seedling phase causes seedling death, which results in poor crop establishment. Poor and uneven crop stands are some of the major causes of yield losses in pearl millet [6–8] in the semi-arid tropics. Stress occurring after crop establishment but within the seedling phase has little effect on grain yield [9] provided it is relieved at the later stages before flowering.

Drought during the seedling phase affects seedling growth in several ways. The rate of leaf appearance in seedling is affected by the timing of available water, that is, an early drought will prolong the seedling phase [10]. It has been further showed that drought affects the close relationship between leaf formation and secondary root development. Secondary roots are formed only when there is soil moisture at the coleoptile node [11]. Genetic variation in the rate of leaf appearance and secondary root formation and their relative rates have been observed under drought [10]. There are no known reports dealing with the genetic manipulation of this trait in a breeding program.

12.2.2
Vegetative Phase

Water stress during vegetative growth may have little adverse effect on grain yield of pearl millet as it has been shown to increase the number of panicles per plant [12–14]. It has been established that only 25% of the tillers produce panicles in pearl millet under normal conditions [15]. The apparent excess production of tillers provides potential compensation for a damaged main shoot or primary tillers [16, 17]. High tillering and asynchrony of tillering contribute to adaptation to drought stress during the vegetative growth phase [18–20]. Water stress during the vegetative phase reduced the dominance of the main shoot and allowed additional tillers to complete their development [12, 21]. Accumulation of abscisic acid under water stress may be responsible for this reduction in apical dominance.

Water stress during the vegetative growth phase delays flowering of the main shoot [12, 13, 20]. This phenological plasticity increases the chances for escape, first from the most sensitive stage of growth until the stress has been relieved,

and second by closing stomata at relatively high water potentials during drought in the vegetative period [22]. The crop thus conserves the limited water resources, increasing the chances to survive the extended periods of stomatal behavior changes. Stomatal opening down to water potentials as low as 2.3 MPa, during stress after flowering, has been observed [23]. Late-flowering genotypes do have a longer GS1 period, that is, the time between seedling emergence to panicle initiation [24, 25] is longer than that for early maturing genotypes. Thus, such genotypes have a higher chance to escape drought stress during the most critical growth phases.

12.2.3 Reproductive Phase

Grain yield losses are highest when stress coincides with the most sensitive stages of crop growth [26]. It has been found that pearl millet is most sensitive to water stress during flowering and grain filling stages [9, 20, 27]. Grain yield and its components are drastically reduced when drought occurs during this stage [12, 13, 28]. Yield reduction is due to both decrease in the number of panicles per plant and decrease in the grain mass. Seed setting that determines the number of grains per panicle is usually less affected if terminal stress occurs after flowering [13, 20].

The reduction in grain mass is mainly due to a shortening of the grain filling period rather than due to a reduction in grain growth rate [29]. This appears to be caused by restriction of the current assimilate supply and not by a reduction in the grain storage capacity [29]. Stomatal closure and a consequent reduction in photosynthetic activity under drought stress have been documented for pearl millet, though only at very low water potentials [23, 30–32]. Pearl millet has the capacity to compensate for such a reduction in the supply of assimilates to the grains by mobilizing stored soluble sugars [28]. This contribution of stored assimilates to the grain growth during drought stress has not been quantified. The link between grain development and the transfer of assimilates from the leaves, with the stems playing a buffering role, appears to be one of the main adaptations of pearl millet to terminal drought stress [33].

12.3 Genetic Improvement in Drought Tolerance

Pearl millet is grown as a rainfed crop in areas where rainfall is too limited for higher yielding cereals such as sorghum or maize. Hence, improving drought tolerance is a priority in pearl millet breeding programs. Breeding for increased adaptation to drought is, however, a challenging task due to various complexities associated with drought adaptation mechanism, uncertainty in timing, intensity and duration of stress, and a large genotype × environment interactions. Conventional approaches to improve drought tolerance in pearl millet have a very short

history and attempts have met with some, though limited, success. Recently, molecular breeding is being viewed as an additional tool to improve drought tolerance with greater precision and efficiency.

12.3.1 Conventional Breeding

Empirical breeding for drought tolerance has mainly addressed the issue of criteria and environment of selection for improving drought adaptation. Various novel approaches have been attempted in pearl millet for enhancing yield under drought environments.

12.3.1.1 Selection Environment

Choosing an appropriate selection environment to improve productivity under drought has been a subject of the major debate in plant breeding, and several theoretical and empirical studies have been reported. Some believe that cultivars targeted for drought conditions can be identified under non-drought conditions (indirect approach), while others think that selection of drought environments should be undertaken under drought stress (direct approach).

The indirect approach involves selection for high yield potential under non-stress conditions with the assumption that genotypes selected under optimum conditions [34–36] would also perform well under drought. In this approach, drought resistance is an unidentified component of performance over different environments and more emphasis is laid on yield potential. The main advantage of this approach is that yield potential and its components have higher heritability in optimum conditions than that under stress conditions [37, 38]. Since yield potential has been reported to be a significant factor in pearl millet in determining the yield under moisture stress [13, 14, 28, 39], improvement in yield potential may have some spillover effects under water stress conditions.

The direct approach recommends that varieties for drought-prone areas must be selected, developed, and tested in the target drought environments [40–42]. Theoretical analyses also indicate that selection for stress environments should be done in stress environments [41, 43, 44]. In this approach, improvement in yield under moisture stress requires dissociation from yield potential under optimum conditions as a major selection criterion [45–47] and the emphasis is placed on drought adaptation and yield under drought conditions.

The subject of selection environments for improving pearl millet in drought environments has received little experimental attention. There are no reports available in pearl millet comparing relative gains in performance under drought conditions through selection in drought vis-à-vis non-drought environments. However, there are indirect inferences. For example, low correlations are often reported between yields of pearl millet measured in stress and optimum conditions [48–50], which indicate that yield performance under drought and non-drought conditions are separate genetic entities and direct selection for yield performance in the target

Table 12.1 Percent contribution of high yield potential, escape, and drought tolerance in determining performance of pearl millet under drought environments.

No. of genotypes	Types of lines tested	Yield potential	Escape	Drought tolerance	Reference
105	Landraces	5	22	73	[126]
30	Hybrids	15	—	—	[50]
14	Cultivars	0.4–9.3	23–81	18–68	[42]
40	Breeding material	4–23	23–37	41–47	[28]
216	Breeding lines	2–12	46–56	34–36	[13]

drought environments would be required to make greater gains in productivity. This is further substantiated by existence of significant crossover genotype × environment interactions observed across optimum and stress environments [51–56]. Using evaluation data from drought stressed and non-stressed environments, many studies showed that drought tolerance and escape were major determinants of performance in drought environments (Table 12.1). On the other hand, high yield potential accounted for 10–15% variation toward performance in drought environments. This has highlighted the importance of evaluation and selection in drought-prone locations and early maturity, and suggested for *in situ* breeding for drought environments. Alternatively, simple and efficient screening techniques might be employed for evaluating large number of genotypes under managed drought conditions.

The work on screening techniques in pearl millet, for adaptation to drought, has primarily focused on terminal drought stress, because it causes higher and irreversible yield losses. Field screening for response to terminal drought can be carried out by withholding irrigation to impose water stress during the rain-free seasons to study the effects of drought stress and to identify whole plant traits associated with adaptation to a particular stress [12–14, 20, 21, 57]. One such technique has been developed at ICRISAT, which compares genotype performance in artificially created terminal stress (flowering to maturity) treatments with performance under fully irrigated, stress-free conditions [13, 14, 28]. Drought resistance is then determined on the basis of genotype performance in the stressed treatment after accounting for differences due to escape and yield potential among genotypes. However, off-season drought screening may not necessarily give results similar to naturally occurring stress in a rainy season crop like pearl millet, as fluctuations in atmospheric conditions or changes in phenology due to different day lengths may alter the results. The technique [58] that involves growing plants in main crop season on sloping plots that are opposite to each other and connected to subchannel lines with polyethylene sheets avoids this problem by increasing the runoff and reducing the water availability. However, this technique has neither been validated nor used in applied breeding programs.

Line-source sprinkler irrigation technique [59], which delivers a continuously declining amount of water, has been extensively used for screening sorghum [60, 61],

rice [62], and pearl millet [63, 64], especially when crop response to moisture stress is non-linear. The major disadvantage, however, with this technique is that even low winds may significantly alter the sprinkler patterns [59] and only a relatively small number of entries can be accurately tested to detect significant differences. It is because of these two factors that line-source sprinklers are no longer used in routine assessments of drought response in pearl millet breeding programs.

Additional limitation of artificially created screening techniques is that they are unable to expose the test material to all the combinations of stress the crop might subsequently experience given that drought stress occurs in a wide range of combinations based on variability in timing, severity, and duration of drought. This necessitates selection in the target environments that are highly prone to terminal drought. There are extensive evidences from other crops that cultivars for stress environments should be selected, developed, and tested under target environments [47, 65–68].

Due to the complexity of drought adaptation, it seems doubtful that any one method or technique will be universally used to measure drought stress [69] because the variability in timing, intensity, and duration of moisture stress is almost infinite and screening methods can expose genotypes to only a few combinations [70]. In empirical breeding programs, evaluation and selection are conducted through multilocation testing of test material in locations that are highly prone to drought stress [47, 68]. The All India Coordinated Pearl Millet Improvement Project (AICPMIP) has carved out a special zone for testing and evaluation of experimental cultivars in locations receiving <400 mm of annual rainfall in order to identify and release cultivars adapted to drought environments and the results are encouraging [71].

Thus, maximum progress can be gained with a good understanding of the predominant patterns of drought occurrence in the target environment, appropriate material that expresses sufficient genetic variability for the most appropriate traits for good adaptation, and reliable conditions for yield testing under drought conditions.

12.3.1.2 **Selection Criteria**

Several efforts have been made to identify traits that can be used as selection criteria in breeding drought tolerant genotypes. Most research has concentrated on the identification of physiological parameters like dehydration tolerance [72–76], dehydration avoidance [77], growth maintenance through stability of cellular membrane [78–80], osmotic adjustment [69, 81, 82], desiccation and heat tolerance [69], leaf gas exchange rate [83–85], and radiation reflectance [77] in various crops, including pearl millet [86]. However, most of these have hardly found any place in routine breeding programs, particularly in developing countries, owing to the lack of simple and easy techniques for selecting such characters on a large scale. On the other hand, morphological characters that can be measured easily appeal most to plant breeders for use as selection criteria.

Growth in greenhouse pots under different soil moisture regimes, germination of seeds and growth of pearl millet seedlings in dimannitol solutions, and stability

of extracted chlorophyll under heat treatments to test drought resistance has been used in pearl millet [87]. Such studies under controlled conditions, however, do not necessarily represent the limiting moisture conditions of the field.

A rapid development of the crop in the initial stages, that is, early vigor, has been correlated with drought tolerance as measured by time taken for wilting initiation and permanent wilting in pearl millet [88]. A crop with more rapid leaf area development could intercept a greater portion of incident radiation and limit water losses by soil evaporation. However, there are apprehensions that greater transpiration from a larger leaf area will exhaust soil water resources and cause severe water deficit in later growth stages [33].

Early flowering, the most important factor determining yield under terminal water stress [14, 28], is recognized as another selection criterion, although its advantage is due to drought escape rather than due to drought tolerance. Genetic variability for earliness is widely available in pearl millet [89, 90] and simple selection has been successful under most circumstances [91]. The most widely used sources of earliness are the *Iniadi*-type landraces from western Africa [92, 93]. New early-flowering cultivars bred by using *Iniadi* landraces have been widely adopted by farmers in India and Africa. However, value of earliness as a selection criterion is significant only if drought predictably occurs toward the end of the growing season.

Panicle threshing percentage is another criterion proposed for improving tolerance to terminal drought that indicates the plants' ability to set and fill grain under water limiting conditions and it integrates the effects of assimilation and translocation under water stress. Research has indicated that it usually explains a large proportion of the variation among genotypes for grain yield under terminal drought stress [28, 77, 94]. Results of a selection study on panicle threshing percentage also indicated that grain yield can be increased under stress conditions [94]. Furthermore, it has been shown that even in a small set of inbred lines, the narrow sense heritability for threshing percentage was sufficiently high to expect significant gain from selection for this trait under drought conditions [95]. Using this selection criterion, an open-pollinated variety (OPV) (ICMV 221) has been developed from a high-yielding and early-maturing composite at ICRISAT.

Low tillering and large panicles are commonly being used as selection criteria in pearl millet breeding. Selecting for these traits results in higher grain yield per panicle [14, 96], which are important yield components in pearl millet [97–99]. These traits are frequently assessed visually, under both drought and non-drought conditions. Variability in panicles size, yield per panicle, and tillering is abundant in pearl millet [90, 96, 100–105]. However, their specific contribution to improved grain yield and stability under terminal drought condition has not been quantified.

Some studies have also used mathematical models to identify crop cultivars that are productive in stressful marginal environments by comparing the change in seed yield between stress and non-stress (optimum) environments [14, 35, 44, 73, 106–108]. Drought susceptibility index [106] that is based on the ratio of yield of individual lines under stress and non-stress conditions to the line means across stress and non-stress has also been widely used in identifying genotypes adapted to

stress [14, 50, 108–111]. Drought response index to provide an indicator of drought tolerance that was independent of escape and yield potential in favorable environments has also been developed [14]. Drought susceptibility index of pearl millet genotypes is a useful criterion to identify genotypes adapted to drought stress conditions, but should be used in combination with yield under stress [50]. It has also been demonstrated that drought response index would be useful to identify genotypes adapted to stress environments, if days to flower don't differ considerably among test entries [50].

12.3.1.3 Yield Improvement

Yield improvement in pearl millet under drought is essential for ensuring high grain yield under stress environments. Though drought tolerance might be perceived differently by physiologists, breeders, agronomists, or biochemists [112], farmers measure the success of new cultivar under drought environments by a known (often >15%) yield advantage. Various genetic approaches have been successfully employed to achieve significant gains in pearl millet productivity under drought conditions. These strategies include use of adapted germplasm, genetic diversification of adapted landraces through introgression of suitable elite genetic material, and exploitation of heterosis.

12.3.1.3.1 **Use of Adapted Germplasm for Stress Environments** The base material required for a successful breeding program may differ for the drought and more favorable environments. Success in drought environments is often much more a consequence of adaptation to environmental stresses than it is of yield potential *per se*, which is not effectively expressed under severe stress. Plant breeders focusing on drought environments are faced with the choice of trying to improve either the adaptation of high yielding, but poorly adapted germplasm, or the yield potential of already adapted germplasm, often in the form of local landraces [113]. Improving adaptation to marginal environments is the more difficult alternative than is improving yield potential, as adaptation is much less well understood, physiologically and genetically, than is yield potential. However, improving yield potential in traditional landraces is constrained by a characteristic plant type that favors adaptation over productivity [16, 17].

The breeding material should provide a good starting point for the program, that is, high productivity under drought conditions, as well as sufficient genetic variation to allow gains from selection. Pearl millet landraces that evolved in dry areas as a result of natural and human selection over centuries exhibit good adaptation to drought and other naturally occurring stresses [55, 56, 114–117] and represent a largely untapped reservoir of useful genes for adaptation to stress environments. A few attempts have been made to exploit them in pearl millet breeding programs in a systematic way. Given that landraces are genetically heterogeneous populations [118, 119], they make very appropriate base genetic material for improving adaptation to drought and other abiotic stresses. A few cycles of mass selection in landraces can increase yield considerably [120, 121]. Landrace-based populations adapted to drought have also been

shown as a useful source material for breeding inbred restorer lines [122]. However, selection needs to be carried out in target environments so that adapted germplasm can express its potential fully in area of its adaptation [41, 123].

A commercial pearl millet variety, CZP 9802, has been developed from selected drought-adapted landraces [124]. In the Indian national testing system for new cultivars, its grain yield performance was 25–58% superior to two national checks (ICTP 8203 and Pusa 266) in drought environments and it also maintained its superiority under near-optimum growth conditions by a margin of 16–47% for stover yield and 4–6% for grain yield. It demonstrated that landrace-derived cultivars can have unique features of adaptation to drought stress in addition to responsiveness to improved conditions. As a result, pearl millet variety CZP 9802 was released by the Government of India for cultivation in drought-affected pearl millet-growing areas in the states of Rajasthan, Gujarat, and Haryana; and it has been adopted very well in drought-prone areas of north-western India [125].

12.3.1.3.2 **Genetic Diversification of Drought-Adapted Germplasm** The traditional cultivars and landraces of pearl millet from drier regions possess good levels of drought adaptation [126], but fail to capitalize on yield-enhancing nutrient and moisture conditions, in the native soils, or externally applied [55, 127]. On the other hand, elite genetic material has a greater yield potential expressed under better endowed conditions, but lacks adaptation to severe drought stress conditions [16, 55, 120, 127]. Detailed physiological studies suggest that these two contrasting groups of genetic materials have differential pathways to yield formation under drought stress [16, 17]. The use of elite breeding material may ensure the yield potential, but leaves behind the difficult task of improving adaptation. Landraces may ensure adaptation to drought stress, but they would need to be improved considerably for productivity. This situation suggests good prospects of breeding for drought-prone environments by diversifying the base of adapted landraces through use of appropriate genetic material to amalgamate the adaptation of landraces with high productivity of elite genetic materials. Given that the Indian landraces are characteristically high tillering and have small-to-medium sized seeds, African elite materials possessing complimentary traits like lustrous and bold grain, compact and large panicles, and rapid grain filling are potential sources for introgression of variation [128].

Several attempts have been made in this direction through hybridization of selected landraces and elite materials (Table 12.2). There was considerable improvement in both grain and stover yield in crosses with individual crosses providing up to 50% higher grain and stover yields under drought stress [56, 114, 116, 129]. Also, crosses had enhanced adaptation range, beyond that of their parental populations as they were better able than their landrace parents to capitalize on the additional resources of good growing seasons and simultaneously have a better capacity than their elite parents to tolerate drought [56, 116, 129–131]. These results suggested that the hybridization between landraces and exotic populations breaks up gene complexes of two contrasting groups of genetic material [41, 132, 133] and is effective in combining drought tolerance and high productivity.

Table 12.2 Mean per cent improvement in performance of crosses between landraces and elite composites over landraces.

No. of landraces	No. of elite lines	Average improvement in crosses for grain yield	Average improvement in crosses for stover yield	Reference
4	5	9	17	[117]
4	3	17	11	[115]
3	4	20	26	[116]
3	6	3	7	[130]
5	5	2	13	[129]

12.3.1.3.3 **Exploitation of Heterosis** Pearl millet is a naturally cross-pollinated crop and several sources of highly stable cytoplasmic genetic male sterility (CMS) systems are now available [134–136]. These two attributes render pearl millet an excellent crop to exploit heterosis through production of commercial hybrids. There are numerous reports of high magnitude of heterosis in pearl millet [48, 49, 137], but a vast majority of them are from drought-free high-productivity environments, leading to argument that heterosis is best exploited under highly productive environments.

The exploitation of heterosis in pearl millet for yield enhancement under drought conditions has been explored through developing hybrids between elite male-sterile lines and pollinators developed from drought-adapted landraces to combine the adaptation of landraces with a higher productivity potential achieved through heterosis expressed in hybrids. Many studies, based on a wide range of male-sterile lines and landraces, have quantified the degree of improvement in hybrids over adapted landraces under water-limited conditions (Table 12.3). Average improvement in grain yield of landrace-based topcross hybrids (TCHs) over their landrace pollinators was 15% with potential benefit of up to 75% in best hybrids. Choice of male-sterile lines had considerable effect on the manifestation of heterosis for grain and stover yields [138, 139] and thus has a great bearing on manipulating grain/stover relationship of hybrids.

Table 12.3 Mean and maximum (in parentheses) heterosis (%) in pearl millet topcross hybrids over landrace pollinators.

No. of male-sterile (A) lines	No. of landrace pollinators	Average heterosis (%) for grain yield	Reference
2	19	31 (75)	[138]
3	6	15 (28)	[182]
12	6	18 (61)	[140]
15	1	3 (26)	[183]
7	7	5 (17)	[139]
1	4	32	[121]
2	15	22	[184]

Considering that in drought-prone regions, livestock (maintained largely on pearl millet dry stover) is an integral component of rural economy, any improvement in grain yield should not be at the cost of stover yield, and hence total biomass productivity needs to be increased. This objective was achieved through landrace-based hybrids. An average of 15% heterosis in growth rate in TCHs based on landrace pollinators [138] has been reported, which translated to a positive biomass heterosis. The partitioning of this extra biomass to either grain or fodder appeared to be controlled by the harvest index of the seed parent, resulting in differential heterosis for either grain or stover yields, depending upon the seed parent used [139].

Research has also shown that variation in biomass heterosis is the major determinant of both grain-yield heterosis and stover-yield heterosis [140]. However, contribution of harvest index heterosis to grain- and stover-yield heterosis has been observed to be of a compromising nature. Harvest index heterosis leads to a positive higher grain-yield heterosis but negative heterosis for stover yield, suggesting that the strategy for increasing grain yield by improving harvest index will not result in the desired outcome in the marginal drought environments where stover yield is also important. There are reports of exploitable genetic differences among the male-sterile lines and landrace-based restorers in their ability to produce heterotic crosses for biomass [140–143] and selection for biomass can be highly effective [144, 145]. These results have clearly demonstrated that it is possible to improve the grain and stover production to meet farmers' needs, while retaining critical adaptation to drought environments by exploiting heterosis between drought-adapted pollinators and carefully selected male-sterile seed parents that partition the extra dry matter to both grain and stover. Thus, exploiting heterosis in hybrids is an effective and rapid way to improve pearl millet production, while retaining critical adaptation to drought environments.

These results have been extended to pearl millet hybrid breeding for drought-prone environments of northwestern India. Since last one decade, a large number of hybrids and open-pollinated varieties have been tested under drought environments and it has been explicitly shown that hybrids provided 25% higher grain yield than OPVs [71]. This magnitude of advantage in grain productivity of hybrids shows that hybrids have greater yielding capacity than OPVs under drought environments and are likely to play a much greater role in enhancing pearl millet productivity in drought-prone regions.

12.3.2 Molecular Breeding

Because of the intrinsic difficulties in breeding for drought adaptation by conventional phenotypic selection [146, 147], this field has become a prime focus for molecular marker-assisted breeding. Efforts in this direction started in pearl millet in the early 1990s with the development of a molecular marker-based genetic linkage map that largely comprised of RFLP loci [148]. This linkage map was short (about 300 cM), but was longer than subsequent maps [149–151] based on crosses of cultivated pearl millet with accessions of its wild progenitors. The linkage map

has been expanded [152] and current genetic linkage map of pearl millet is 1148 cM long [153].

Genetic mapping has targeted terminal drought tolerance. Research at ICRISAT has identified quantitative trait loci (QTL) that had significant effects on pearl millet yield in drought stress environments [105, 154, 155]. Comparison of hybrids with and without these QTL showed that QTL-based hybrids were significantly, but modestly, higher yielding in a series of terminal drought stress environments [154]. However, this gain under stress was achieved at the cost of a lower yield in the non-drought environments. A major QTL mapped on Linkage Group (LG) 2 accounted for up to 32% of the phenotypic variation in grain yield under post-flowering drought stress environments [155, 156]. In addition, a number of other QTL were detected that were associated with maintenance of grain yield-determining component traits [157].

The QTL with little interaction with environment [158] has been transferred to drought-sensitive pearl millet lines through marker-assisted backcross breeding [159]. Several introgressed lines carrying LG 2 genomic region exhibited positive general combining ability (GCA) for grain yield under terminal stress that was associated with a higher panicle harvest index [157]. Physiological dissections indicated that lines having QTLs had lower transpiration rate compared to lines not carrying this QTL. There are reports that LG 2 QTL is also associated with salinity tolerance [160].

12.4 Heat Tolerance

Several growth processes like the rate of germination, rate of coleoptile elongation, or the rate of photosynthesis require rather high optimum temperatures, for example, 35 °C in pearl millet [161], which is indicative of good adaptation of pearl millet to the hot growing conditions in the Sahel and in many parts of India. The high temperature tolerance has relevance at both seedling and reproductive stages of crop.

12.4.1 Tolerance at Seedling Stage

Germination rate and final germination percentage are reduced following short exposure to 50 °C, but not at 45 °C [161, 162]. At constant exposure to 47 °C, no germination has been observed under controlled environment conditions. Field studies in the Sahel [10] indicated that pearl millet seedlings are most vulnerable to high temperatures during the first 10 days of sowing. This was confirmed by field studies in the Indian Thar Desert [163]. During other stages of seedling growth, the effect of high temperatures is small when the available water is sufficient for transpiration that cools the leaves [10, 163]. Controlled environment studies with young seedlings have shown that pearl millet responds to

supraoptimal temperature conditions with the production of a series of heat shock proteins [164]. A conditioning or hardening effect of intermediate temperatures has also been observed.

High seedbed temperature (>45 °C) is one of the most important factors causing poor plant stands of pearl millet [165]. Poor seedbed preparation, inappropriate sowing methods, poor seed quality, and low soil fertility are other factors responsible for low and variable plant populations. Plant stand losses due to these factors can be minimized by better agronomic management, but losses due to high soil surface temperature are difficult to control by cultural methods. Therefore, genetic improvement in tolerance of high seedbed temperature assumes importance.

A rapid screening procedure for seedling emergence under high temperatures, using a large steel tank and infrared heat lamps mounted on an adjustable rack suspended over the tank is in place [8]. Temperatures can be adjusted by raising and lowering the lamp rack. This procedure was used for a selection experiment in two populations for two cycles and found that it was effective in increasing emergence under high temperature conditions in the absence of water stress [166].

Peacock *et al.* [163] identified genetic differences in seedling survival under high soil surface temperatures using a field screening procedure during the hot and dry seasons in sandy soils in the Thar Desert in India. The method is rapid and inexpensive and can be used with a large number of genotypes. Its usefulness, however, is limited because tests can be conducted only during 2 months in a year, and experiment's failure due to occasional rains is possible. The present use of this method in a selection study indicates that it is effective in identifying genotypes with superior seedling heat tolerance [167, 168].

To overcome limitations of the field screening procedure, a controlled environment method using a sand bed that can be heated electrically and a laboratory method based on measuring membrane thermostability have been developed [169]. Initial results from a selection study in variable populations show that both procedures appear to be effective in increasing seedling survival under heat stress. Results from these two procedures show good correlations with field results. Their advantage appears to be higher heritabilities and more flexibility in their application [167, 168].

12.4.2
Tolerance at Reproductive Stage

In view of climate change and rising temperatures, tolerance of crops to high temperature during their reproductive stage has recently assumed high significance. A temperature rise of 0.5–1.2 °C by 2020, 0.88–3.16 °C by 2050, and 1.56–5.44 °C by 2080 has been projected for South Asia [170]. It has also been projected that by the end of twenty first century, mean annual temperatures in India will increase by 3–6 °C [171]. Climate change models have indicated drastic reductions in yield of cereal crops in tropical regions with moderate increase (1–2 °C) in temperature. This is likely to result in significant changes in cropping pattern and areas of crop

production, and maize and sorghum might be replaced by pearl millet in some of the semi-arid regions of Asia and Africa.

The impact of high temperature stress during reproductive period has been studied in many crops. High temperature stress (>35 °C) for 1 h has been found to induce spikelet sterility in rice [172, 173]. Similarly, temperature higher than 36 °C is reported to reduce pollen viability in maize [174] leading to reduction in yields. Similar effects of short spells of high temperature during flowering on fertility have been reported in sorghum [175] and wheat [176]. Contrary to this, pearl millet has good degree of tolerance to high temperatures of up to 42 °C during flowering. Hence it has occupied considerable areas (>600 000 ha) in the hot and dry post-rainy season (locally referred to as summer) in the northern and western parts of India. In summer season, pearl millet hybrids of 80–85 day duration can provide 4–5 tons/ha of grain and 8–10 tons/ha of dry stover under irrigated and well-managed conditions. Owing to higher air temperatures (often above 42 °C) coinciding with flowering in this region, the summer crop suffers from spikelet sterility leading to drastic reductions in grain yield. Only a few hybrids have shown good seed set under such high temperature conditions, leaving a limited choice of cultivars that always runs the risk of such cultivars breaking down to downy mildew. Thus, there is a need to identify sources of flowering-period heat tolerance to strengthen the hybrid breeding program for summer season.

ICRISAT made initial efforts in this direction by conducting some pilot studies under both controlled environmental and field conditions in target environments of northwestern India. In the controlled environments, screening for heat tolerance is conducted under growth chambers (simulated for a normal day where maximum temperature reaches 43 °C) by exposing pearl millet to high-temperature stress at boot leaf stage [177]). Screening for heat tolerance is also conducted under field conditions in target environment of northwestern India. Since the occurrence of high temperatures is unpredictable and breeding lines generally have a wide range of maturity, material is planted at three different dates during February–March at about 10 days interval at three–four locations with high temperatures so that the temperatures of ≥42 °C coincide with flowering of all the entries at least in one of the planting dates at each location. Weather loggers are installed in the experimental field to record air temperatures on hourly basis. The nursery is irrigated at regular intervals to avoid moisture stress in the field.

Dates of emergence of boot leaf and flowering are recorded in each planting date, and panicles are bagged after pollination to protect them from bird damage. At dough stage, seed set is recorded and data on seed set of plants that got exposed during flowering to air temperatures of ≥42 °C across the three dates are used to identify those with higher seed set (>60%) and presumably with high levels of heat tolerance.

Large genetic variation in tolerance to heat at reproductive stage among pearl millet breeding lines and populations has been observed, and heat-tolerant sources have been identified. Based on multilocational screening during the 2009–2010 summer season, two maintainer lines ICMB 92777 and ICMB 05666 were found to have >60% seed set when the air temperature during flowering exceeded 42 °C.

In addition, four B-lines (ICMB 00333, ICMB 01888, ICMB 02333, and ICMB 03555) were found as heat tolerant on the basis of 2010 screening that needs further validation in multiyear and multilocation testing. Populations like ICMV 82132, MC 94, ICTP 8202 and MC- Bulk have also been identified as sources of heat tolerance for further selection. Three germplasm accessions (IP 19799, IP 19877, and IP 19743) were also identified as heat tolerant (seed set of >50%), and can also be further utilized for diversifying the genetic base of heat-tolerant materials in pearl millet. However, the mechanism of heat tolerance is yet to be investigated in these materials.

The lines identified for heat tolerance need to be validated for this trait and those found stable can be used for developing mapping populations to identify QTL for use in marker-assisted breeding. Among the three major abiotic production constraints presented in this chapter, marker-assisted breeding is likely to be more successful for flowering period heat tolerance because the upper limit of temperature is known well predictably unlike salinity and drought stress. The main challenge would be to identify reliable QTL because the temperatures during flowering time below the predicted maximum might vary (sometime below 40 °C) due to occasional rains and cloud cover, and thus pose a challenge to reliable phenotyping for this trait. Development of a controlled environment facility for high temperatures maintenance during flowering would accelerate the process of QTL identification.

12.5 Salinity Tolerance

Salinity is a major constraint to crop production, especially in the arid and semiarid regions of the world, where low precipitation, high surface evaporation, irrigation with saline water, rising water tables, and poor irrigation practices generally increase the levels of soluble salts. Increased frequency of drought events over most land areas [178, 179], coupled with higher temperature, will intensify salinization due to increasing upward capillary transport of water and water-soluble salts from the groundwater to the root zone with no or negligible leaching under water-limiting environments [180, 181]. Thus, salinization is expected to be increasing in the future climate change scenario. At present, about 77 m ha (5–7% of the cultivable lands) are affected by salinity across the globe. Management of saline soils by flushing out of salts using fresh water is costly, and is limited by availability of fresh water. Thus, crop production by using salinity-tolerant crops is one of the best options. Pearl millet having high in-built tolerance to saline soils will be in advantageous position and can be deployed in saline lands for grain and forage production.

Some preliminary research work has been done at ICRISAT on salinity tolerance in pearl millet in collaboration with the International Center for Biosaline Agriculture (ICBA), and its NARS partners in both India and WANA region. Advanced breeding materials have been screened for salinity tolerance at ICRISAT, Patancheru, India, and ICBA, Dubai, initially through pot culture method, which is followed by screening of these identified materials in salinity-affected fields. In pot

Table 12.4 Performance of salinity-tolerant pearl millet breeding material under saline fields at Gangavathi, Karnataka, India during 2004–2005 [185].

Type of breeding material	Range	
	Grain yield (kg/ha)	Dry fodder yield (kg/ha)
B-Lines: ICMB 01222, ICMB 96333, ICMB 95222	940–1265	3980–6940
Sensitive B-line (control)	797	2486
R-lines: HTP 94/54, CZI 9621, ICMP 451	1081–1311	4113–6721
Sensitive R-line (control)	974	3794
Germplasm accessions: IP 6105, IP 6098, IP 22269	1155–1411	4196–6117
Improved populations: Dauro genepool, Sudan Pop III, HHVBC Tall	1452–1996	4009–6117

culture method, breeding material is grown in pots with salinity treatment in an outdoor environment equipped with a rainout shelter along with controls. At ICRISAT, a 200 mM NaCl treatment is provided for screening tolerant genotypes, whereas screening is done at 5, 10, and 15 dS/m salinity levels at ICBA. This salinity-tolerant material identified under controlled environments then undergoes testing in salinity-affected fields at Gangavathi (Karnataka, India), Rumais (Sultanate of Oman), Dubai (UAE), and some other locations in WANA region. The trials are drip irrigated with saline water (7.5–8.25 dS/m) at Dubai and Rumais, while they are conducted under saline rainfed conditions at Gangavathi.

Screening resulted in identification of advanced breeding lines, parental lines of potential hybrids, improved population (including open-pollinated varieties), gene pools and composites, and germplasm accessions with high biomass (forage) presumably with high degree of salinity tolerance under salinity levels up to 15 dS/m (Table 12.4). In the short-to-medium terms, some of these materials can be released for cultivation after extensive validation of their yield performances in on-farm trials. Working on these lines, a pearl millet variety "HASHAKI 1" has been identified for release in Uzbekistan in 2012 as a high-forage variety for salt-affected areas. The identified salinity-tolerant pearl millet lines should be utilized in breeding programs to develop salinity-tolerant locally adapted cultivars (both OPVs and hybrids). This will enable farmers in salt-affected areas to adopt and grow a new crop such as pearl millet in lands that otherwise are fallow most of the years.

Since populations have shown large intra-population variability for forage yield in saline soils, and forage yield under saline conditions has been shown to have significant and high positive correlation ($r^2 = 0.92$) with salinity tolerance index (STI), direct selection for forage yield in saline soils can effectively enhance not only forage yield but also salinity tolerance. Direct selection for grain yield under saline conditions can also enhance salinity tolerance for this trait, although it would be less effective than selection for tolerance with respect to forage yield because the correlation between grain yield under salinity conditions and salinity tolerance index, though significant and positive, is smaller in magnitude.

Several parental lines and populations with both high grain yield ratio (ratio of yield under salinity versus control) and stover yield ratio were identified in pearl millet and there was highly significant and positive correlation between grain yield ratio and stover yield ratio ($r^2 = 0.80$), implying that selection for high stover yield ratio is likely to also lead to concomitant genetic improvement in grain yield ratio, and that simultaneous selection for both productivity and STI will be highly effective. Parental lines with large contrasts for grain yield ratio have been identified to develop mapping populations to identify QTL for salinity tolerance.

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13
Bamboo: Application of Plant Tissue Culture Techniques for Genetic Improvement of *Dendrocalamus strictus* Nees

C.K. John and V.A. Parasharami

Abstract

Bamboos are valuable natural resources important for both economy and environment. Large-scale propagation of bamboos is difficult because of their peculiar flowering behavior only at very long intervals, and sterility in some species. Bamboos are generally propagated by vegetative methods. Most of these methods pose difficulties such as large propagules, difficulty in extraction and transport, lack of sufficient numbers for raising large-scale plantations, and extraction of propagules lowering the productivity of parent clumps. In India, *Dendrocalamus strictus* has wide distribution, abundance, and economic importance. *D. strictus* occupies 53% of total bamboo area in India. In *D. strictus*, methods are available for (i) *in vitro* propagation from nodal bud explants from mature clumps, (ii) *in vitro* induction of microrhizomes, and (iii) selecting NaCl stress-tolerant variants. *D. strictus* is a suitable candidate for further work on developing abiotic stress-tolerant variants.

13.1
Introduction

Bamboos are perennial, woody grasses, and are valuable natural resources important for both economy and environment [1]. They have more than 1500 documented uses. Important traditional uses include housing, food, fuel, and raw material for household utensils, agricultural implements, and handicrafts [2, 3]. Bamboos have great potential for employment and income generation for rural communities [4]. Bamboos are among the fastest growing plants on Earth [5]. Bamboo is substitute for wood in pulp and paper manufacturing. Worldwide, more than 2.5 billion people trade in or use bamboo [6]. Annual trade in bamboos (both raw and finished products) is estimated at more than US$ 5.0 billion. This represents only a small proportion of total bamboo usage. Domestic use is estimated to account for at least 80% [4]. Modern manufacturing techniques allow the use of bamboo in timber-based industries, to provide bamboo flooring, board products, laminates, and furniture [4]. Bamboo shoots are an important processed food product on the

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international market. China is the leading exporter with an annual export value of nearly US$ 140 million [7]. Bamboo furniture is an expanding business in many countries [4, 8]. Worldwide, domestic trade and subsistence use of bamboo are estimated to be worth US$ 4.5 billion per year. Global export of bamboo generates another US$ 2.7 billion [4, 6]. The vulnerability of some animal species is increased by the simultaneous flowering and subsequent death of entire populations in supra-annual cycles ranging from 7 to 120 years or more. The best known among these animal species is the giant panda (*Ailuropoda melanoleuca*). The red panda (*Ailurus fulgens*) and the Himalayan black bear (*Selenarctos thibetanus*) are also heavily dependent on bamboo [4]. In recent years, with the looming threat of global warming, there is much interest in bamboo as a carbon sink [9, 10].

Worldwide, there are about 1400 species of bamboos under 101–118 genera. They are classified into three tribes: Bambuseae, Arundinarieae, and Olyreae. According to Ohrnberger [11], the subfamily Bambusoideae comprises both woody and herbaceous bamboos with 1575 species altogether.

Bamboos can be categorized into two broad categories: sympodial (pachymorphic) and monopodial (leptomorphic) based on the rhizome system [1]. The former is clump forming and the latter spreading type. Most clump forming bamboos are tropical, and spreading type temperate. Clump formation in sympodial bamboos is believed to be an adaptation to growth in drought-prone tropical conditions, as compact clumps and leaf shedding during summer help the plant in controlling water loss and survive adverse climatic conditions.

Ever increasing rate of deforestation makes search for alternative natural resources an urgent need. Bamboos are suitable for large-scale cultivation in marginal and degraded lands under agroforestry [12]. The extensive rhizome and root systems help in effectively controlling soil erosion and in reinforcing embarkments, and accumulation of leaf mulch helps in conserving soil moisture. Faster growth and biomass production help in generating large amounts of oxygen and effectively sequestering atmospheric carbon dioxide.

13.2 Vegetative Propagation

Large-scale propagation of bamboos is difficult because of their peculiar flowering behavior only at very long intervals, and sterility in some species. Bamboo seed is infrequently produced and rapidly loses viability. Most woody bamboos are semelparous: gregariously flower/seed only at the end of very long periods of vegetative growth and die *en masse* [1, 13]. Brandis [14] classified bamboos into three categories on the basis of their flowering behavior: (i) species that flower annually or nearly so, (ii) species that flower gregariously and periodically, and (iii) species that flower irregularly. Blatter [15] considered these three categories as fairly complete. A vast majority of the woody bamboos belong to the second category in which the intermast periods range between 10 and 120 years (or more). This peculiar flowering behavior has intrigued mankind for long and still remains as a botanical

enigma. In general, the intermast periods of woody bamboos are thought of as species specific and rigid. However, it is not the case in many bamboos [13, 16, 17].

Bamboos are generally propagated by vegetative methods. A vegetative propagule of a bamboo plant must have shoot, root, and rhizome. Failure to develop any of these leads to failure of the propagule [18]. The most widely used vegetative propagation methods are clump division (offsets/rhizome), whole culm cuttings, layering, culm segment cutting, and branch cutting [19]. Most of these methods pose difficulties such as large propagules, difficulty in extraction and transport, lack of sufficient numbers for raising large-scale plantations, and extraction of propagules lowering the productivity of parent clumps. In species such as *Bambusa arundinacea* and *Dendrocalamus strictus* in which seeds are available once in a while, macroproliferation of seedlings can be another method [20, 21].

Bamboos in general are openly wind pollinated. They have physical/physiological barriers that prevent self-pollination/favor cross-pollination [22–25]. Many bamboos are known to be highly heterozygous [26–28]. This makes them suitable candidates for selection [29]. Most of the characters contributing to increased biomass production are expressed in the adult stage. Hence, propagation from adult clumps has some advantages.

13.3 Micropropagation

Micropropagation methods are available in many species of bamboo [30–40]. Plant tissue culture work in bamboos started as early as 1969 [41]. During the past 40 years, a vast number of reports on *in vitro* propagation methods for a large number of species have appeared. Three tissue culture approaches of practical use for mass multiplication of bamboos are (i) enhanced shoot multiplication by axillary/adventitious branching from juvenile and mature explants, followed by *in vitro*/*ex vitro* rooting of these shoots [42–44]; (ii) somatic embryogenesis from mature embryo (seed) and nodal bud explants from both seedling and mature culms [45–47]; and (iii) *in vitro* induction of microrhizomes [48, 49].

13.4 Genetic Improvement for Abiotic Stress Tolerance

Abiotic stresses, extremes of temperature (heat and cold), extremes of water availability (drought and flood), and soil salinity pose serious threats to the growth and yield of crop plants. Vast areas of land in India and other countries are abiotic stress prone. To use these lands for cultivating crop plants, resistant varieties are required. There have been attempts to develop abiotic stress-resistant varieties. Earlier, this has been through selection and breeding [50–52] or through tissue culture [53, 54], and in recent years through gene transfer [55–58]. Two main approaches being used to improve stress tolerance are (i) the exploitation of natural

genetic variations, either through direct selection in stressful environments or through mapping quantitative trait loci and subsequent marker-assisted selection; and (ii) the generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of stress tolerance [59]. The limiting factor in extension of biotechnology to abiotic stresses is the lack of information on what are the "useful genes" – genes that would lead to better stress tolerance [56]. Transgenic approaches to developing stress-tolerant crop varieties are based on the introgression of genes that are known to be involved in stress response. The task of generating transgenic cultivars is not limited to the success in the transformation process. Proper incorporation of the stress tolerance is essential. Evaluation of the transgenic plants under stress conditions and understanding the physiological effect of the inserted genes at the whole plant level are challenges remaining to be overcome [58]. Among plant biotechnologies, plant tissue culture techniques offer easier and reliable methods of developing abiotic stress-tolerant varieties [53, 54, 60, 61]. Bamboos are suitable candidates for development of abiotic stress-tolerant varieties given their usefulness in large-scale cultivation in marginal and degraded lands under agroforestry.

13.5 *Dendrocalamus strictus*

In India, *D. strictus* is one of the bamboo species having wide distribution, abundance, and economic importance. *D. strictus* is a compact clump forming bamboo. Clumps are deciduous and densely tufted (Figure 13.1). *D. strictus* occupies 53% of total bamboo area in India. It is widely distributed in semi-dry and dry zones along plains and hilly tracts up to an altitude of 1000 m and commonly cultivated throughout the plains and foothills. *D. strictus* grows under a wide temperature range from −5 to 45 °C. Most important use of *D. strictus* is as raw material in paper manufacture. It is also used for construction, agricultural implements, musical instruments, furniture, and so on.

Among the economically important bamboos of India, *D. strictus* shows much diversity for intermast periods [13]. Intermast periods reported from different parts ranged between 8 and 47 years. Since this species also shows sporadic flowering, some of these reports may be that of sporadic flowering and may not represent the intermast periods. These reports point toward the existence of many land races of *D. strictus*.

In *D. strictus*, methods are available for *in vitro* propagation through enhanced shoot multiplication from juvenile and mature explants [42, 62], somatic embryogenesis [45, 46], and *in vitro* induction of microrhizomes [48, 49] (Table 13.1). Standard procedure for micropropagation involves *in vitro* multiple shoot production, followed by *in vitro*/*ex vitro* rooting and acclimatization (Figures 13.2 and 13.3).

Nadgir *et al.* [42] obtained multiple shoots from seedling explants of *D. strictus* in liquid MS medium supplemented with 2.22 μM BA and 5% coconut water. These

Figure 13.1 Clump of *D. strictus* Nees.

multiple shoots were rooted by treating them for 48 h in the dark in half-strength MS medium supplemented with 0.49 μM IBA. Nadgir *et al*. [42] could obtain multiple shoots from nodal segment explants from mature clumps on semi-solid MS medium supplemented with 2.22 μM BA, 0.92 μM Kn, and 10% coconut water. These multiple shoots were rooted in half-strength semi-solid MS medium containing 0.25% activated charcoal after treatment for 96 h in the dark on half-strength semi-solid MS medium supplemented with 0.49 μM IBA. After this step, the plantlets turned pale green and appeared in poor health. This could be avoided by transferring the plantlets to liquid MS medium containing 0.44 μM BA and 0.46 μM Kn + 5% coconut water. Rooted plantlets from seedling explants as well as mature

Table 13.1 Reports on micropropagation protocols on *D. strictus* Nees.

No.	Explant	Medium	Response(s)	Reference
1.	Seedlings	MS + 0.88 μM BAP + 5%CM + 2% sucrose (liquid medium)	Multiple shoots	[42]
	In vitro rooting of multiple shoots	1/2 MS + 0.49 μM IBA (in 48 h dark followed by transfer to light)	Rooting 80%	
	Mature shoots	MS + 2.22 μM BAP + 0.92 μM Kn + 10% CM + 2% sucrose (semi-solid medium) + 0.25% activated charcoal		
2.	Seed	B5 + 30 μM 2,4-D + 2% sucrose + 0.8% agar	Callus formation in 10–12 days at embryonal end; somatic embryogenesis in 67%	[45]
	Callus (i)	B5 + 0.5 μM IBA + 1 μM NAA + 2% sucrose (liquid medium)	Conversion of somatic embryos; plantlet formation in 40%	
	Plantlets	1/2 B5 + 0.5 μM IBA + 1 μM NAA + 1% sucrose (liquid medium with filter paper raft)	Growth of converted plantlets	
3.	Shoot apices	2,4-D medium	Creamy white callus	[63]
	Subcultures from (i)	Varied auxin/cytokinin levels	No differentiation	
	Subcultures from (i)	Varied NAA/BA combinations	Green, partially organized, granular or nodular callus; it was later converted to adventitious shoots that were rooted readily	
4.	Production of somatic embryos	MS + 13.58 μM 2,4-D + 2.32 μM Kn	Somatic embryos	[64]
	Encapsulation of somatic embryos	Encapsulated inside Ca alginate beads	Germination frequencies of 96 and 45% were achieved *in vitro* and in soil, respectively	
	Ca alginate beads	Additional mineral oil coating of Ca alginate beads	95–96% plantlet germination under *in vivo* conditions	
5.	Single-node stem segments	MS + 2.85 μM IAA + 0.5 mg/l adenine sulfate (Ads) (modified medium)	Two to four axillary shoots/explant	[65]
	Inversely oriented axillary shoots	4.9 μM IBA + 5.37 μM NAA + 2.26 μM 2,4-D + 1 mg/l phloroglucinol	Rooting 30%	

6.	Nodal explants from seedling culture	MS + 2.32 μM Kn + 9.0 μM 2,4-D + 10 mg/l Ads + 3% sucrose	Somatic embryos germinated (95–98%) into normal plants and 95% successful rooting	[47]
	Mature zygotic embryo (MZE)	MS + 2.32 μM Kn + 9.04 μM 2,4-D + 10 mg/l Ads + 3% sucrose	Somatic embryos germinated (95–98%) into normal plants and 95% successful rooting	
	Nodal explants of somatic embryo regenerated plants	1/2 MS +1.22 μM IBA + 0.5 mg/l Ads + 1.44 μM GA3 + 3% sucrose	*In vitro* flowering induction	
7.	Seedlings	1/2 MS + 2.22 μM BA + 2% sucrose	Plantlets initiated	[48]
	Proliferation and production of plantlets	Simple minimal media – growth regulators	*In vitro* rhizome formation 80%	
8.	Seedlings	MS (liquid medium) + 0.88–8.88 μM BA + 2.32–4.65 μM Kn + CW	Multiple shoots	[66]
	Axillary buds	MS (liquid medium) + 0.88–8.88 μM BA + 2.32–4.65 μM Kn + CW	Multiple shoots	
	Propagules of (i) and (ii)	MS (liquid medium) + 1.22–9.80 μM IBA + 2% sucrose	Root induction	
9.	Seed	MS + 30 μM 2,4-D	Embryogenic callus	[43]
	Embryogenic callus	MS + 30 μM 2,4-D + 5 μM Kn + 2 μM IBA + 250 mg/l PVP + 3% table sugar	Secondary embryogenesis (two- to fivefold/5 weeks)	
	Embryogenic callus	MS + 30 μM 2,4-D + 10 μM BAP + 250 mg/l PVP + 3% table sugar	Secondary embryogenesis (two- to fivefold/5 weeks)	
	Somatic embryos	MS + 30 μM 2,4-D + 5 μM NAA + 5 μM kinetin + 250 mg/l PVP + 3% sucrose + 0.2% Gelrite	Conversion to plantlets	
10.	Embryogenic callus	0–200 mM NaCl + MS + 3% sucrose + 13.6 μM 2,4-D + 2.32 μM Kn + 0.8% agar	100 mM NaCl-tolerant callus initiated	[67]
	Stable 100 mM NaCl-tolerant embryogenic callus	MS (maintenance medium) + 3% sucrose + 0.8% agar + 9.04 μM 2,4-D + 2.32 μM Kn + 0–200 mM NaCl	Differentiated into somatic embryos	
	Somatic embryos tolerant to 100 mM NaCl	1/2 MS + 2% sucrose + 0.1 μM NAA + 0.49 μM IBA + 100 mM NaCl	39% of mature somatic embryos tolerant to 100 mM NaCl germinated and converted into plantlets	

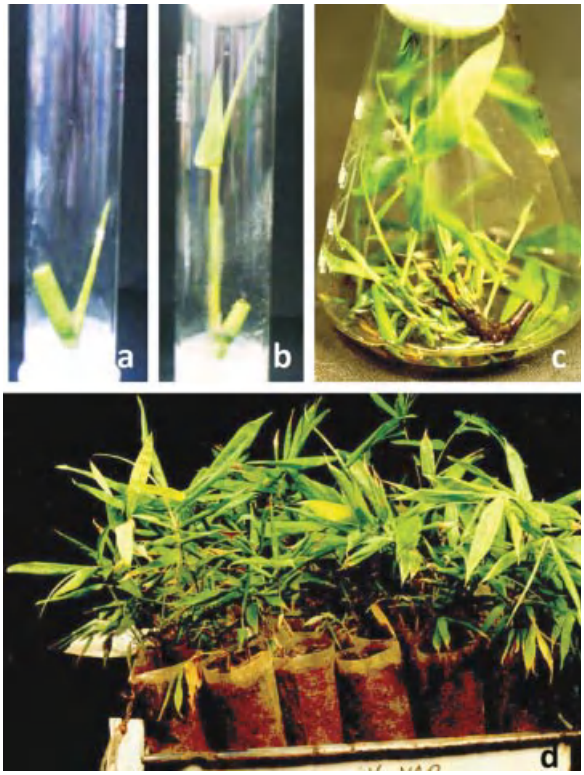

Figure 13.2 *In vitro* propagation of *D. strictus* Nees. (a) Sprouting of mature clump-derived nodal bud explant on initiation medium. (b) Further elongation of the sprouted bud on the same medium. (c) Further multiplication and elongation of shoots on shoot multiplication medium. (d) Micropropagated plantlets.

clump-derived explants were first transferred to 1: 1 sterile sand/soil mixture in pots. These plantlets were covered with glass beakers to maintain humidity and incubated at 25 °C and 16 h light and 8 h dark regimes. After new leaves emerged, these plantlets were transferred to greenhouse and then to field. The work by Nadgir *et al.* [42] is one of the first reports of successful *in vitro* regeneration of plantlets from a mature bamboo clump.

Rao *et al.* [45] obtained plant regeneration through somatic embryogenesis of *D. strictus*, by culturing seeds on B5 medium supplemented with 2,4-D. Callus cultures obtained from the embryonal end of the seeds differentiated into green embryoids. On transfer to a liquid B5 medium + sucrose + IBA + NAA, 40% of the embryoids developed into plantlets. Further development of the plantlets occurred in half-strength liquid B5 medium + 1% sucrose + 0.5 μM IBA + 1 μM NAA.

Huang *et al.* [63] cultured shoot apices of *D. strictus* in 2,4-D-containing medium, and developed a creamy white callus. Subculturing into media containing different

Figure 13.3 Field trial of micropropagated and hardened plants of *D. strictus* Nees.

concentrations of auxins/cytokinins did not result in differentiation of shoots. Subculturing on media containing combinations of NAA and BA resulted in a green, partially organized, granular or nodular callus. This callus, on further culture, maintained its partially organized nature and produced adventitious shoots. These shoots were rooted and transplanted in soil.

Mukunthakumar and Mathur [64] produced artificial seeds of *D. strictus* by encapsulating somatic embryos obtained on MS medium containing 13.58 μM 2,4-D and 2.32 μM Kn, in calcium alginate beads. Germination frequencies of 96 and 45% were obtained *in vitro* and in soil, respectively, for these artificial seeds. The germination frequency *in vivo* was increased to 56% when an additional coating of mineral oil on the alginate beads was tried. Germinated artificial seeds could be raised into plantlets.

Chaturvedi *et al.* [65] cultured single-node stem segments excised from newly regenerated branches of approximately 10-year-old field-grown culms of *D. strictus* in a modified MS medium supplemented with 2.85 μM IAA and 81.43 μM Ads. This resulted in the production of two to four axillary shoots per explant. These shoots did not survive because no roots were produced. Rooting was induced when the explants were cultured in an inverted position, in the rooting medium containing 4.9 μM IBA + 5.37 μM NAA + 2.26 μM 2,4-D + 1 mg/l phloroglucinol at pH 5.2. A seasonal effect of response was noticed by the authors, with nodal explants collected during July–August responding most favorably. The *in vitro* raised plantlets were transplanted with about 80% success. Transplanted plantlets grew normally in soil under glasshouse as well as field conditions.

Rout and Das [47] obtained plant regeneration via somatic embryogenesis in callus cultures derived from nodal explants of *in vitro* grown seedlings and excised mature zygotic embryos of three bamboo species (*Bambusa vulgaris, Dendrocalamus*

giganteus, and *D. strictus*) on MS medium supplemented with 2.32 μM Kn, 9.5 μM 2,4-D, 54.29 μM Ads, and 3% sucrose incubated either in the light or in the dark. Almost all (95–98%) somatic embryos germinated into normal plants and when transferred to soil 95% of the plants survived.

Shirgurkar *et al.* [48] developed a micropropagation technique for the large-scale production of *D. strictus* plantlets. Seedling cultures were initiated on half-strength MS medium supplemented with 2.22 μM BA and 2% sucrose. Further proliferation and production of plantlets occurred on MS medium + 2% sucrose. In about 80% of cultures, rhizome formation also occurred. Nearly 90% of these plantlets survived when transferred to 1: 1 sand/soil mixture in pots.

Ravikumar *et al.* [66] could induce multiple shoots from seedlings and axillary buds of mature plants of *D. strictus* on MS medium supplemented with BA and Kn. In the primary culture, in a span of 20–25 days about 35–45 shoots were obtained from a nodal explant of seedling and 3–8 shoots from a nodal explant of mature plants. These multiple shoots were rooted under *in vitro* and *ex vitro* conditions. By *ex vitro* method using 1080 μM IBA 85–90% rooting was achieved, when the shoots were kept at 85–90% humidity and 27–30 °C. The shoots required 20–25 days for root initiation. Rooted plantlets performed well during acclimatization.

Saxena and Dhawan [43] developed a complete protocol for large-scale propagation of *D. strictus* by somatic embryogenesis. Seeds cultured on semi-solid MS medium + 30 μM 2,4-D produced embryogenic callus from the embryo. Somatic embryos formed *in vitro* multiplied rapidly (two- to fivefold every 5 weeks) on semi-solid MS medium + 10 μM 2,4-D + 2 μM IBA + 5 μM Kn + 250 mg/l soluble PVP, or MS medium + 10 μM 2,4-D + 10 μM Kn + 250 mg/l soluble PVP. Upon transfer to MS medium + 5 μM NAA + 5 μM Kn + 250 mg/l soluble PVP, the dark green embryos developed into healthy plantlets. Unrooted shoots were rooted on half-strength MS medium + 3 μM NAA + 2.5 μM IBA. When these rooted plantlets were transferred to soil in polythene bags, more than 80% of them survived. Using this method, Saxena and Dhawan [43] produced more than 100 000 plants. Saxena and Dhawan [43] noticed a strong genotypic effect at all stages of regeneration, that is, initiation, multiplication, and germination of somatic embryos.

Saxena and Dhawan [43] also studied various factors such as potting mixture, humidity regime, and seasons, which are known to influence the transplantation process. The seasonal effect was found to be very pronounced. Saxena and Dhawan [43] encountered higher rates of mortality during winter (October–February). They attributed this to *D. strictus* being more susceptible to low temperatures because the species is well adapted to growth in warm areas. The tissue-cultured plants usually take 6–8 weeks after transplantation to form rhizomes. According to Saxena and Dhawan [43], plants transplanted in July survived winter as the plants produced rhizomes, and plantlets transferred to soil in October or later died because they were exposed to low temperatures before they formed rhizomes.

Singh *et al.* [67] successfully regenerated NaCl-tolerant plantlets of *D. strictus* via somatic embryogenesis from NaCl-tolerant embryogenic callus. Selection of embryogenic callus tolerant to 100 mM NaCl was made by exposing the callus

cultures to 0–200 mM concentrations of NaCl in callus initiation medium (MS medium supplemented with 13.6 μM 2,4-D + 2.3 μM Kn + 3% sucrose + 0.8% agar). The NaCl-tolerant embryogenic callus differentiated somatic embryos on maintenance medium (MS medium + 9.0 μM 2,4-D + 2.3 μM Kn + 3% sucrose + 0.8% agar) and containing 0–200 mM NaCl. About 39% of mature somatic embryos were tolerant to 100 mM NaCl. These somatic embryos germinated and converted into plantlets in half-strength MS medium + 0.1 μM NAA + 0.49 μM IBA + 2% sucrose + 100 mM NaCl. About 31% of these plantlets established well on transplantation into a garden soil and sand (1: 1) mixture containing 0.2% (w/w) NaCl. These results suggested that plantlets regenerated from 100 mM NaCl-tolerant embryos can survive on soils rich in salt.

13.6 Future Prospects

D. strictus is a clump forming tropical bamboo adapted for growth in a wide range of climatic conditions. In *D. strictus*, methods are available for *in vitro* propagation through enhanced shoot multiplication from juvenile and mature explants, somatic embryogenesis, and *in vitro* induction of microrhizomes. *In vitro* selection procedure for developing salt-tolerant variant is also available. Developing a frost-tolerant variant of this bamboo as well as developing drought-tolerant monopodial (leptomorph) temperate bamboos, frost-tolerant sympodial (pachymorph) bamboos, and salt tolerance in both the groups will be very useful in extending the range of growth habitats of bamboos.

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Section IIIB
Leguminosae

Improving Crop Productivity in Sustainable Agriculture, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, and Renu Tuteja.

14
Groundnut: Genetic Approaches to Enhance Adaptation of Groundnut (*Arachis Hypogaea*, L.) to Drought

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Abstract

Groundnut or peanut is grown as an oilseed or food crop in more than 100 countries under diverse agroclimatic conditions. About 80% of the world's groundnut is grown in drylands of semiarid tropics that experience erratic droughts and high temperature variability. The impending climate change characterized by an increase in frequency and severity of droughts and elevated temperatures has accentuated negative impacts on the groundnut productivity. Therefore, it is essential to put in place genetic and management interventions in an environmentally sustainable manner to combat negative impacts of climate change. The chapter focuses on the abiotic constraints to groundnut production and describes the current understanding of the crop improvement, under each chapter, with reference to enhancing the adaptive capacity of groundnut to the targeted abiotic stresses.

A significant progress has been made in characterizing the genetic variability for various adaptive traits in cultivated "tetraploid" groundnut. Conceptual approaches tested have demonstrated the potential for trait-based breeding by introgressing discrete components of Passioura's yield model. The existing literature suggest that success of crop improvement for drought by introgression of several traits can be achieved through exploiting the existing genetic variability, identification, and characterization of most relevant traits and genes from the germplasm collections and promising trait donor wild races. However, the introgression of multiple traits of interest through conventional breeding has been slow, costly, and laborious due to a lack of rapid and cost-effective selection tools. Recent advances in DNA technologies have opened up novel opportunities to enable marker-assisted selection and breeding in a number of crops. Besides, transgenics technology has emerged as an alternative option for trait pyramiding. However, the challenge in groundnut has been to generate genomic resources to utilize them in molecular breeding program. This chapter provides insights into current research knowledge on the

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groundnut crop improvement for drought adaptations and a few potential options, which are promising for challenges to be addressed.

14.1 Introduction

14.1.1 Importance of Groundnut

Groundnut or peanut (*Arachis hypogaea*) is an annual legume grown in over 100 countries between 40°N and 40°S, under diverse agroclimatic conditions. Groundnut kernel is the most economically important commodity with an oil content of 50% and protein content of 25%. Other parts of groundnut plant (stems and foliage) are economically important as hay for livestock, while the by-products of oil extraction, that is, shells, peanut meal, and residues, are used either as livestock feed or as fertilizer in crop husbandry. Groundnut being a leguminous plant, it can fix atmospheric N (up to 300 kg N/ha) and crop residues incorporated into soil can potentially contribute up to 120 kg N/ha.

Groundnut kernel is a prime source of cooking oil in many Asian and African countries. However, groundnut is also an important source of vegetable protein in addition to 30 essential nutrients (niacin, folate, fiber, magnesium, vitamin E, manganese, and phosphorus). The use of groundnut kernels and its value-added products such as peanut butter and snack food is on the rise due to growing vegetarianism and demand for healthy foods. More recently, groundnut has been identified as a source of reservatrol, an antioxidant linked with antiaging effects, reduced cardiovascular disease, and reduced cancer risk. It is reported that about 73 μg of reservatrol is available in 1 ounce of eaten groundnut [1].

Despite multiple benefits of groundnut and its products, some human health concerns of groundnut are viewed seriously [2]. Consumption and exposure to groundnut/by-products can cause mild to severe allergic reactions or fatal anaphylactic shock [3]. Aflatoxin (AF) contamination of groundnut that occurs under drought and elevated temperature conditions is yet another serious health risk for humans. Aflatoxin is a carcinogenic, immune-suppressing, and antinutritional natural contaminant produced in a number of crops including groundnut. It is produced in the kernels infected by *Aspergillus flavus* or *Aspergillus parasiticus* soil fungi under prolonged water deficit and elevated temperature regimes. The worldwide increase in the incidence of hepatitis B and hepatitis C virus is increasing the importance of aflatoxin as a potential health risk since the toxin is implicated in predisposing people who ingest large quantities of aflatoxin to liver diseases. These health concerns are driving a desire to significantly decrease the levels of aflatoxin allowed in foods worldwide. Research efforts are currently directed to develop varieties resistant to aflatoxin production and also adaptation to drought conditions.

14.1.2
Origin and Diversity

The groundnut originated in the region of Southern Bolivia and Northern Argentina [4–6]. The first domestication of the groundnut is believed to have been in the valleys of the Parana and the Paraguay River Basins of the Gran Chao area in South America. Early European explorers found native Indians cultivating the crop in many islands in the Antilles on the northeastern and eastern coasts of Brazil, in all warm regions of the Rio de la Plata basin, extensively in Peru, and sparely in Mexico.

The genus *Arachis* is a member of family Fabaceae (Leguminosae) [7] and it was the only member of the genus when Linnaeus names the cultivated groundnut *A. hypogaea*. The taxonomic classification and validation have been described in earlier works [8].

Currently, large numbers of groundnut cultivars are grown across the world with four major cultivar groups, namely, Spanish, runner, Virginia, and Valencia types. These are characterized by their morphological and phenological characteristics [9]. Several of varieties in each type have potential to exploit for their genetic variability and relevant traits amenable for crop improvement for drought tolerance.

14.1.3
Area, Production, and Productivity

Groundnut is grown in most tropical, subtropical, and temperate regions, especially in Africa, Asia, and North and South Americas, between 40°N and 40°S (Table 14.1). In Asia, groundnut is a major crop in China, India, Indonesia, Myanmar, Thailand, and Vietnam. China is currently leading the production of groundnut with a share of about 41.5% of overall world production, followed by India (18.2%) and the United States (6.8%). Interestingly, developing countries account for 96% of the global area under groundnut cultivation contributing 92% to the global production. About 58% of the global groundnut area is in Asia, which contributes 67% to the groundnut production with an annual growth rate of 1.28%, 2.00%, and 0.71% for area, production, and productivity, respectively. But 40% of global groundnut-growing area is in African countries, which contribute 26% of global production.

14.1.4
Major Abiotic Stresses

About 80% of the world's groundnut production comes from dryland regions of the semiarid tropics, where soil fertility is poor and climatic variables such as current rainfall, seasonal temperatures, determine crop productivity [10–13]. In addition to drought and high temperatures, foliar diseases such as leaf spots (*Cercospora arachidicola*) and rust (*Phaeoisariopsis personata*) are also major constraints of groundnut

Table 14.1 Area, production, and productivity of groundnut-cultivating countries.

Country/region	Area (m/ha)		Yield (m/ha)		Production (mt)	
	2009/2010	Projection 2010/2011	2009/2010	Projection 2010/2011	2009/2010	Projection 2010/2011
China	4.38	4.45	3.36	3.39	14.71	15.1
United States	0.44	0.51	3.83	3.71	1.68	1.89
South Asia						
India	5.3	6	0.92	1	4.9	6
Pakistan	0.11	0.11	0.9	0.9	0.1	0.1
Sub-Saharan Africa	7.8	7.84	0.95		7.39	
Nigeria	1.25	1.25	1.24	1.24	1.55	1.55
Senegal	1.06	1	0.97	1.1	1.03	1.1
Chad	0.35	0.35	1.32	1.32	0.47	0.47
Ghana	0.47	0.47	0.94	0.94	0.44	0.44
Sudan	1	1	0.85	0.85	0.85	0.85
Congo	0.48	0.48	0.78	0.78	0.37	0.37
Burkina	0.35	0.35	1.01	1.01	0.35	0.35
Cameroon	0.31	0.31	0.77	0.77	0.24	0.24
Mozambique	0.29	0.29	0.38	0.38	0.11	0.11
Niger	0.26	0.26	0.42	0.42	0.11	0.11
Mali	0.25	0.25	1.1	1.1	0.28	0.28
Malawi	0.22	0.22	1.2	1.2	0.27	0.27
Uganda	0.21	0.21	0.7	0.7	0.15	0.15
Guinea	0.21	0.21	1.24	1.24	0.26	0.26
Benin	0.16	0.16	0.81	0.81	0.13	0.13
Cote d'Ivoire	0.15	0.15	1	1	0.15	0.15
Central African Republic	0.13	0.13	1.12	1.12	0.14	0.14
South Africa	0.06	0.06	2.05	2.09	0.12	0.12
Southeast Asia						
Indonesia	0.75	0.75	1.67	1.67	1.25	1.25
Burma	0.67	0.67	1.49	1.49	1	1
Vietnam	0.25	0.24	2.11	1.99	0.53	0.49
Thailand	0.07	0.07	1.77	1.77	0.12	0.12
South America						
Argentina	0.22	0.27	3.8	3.15	0.84	0.85
Brazil	0.1	0.1	2.47	2.5	0.24	0.25
Egypt	0.06	0.06	3.17	3.17	0.19	0.19
Mexico	0.05	0.05	1.56	1.56	0.07	0.07
Others	0.19	0.19	2.37	2.34	0.44	0.44
World	20.4	21.26	1.64	1.65	33.36	35.12

Source: USDA (2010).

productivity. Although subsoil constraints such as pH, salinity, and nutrient disorders occur in some parts of the groundnut-growing regions, drought and temperatures are the most widespread abiotic constraints. The ongoing climate change characterized by an increase in frequency and severity of droughts and elevated temperatures is likely to have major negative impacts on the peanut productivity and food safety. Therefore, it is absolutely essential to put in place genetic and management interventions in an environmentally sustainable manner to combat negative impacts of climate change.

Historical analysis of climate and yield performance suggested that total seasonal rainfall [14] and its distribution [15] are major determinants of groundnut yield in rainfed region. Evidently, among the environmental stresses, water deficit is the most important factor that limits the production of groundnut. Water deficits alone or in combination with elevated temperatures can result in significant crop loss or even crop failures, depending on timing, severity, and duration of the stress.

14.2
Response to Water Deficits at the Crop Level

14.2.1
Effects of Water Deficits on Yield

Considerable information has been reported on the effects of water deficit at different stages of groundnut [16–25]. The yield response can vary considerably depending on the timing of water deficit, genotypes, and growing conditions. While water deficits during preflowering phase can result in yield advantages, water deficits during the reproductive phase can result in significant yield losses (Table 14.2).

14.2.2
Effects of Multiple Water Deficits

Rainfed groundnut can experience water deficits at any or many times during the growth cycle. There is limited information on the effects of multiple droughts on groundnut. Williams *et al.* analyzed effects of 12 different drought patterns that varied both in the duration and the timing of single and multiple drought phases on 22 genotypes [21]. The results showed that occurrence of water deficit during the early phase in crops' life (until shortly after the first flowers had been produced) had improved crops endurance to any subsequent droughts during the reproductive phase. The responses of genotypes to drought were influenced by the timing of drought relative to phenological development and by the yield potential of the genotype under nonstressed conditions. Undoubtedly, early maturing character play an important role in escaping end-of season drought with reasonable yield compared to long season genotypes.

While short episodes of water deficits had little effect on the crop, >90% of the yield variation was attributed to the intensity of drought and cumulative duration of droughts in the crop's life [19]. The negative relationship between the yield

Table 14.2 Effects of droughts at different growth stages on pod yield of groundnut.

Drought phase	Yield under nonstress condition (kg/ha)	Duration or stage of drought in the crops growing cycle	% Change in yield due to drought	Comments	References
Emergence to flowering	4615–4720	22–52 DAS	+12.3 to +18.7	30 d of drought with mean temperatures of 22 °C Var. Robut 33-1	[26]
	3258–4720	7–40 DAS	+12.3 to +34.9	Data from 3 year trial Var. Robut 33-1	[22]
	2420–3450		−11% to +56.5%	Range in 11 genotypes	[25]
Emergence to start of pegging	4615–4720	43–103 DAS with a recovery irrigation at 73 DAS	−18 to −29%	Two episodes of drought with one single recovery irrigation Var. Robut 33-1	[19]
Flowering to pod settling	3130–5200	Beginning of flowering to harvest	−73% to −96%	Water supply withheld. Data from two cultivars, Floriman and ASEM	[24]
	4460–5040	Beginning of flowering to harvest	−48 to −55%	5 mm of water applied thrice weekly during the stress period	[24]
	5165–7589	36-70 DAS	−15.8% to −26.8%		[18]
Pos set to early seed filling	5165–7589	71-105 DAS	−25.0% to −37.4%	Range from 4 year trials with florunner	[18]
Active seed filling	4615–4720	93–155 DAS	−23.0% to −68.5%	53 days of end-of-season drought coupled with mean air temperatures above 28 °C	[19]
	5165–7589		−5.4% to −49.0%	Range from 4 yr trials with florunner	[18]

potential (yield under nonlimiting conditions) and drought resistance (defined as difference between yield potential and yield achieved under a given drought situation) became stronger as the severity of the end-of season drought increased. These studies have also shown that the yield potential under nonlimiting conditions is highly correlated with yields achieved under some drought patterns (particularly

end-of- season droughts), there was no correlation between yield potential under nonstress condition and the yield under mid-season drought that involved renewal of water supply following the drought [19].

The major sources of genotypic variation reported in the literature have been associated with the reproductive physiology, the ability to initiate pods despite water deficit or to recover rapidly when drought was released by irrigation or rain. While these studies enable irrigation management to optimize yield per unit water input, significant genotypic variation in recovery from early and mid-season droughts presents scope for selection of genotypes better adapted to different drought-prone environments and to breed for genotypes with rapid recovering ability and high yield potential under nonlimiting conditions [25, 27].

14.2.3 Effects of Water Deficit at Different Stages of Crop Growth

The water deficits may occur anytime or many times during life cycle of a rainfed groundnut crop as and when soil moisture depleted below a threshold limit and the supply is not renewed. The sensitivity of groundnut plant to water deficit depends on plant's developmental stage at which water deficit occurs. Progressing water deficit typically results in reduction in plant's transpiration by partial or complete stomatal closure. Severe water deficit can drastically reduce photosynthetic rate and therefore carbon assimilation, leading to inhibition of plant growth and developmental processes and biomass accumulation. The following section describes the effects of water deficits at various stages of the plant growth and its implication to the aboveground biomass and economic yield.

14.2.3.1 Germination and Emergence

Seed germination in groundnut is regulated by a combination of dormancy and environmental factors such as light, water, oxygen, and temperature. While the seed dormancy of groundnut is closely associated with taxonomical characteristics [9] and can be released using a plant growth hormone ethylene [28], environmental factors such as soil water and temperature remain the main limiting factors for seed germination. When temperatures are nonlimiting, imbibition of water by the seed under dark conditions will initiate a chain of metabolic processes that trigger germination of the seed. Studies revealed that an average seed moisture of about 35% was necessary for groundnut seed to germinate (seed is defined as germinated when length of radical reaches 2 mm). However, 100% germination in a sample was obtained at about 55–60% average seed moisture [29].

There is not much information on the effect of temperature of imbibed water on germination in groundnut. However, close analogies can be drawn from Refs [30–32], which defined the temperature thresholds for germination of a number of groundnut genotypes (Table 14.3). Results of these studies showed that the base temperature (T_base) ranged from 8 °C to 11.5 °C, optimum (T_opt) from 29.0 °C to 36.5 °C and maximum (T_max) from 41 °C to 47 °C for germination. These limits meant that the temperatures of imbibed seeds above

Table 14.3 Temperature limits of various growth and developmental processes in groundnut.

Process	T_base (°C)	T_opt (°C)	T_max (°C)	References
Germination	8–11.6	29–36.5	41–47	[33]
Leaf appearance	10	30		[34]
Leaf photosynthesis		36		
Onset of anthesis	11	29–33	38	[34, 35]
Pollen viability and seed setting		27–32	37–43	[36]
Pod size and pod growth rate		24		
Harvest index		27–32	37–44	[36]

or below the T_opt can affect the germination and the ambient temperatures near or above T_max may inhibit germination of the seed under well-watered conditions. The significant genotypic difference in the ability to tolerate supra and suboptimal temperatures in groundnut presents scope for improving genotypic adaptation to temperatures.

However, groundnut seed germination and emergence in the field are two separate processes. The emergence in field is the combined effect of seed germination and emergence of the seedling out of soil, which is an important factor contributing to plant stand and subsequently plant density in the field. While seed germination is governed by microenvironment factors immediately around the seed, the seedling emergence in the field can be influenced by other soil-related constraints such as soil texture, structure, and bulk density, and pH, which sometimes may override the environmental factors and vigor of germinating seed.

14.2.3.2 **Vegetative Phase**

Severe water deficits during vegetative phase reduce leaf and stem growth through effects on stomatal conductance and photosynthesis. A number of independent studies have shown positive effects of mild water deficits during the preflowering phase on pod yield [19, 20, 25] and hence warrant some discussion.

Detailed crop growth analysis conducted on a Spanish genotype "Robut 33-1" suggested that water deficit after seedling emergence until the first-flower appearance followed by adequate water supply through the crops life triggered a number of changes in developmental processes such as increase in leaf area, reduced main stem elongation, increased peg numbers penetrated into soil, and more mature pods per unit land area. These changes in turn led to yield benefits compared to a crop that received regular irrigation [26].

Puangbut *et al.* investigated effects of preflowering drought on 11 genotypes and found that improved yield under preflowering drought was associated with increased root growth nodulation, and N fixation [25, 37]. Interestingly, the increase in root length density of groundnut at soil depths below 30 cm in response to the imposed water deficits was not observed in other legume crops such as soybean or pigeonpea [38]. The maintenance of leaf water status, partial stomatal conductance, and nitrogenase activity by groundnut during soil water deficits compared to the

other legumes was attributed to greater density of roots in the lower depths of the soil profile (see Section 14.2.4.3 for more details).

14.2.3.3 **Reproductive Phase**

Water deficits during the reproductive phase, depending on the severity, can drastically reduce the economic yield. Water deficits during flowering phase can result in reduction in flower number [31] or delay in time to flower [39]. However, since only up to 20% of flowers result in pods that contribute to final yield, a reduction in flower number due to water deficits rarely influence yield [20]. Groundnut, with an indeterminate growth habit, can compensate for reduced flower numbers under water deficits by producing flush of fresh flowers once the water deficit has been alleviated through irrigation or rain [26, 40]. Likewise, water deficits during pegging affect yield primarily by reducing pod number [17, 41, 42] unless sufficient water supply is resumed (upon release of drought) to allow carbon assimilation and supply to fill kernels [40].

The subterranean fruiting habit of groundnut makes it sensitive to soil water deficits occurring during pod filling in two ways: (a) by affecting plant water status (and thus carbon assimilation) through depletion of soil water in the root zone, and (b) by directly affecting the pod growth independent of root zone moisture when podding zone (to a depth of 5–7 cm from soil surface) is dry. The podding and rooting zones effects may not be always readily separable as root hair would also be present on the root in the podding zone. However, dry podding zone soil despite the presence of plentiful subsoil moisture in deeper zone is of common occurrence under rainfed conditions as well as under poor irrigation management conditions, where water requirements of plants during pod filling phase are met solely by subsoil moisture.

The water deficits in pod zone can also affect calcium uptake by pods [43]. It is well established that calcium is passively absorbed by developing pods directly from the pod zone soil solution as calcium uptake by roots is not usually available for pod growth and development [44, 45]. However, cultivar variation in reproductive development under water deficit [32] and calcium uptake [46] suggest scope for varietal improvement to minimize effects of pod zone water deficits during pod filling.

There are also some indirect effects of water deficits on the peg and pod development in groundnut. Groundnut pegs are positively geotropic and can penetrate up to 7 cm deep in the soil to allow normal pod development. In soils with high clay content, even mild water deficits can present a physical barrier for the peg penetration and growth of penetrated pegs [47]. Thus, the subterranean fruiting habit of groundnut adds another layer of complexity in understanding and managing the effects of water deficits.

14.2.4
Effects of Water Deficits on Some Physiological Processes

The yield reduction under water deficits is directly related to inability of roots to supply water needed for a series of physiological processes like photosynthesis,

stomatal conductance, and plant–water relations resulting in a negative impact on plant growth and development [48]. The ongoing climate variability is likely to accentuate the negative impacts of droughts and high temperatures. In order to improve adaptation of groundnut to water-limited environments, it is essential to understand the interactions of water deficits with temperature, and their effects on the physiological processes leading to yield formation.

14.2.4.1 Water Deficit and Temperature Interaction

The importance of water to plant growth was recognized by prehistoric farmers because irrigation systems already existed in Egypt, Babylonia (modern Iraq), India, and China at the beginning of recorded history. The historic evolution of classical concepts, which dates back to seventeenth or eighteenth century, of plant–water relations can be appreciated from a number of review articles [49, 50].

As the understanding of the influence of environmental factors on crop production improved with time, it became possible to quantify the water deficits and distinguish between effects of other environmental stresses such as high or low temperatures and soil and atmospheric stresses on plant–soil–water relations, plant growth, and yield.

Even under the best crop husbandry, crops can fail to achieve their genetic potential because of environmental constraints. Improvements in crop adaptation to environmental stresses with eventual higher crop yield can be attained if the specific environmental constraints for yield reduction are identified and resolved through either or combination of genetic enhancement and management approaches.

The main environmental variables, saturation vapor pressure deficit, ambient temperatures, and irradiance, have profound interactions and influence sensitivity of plants to water deficit significantly. In many climatic conditions, these variables are closely coupled to the plant water status, which is a function of soil water availability. It is usually impossible to control these parameters effectively in the field, so most of the physiological studies on these variables have been restricted to controlled environments.

While water deficit is the main theme of the chapter, the interactions of water deficits with temperature cannot be ignored as it can accentuate negative impacts of water deficit [51]. Several aspects of plant growth regulation are affected when the ambient air and soil surface temperature exceeds 40 °C in the crop environment. In fact, the subterranean fruiting habit of groundnut renders it more sensitive to high soil temperatures even under well-watered conditions [36, 52]. It is necessary to interpret water deficit effects in conjunction with temperature limits as the ambient temperatures below or above the optimum range would confound the effects on water deficits. As described earlier, sub-or supraoptimal temperatures can limit plant growth and development even under nonlimiting water conditions (Table 14.3).

Varaprasad *et al.* [36] concluded that while increasing CO_2 levels increased leaf photosynthesis and seed yield, there are no beneficial interactions between elevated CO_2 and temperature, and that seed yield of peanut will decrease under future

warmer climates, particularly in regions where present temperatures are near or above optimum [36].

14.2.4.2 Water Uptake and Plant–Water Relations

The ability of plants to mine stored water from deeper soil layer is an important mechanism to sustain plant growth and development. Short episodes of water deficit during the early vegetative stage are known to promote root growth in groundnut [19, 23], however, prolonged and severe water deficit can reduce root growth rate [53]. Rucker *et al*. reported that groundnut genotypes with large root system were able to tolerate drought conditions better than those with small root systems [54]. A strong correlation between root and shoot dry weight has also been reported in groundnut [27]. Puangbuti *et al*. [25] found that genotypes with large root systems were able to recover faster when water supply is renewed by irrigation or rain [25].

Under drought stress, deep root system can be an adaptive strategy to sustain prolonged period of water deficits. In groundnut, root length density (RLD) (root length per unit volume of soil) was found to increase in lower soil profiles [55] highlighting adaptive nature of the crop to drying soils. However, genotypes with increased greater RLD in deeper soil profiles showed smaller reduction in pod yield and HI under water deficit conditions [56]. Maintaining root growth and manipulating root distribution to access water from deep soil layers seem to be two important drought avoidance mechanisms in groundnut that could be further explored for identifying genotypes with resilience against droughts.

The water status of plants represents an integration of a number of variables such as atmospheric demand, soil water potential, rooting density, and distribution and therefore is a true measure of water deficit in plants [57]. The water status of plant has been defined in terms of leaf relative water content and leaf water potential or its components, that is, osmotic and turgor potential [50]. A detailed review by Wright and Nageswara Rao describes the use of these parameters to define the water deficit in groundnut [58].

A number of studies have reported significant genotypic variability in maintenance of leaf water potential and stomatal conductance under diverse water availability situations [59–61]. Clavel *et al*. [62] identified two different plant–water relation strategies to cope with water deficit in groundnut. The first strategy was characterized by delayed stomatal closure and low cell membrane damage during drought. Genotypes with these characters had ability to maintain high water uptake even under water deficit conditions. In the second strategy, genotypes maintained higher RWC by early stomatal closure. However, genotypes with these two divergent strategies produced similar yield.

14.2.4.3 N Fixation

Nitrogen is vitally important for leaf area development and photosynthesis in all legumes including groundnut. The symbiotic nitrogen fixation by the *Bradyrhizobium* bacteria present in root nodules is the most significant natural pathway for the introduction of atmospheric nitrogen into the plant. The ability of groundnut to

fix atmospheric nitrogen reduces the need for chemical fertilizer inputs and provides important economic and environmental benefits [63, 64]. Nitrogen fixation activity in groundnut has been assessed using various indirect measurements such as nodule weight and number, biomass production, shoot dry weight, and nitrogenase activity using acetylene reduction assay [65, 66].

A number of studies revealed that N fixation is extremely sensitive to soil water deficits [66–68]. Several mechanisms have been proposed to describe effects of drought on N fixation by legumes: (a) reduced carbon supply, (b) shortage of oxygen, (c) feedback regulation of nitrogen accumulation, and (d) oxidative stress damage at cellular level. These processes individually or collectively affect the nitrogenase activity in the nodule [68–72]. Nitrogenase activity has been shown to be severely reduced as leaf and nodule water potentials decreased below −1.4 Mpa [38]. Changes in leaf turgor pressure could cause rapid changes in nodule activity and thus N fixation, which could explain the sensitivity of N fixation in drying soils [73]. In comparison to photosynthesis, N fixation process is more sensitive to water deficits [74, 75].

Significant genotypic variation in N fixation was found under irrigated as well as water deficit conditions. Although biomass production under the imposed drought was not significantly different, significant genotypic variation was observed for N fixation [66]. Sinclair *et al.* reviewed relative merits of various methods used to measure N fixation and suggested that nitrogenase activity can be expressed as a function of plants transpiration [65].

There is limited information on the link between transpiration and N fixation under progressive soil drying cycles. Devi *et al.* reported significant genotypic variation in N fixation in progressively drying soil [76]. While N fixation decreased rapidly after a threshold level of fractional available soil water (FASW), there was a significant variation among genotypes in the threshold FASW at which N fixation started to decrease. Substantial variation in the FTSW threshold for transpiration was previously reported for these genotypes [77].

14.2.4.4 **Photosynthesis and Transpiration**

Photosynthesis and transpiration are critical for plant's growth and development. Both the physiological processes are mediated simultaneously by stomatal pores present in the epidermal layers of leaf. Groundnut has stomata on both sides of the leaf. While there exists a significant genotypic variation in the density and size of stomata in groundnut [78], there is little information about the significance of this "amphistomatal" characteristic in adaptation of groundnut. Studies have shown that two-thirds of the groundnut leaf's total net photosynthesis accounts for diffusion of CO_2 through the adaxial leaf surface, which explains the high photosynthetic efficiency of groundnut compared to certain other C_3 species [79].

The saturation vapor pressure deficit (the difference between saturated vapor pressure at an air temperature and actual vapor pressure) is a principal mediator of CO_2 and water vapor exchange through the stomata and thus a major determinant of potential rates of evaporation and photosynthesis. Controlled environment studies have shown that stomatal conductance of well-watered groundnut plants can be

affected strongly by changes in saturation deficit. The response was stronger at higher irradiances when variations in saturation deficit between 1.5 and 3.0 kPa caused three–fourfold changes in leaf conductance [80]. However, the stomatal conductance was greatly reduced or absent under severe water deficits and accompanied with high saturation deficits. A number of studies have found that both time of the day and plant water status influence both gas exchange and the stomatal conductance resulting in a tight inverse relationship between the rate of photosynthesis (p_n) and stomata conductance (C_s) in groundnut [22, 81, 82].

Groundnut crops under semiarid environments can experience stomatal closure very frequently (usually during midday) when saturation vapor pressure deficit can often exceed 3 kPa and thus potentially limiting period of active photosynthesis during the growing cycle [22]. Prolonged water deficits coupled with high temperatures can progressively reduce the duration of active gas exchange through stomata affecting growth and developmental processes. However, the rapid and complete recovery to normal stomatal conductance following renewal of water supply following severe stress has been widely reported in groundnut [37, 38, 83]. This capacity to recover rapidly to normal transpiration and CO_2 assimilation represents an important mechanism underpinning the adaptive response of groundnut.

A number of studies have investigated diurnal trends, effects of ambient temperature, irradiance, and plant and leaf water status on net photosynthesis and stomatal conductance and reported a significant genotypic variation [38, 79, 81–84]. However, there is limited published information on the water deficits affecting the net photosynthesis in groundnut at canopy level.

Although CO_2 assimilation integrated over the whole season accounts for more than 90% of dry matter production is, studies in many crops have failed to establish a convincing evidence for a relationship between the instantaneous measurement of photosynthesis and crop's performance. However, in case of groundnut, Bhagsari and Brown [81] using instantaneous assays of photosynthesis for extended periods of sampling throughout the day demonstrated that the cultivated groundnut had a higher net photosynthesis (range 18–30 mg CO_2/dm^{2-}/h) and high translocation rates compared to the *Arachis* wild species (range 11–26 mg CO_2). Variations of up to 100% in photosynthetic rates per unit leaf area and heterosis for this trait have been reported in groundnut genotypes [81, 82, 85–87].

Although these studies present a good example to demonstrate scope for crop improvement using Pn, there is a lack of published information on how Pn at plant level influence the crop productivity. Zelitch reviewed complexities in extending instantaneous measurements of photosynthesis to crop performance and suggested scope for increasing the rates of net photosynthesis per unit leaf area, translocation, and enlarging the storage capacity by selection to bring yield improvements, especially in C_3 species [88].

14.2.4.5 **Partitioning of Dry Matter to Pods and Harvest Index**

The water deficits during reproductive growth phase also impact partitioning of assimilates to pods depending on the genotypes and duration of stress [19, 26, 40, 89, 90]. Earlier studies in groundnut concluded that translocation of

assimilates to pods occurs only from leaves and there is no remobilization of assimilates from other storage organs such as stem and roots. These conclusions were based on the observations that the partitioning ratio (PR – ratio of pod growth rate to crop growth rate adjusted for high-energy content) was always less than 100% [33, 91]. However, Bell [32], Chapman [35], and Wright *et al.* [89] reported evidence for remobilization in groundnut with PR in excess of 100% in some cultivars growing under water deficits during pod filling phase.

Although not related directly, water deficits can affect soil nutrient availability, especially calcium and boron, which in turn influence kernel growth and quality [92]. Calcium especially is distinct from other major nutrients in that it is largely phloem-immobile and enters the kernel through outer shell wall of pod mainly by direct diffusion from the soil and there is little downward redistribution of Ca after transpiration-driven uptake through the xylem [93]. Water deficits in the pod zone can thus directly influence pod and seed development in groundnut plants that otherwise have adequate water status as a result of water uptake from lower depths in the root zone [43].

14.2.5 Effects of Water Deficit on Seed Quality

End-of season water deficits can also negatively impact nutritive value and food safety of groundnut. The effect of water deficits on some of the kernel quality and food safety factors is described in the following sections.

14.2.5.1 Protein

Protein constitutes up to 18–23% [94] in groundnut kernel and values of up to 36% have been reported [95] with protein content increasing as the seed matures. Water deficits occurring during seed filling phase would affect the seed filling and thus interfere with protein accumulation [96, 97].

14.2.5.2 Oil Content and Quality

Total oil content of mature groundnut kernel may range from 42% to 54% depending on the genotype and environmental conditions [94, 98]. An important factor associated with groundnut oil quality is the "peroxidase value," which is used as a measure of unsaturation of the oil. Groundnut oil is made of 12 free fatty acids with oleic and linoleic acids accounting for more than 80% of the total oil [95]. The degree of unsaturation of groundnut oil is almost entirely due to the ratio of oleic and linoleic acid content, which is generally expressed as oleic/linoleic acid ratio. Conventional groundnut varieties grown for oil purposes typically contain 50% of oleic and 35% of linoleic acids. However, recent breeding efforts have been successful in developing genotypes with high oleic acid (>85% compared to 50% in normal types) and low linoleic acid <5% compared to >35% in normal types) [99]. The dramatic change in the oleic/linoleic acid ratio meant significantly better shelf-life for high oleic groundnut and groundnut products (3–15 times longer) compared to regular groundnut cultivars.

The information on the effects of water deficits on fatty acid composition in groundnut has been inconsistent. Holaday and Pearson demonstrated highly significant effects of seasonal temperatures, location, and varieties on oil production from commercial production areas with interactions between location and year of production [100]. Dwivedi *et al.* reported little effect of drought on total oil or oleic acid content, while drought reduced the content of linoleic acid [101]. A recent study reported that there were significant G × E interactions for O/L ratio for genotypes with low and intermediate O/L ratio (1–3), but not for those with high O/L ratio (22–25) [102]. An analysis of a multilocation study in Australia involving 133 breeding lines over 20 diverse environments revealed significant variation in oil content (ranging from 44 to 55%) and kernel yield (2–8 t/ha) with low G × E for oil content. A lack of relationship between oil content and kernel yield in these studies suggested that selection for high oil content can be achieved without compromising kernel yield [103]. While the genetic potential for oil content is largely untapped, the lack of any correlation between oil content and levels of fatty acid composition [102, 104] suggests that selection for improved (Hi–oleic) fatty acid composition as well as total oil content could be progressed simultaneously. However, it should be noted that water deficits during seed filling can significantly reduce total oil yield of the crop by affecting seed filling and reducing kernel yield.

14.2.5.3 **Aflatoxin**

Aflatoxins are a group of naturally occurring toxins produced in groundnut (many other nuts including maize) kernels by the common fungi, *A. flavus* and *A. parasiticus,* under specific moisture and temperature regimes. The major aflatoxins of concern are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) with AFB1 being predominant and the most toxic. The worldwide increase in the incidence of the hepatitis B and C viruses is increasing the importance of AF as a potential health risk since the toxin is implicated in predisposing people who ingest large quantities of AF to liver diseases and hence their presence in groundnut is heavily monitored and regulated to ensure a safe food supply [105, 106]. Groundnut with aflatoxin level above the minimum regulated limits cannot be used for human consumption where groundnut are consumed as food and therefore represent great economic losses for the groundnut industry [107, 108].

Aflatoxin contamination of groundnut can occur in the field (preharvest) under a severe end-of-season drought stress condition and/or under poor postharvest storage conditions where kernel moisture and temperature are not controlled [109], as shown in Figure 14.1.

In principle, the aflatoxin production in groundnut kernel infected by *A. flavus* or *A. parasiticus* is mainly controlled by kernel water activity, temperature, and maturity of kernel [110]. Groundnut crops experiencing prolonged end-of-season drought coupled with elevated temperatures (22–35 °C) (broken line in Figure 14.1) have greater probability of infection by *A. flavus* and subsequent aflatoxin production. While irrigated groundnut crops are less prone to aflatoxin production (due to

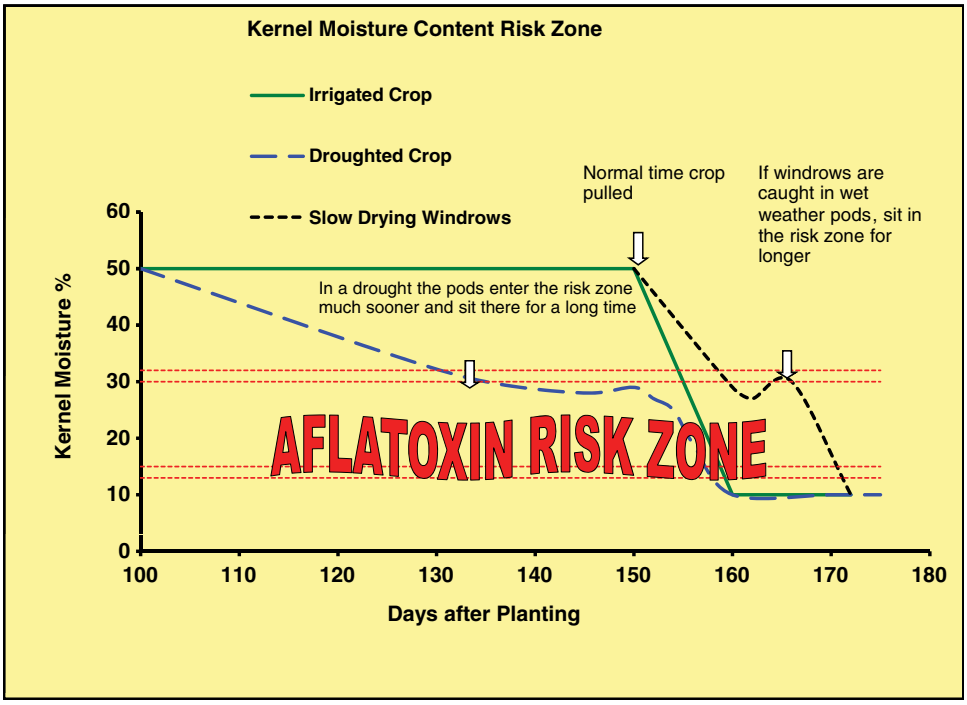

Figure 14.1 Possible risk zones of aflatoxin contamination in crop growth stages in groundnut.

high kernel moistures), the poor postharvest practices (through suboptimal drying) can result in postharvest aflatoxin production. It is beyond the scope of this chapter to go into details of aflatoxin contamination in groundnut.

14.3 Some Physiological Mechanisms Contributing to Drought Tolerance in Groundnut

Relevance of several drought tolerance traits have been described in a number of crops [111–115]. To enhance the effectiveness of trait-based selection, various traits were grouped on the basis of their primary influence on growth as well as secondary influence through modulating some process(es) that contribute to plants' tolerance or resilience. As such traits associated with imparting tolerance, a stress can be categorized as (a) primary traits (constitutive and induced/acquired); (b) secondary traits; and (c) integrated traits. Primary traits, mostly, are heritable, hence amenable for introgression to elite genotype background. However, characterization of physiological and biochemical mechanisms controlling these traits is complex [116–120]. Moreover, these traits have significant relevance to crop improvement for drought adaptation as they are intrinsic to plant species.

In general, traits can be visualized and further classified to indicate their major functions while conferring the drought tolerance:

1) **Traits associated with cell turgor:** Superior water relations: (a) water mining, (b) water conservation, and (c) water use efficiency.
2) **Traits associated with positive carbon balance:** (a) Optimum leaf area/light extinction coefficient, (b) stay green, (c) leaf nitrogen, and (d) mesophyll efficiency associated with chloroplast.
3) **Traits associated with cellular level tolerance (survival and recovery):** (a) Osmotic adjustment, (b) membrane stability, (c) scavenging of plant cytotoxic 160 compounds including ROS, (d) protein turnover/protein folding, (e) transporters, and (f) pollen viability and sterility.
4) **Traits associated with plant type:** (a) Phenology and (b) remobilization of resources.

There are general consensuses among the groups working in this area that drought tolerance of crops can be improved only by bringing together the diverse adaptive mechanisms.

This section summarizes the information available in groundnut on genotypic variations in physiological traits of both constitutive and adaptive nature and their significance in imparting drought tolerance in groundnut.

14.3.1
Water Extraction Efficiency

Water extraction by the plants in general depends on the root mass, length and its distribution in soil. Groundnut genotypes with larger root system were able to maintain higher transpiration efficiency (described in Section 3.2) under water deficit conditions [37, 121]. Groundnut genotypes that showed yield benefits under preflowering drought stress had more root dry weight and root length density in the deeper soil layers compared to nonstress treatments [115]. The root traits have been identified as being constitutive as well as adaptive that can be used as selection criteria for identifying genotypes for limited water availability [50, 122, 123].

Developing varieties with deeper rooting ability is one of the important avenues for improving productivity under wide range of environments where water is available in deep soil layers.

14.3.2
Transpiration Efficiency

While variation in photosynthetic rate (Pn) per unit leaf area represents a measure of carboxylation efficiency, Pn per unit of transpiration (T) at cellular level, dry matter production per unit transpiration at plant level represents a measure of transpiration efficiency (TE). TE at plant level is one such trait that can contribute to productivity under water-limited environments. Erratic droughts and rapidly

contracting aquifers across the world necessitated researchers to look for ways to improve TE of crops.

Two major potential sources of variation in TE are the capacity of leaf mesophyll tissue for photosynthetic carbon fixation and the conductance of water vapor by stomata. A relationship between the ratio of internal to ambient CO_2 pressures (p_i/p_a) and TE in C_3 plants means that any factor affecting stomatal conductance and CO_2 assimilation rate in a disproportionate manner thereby affecting p_i/p_a, will also affect TE [124]. In groundnut, many studies have found that genotypic differences in TE were associated with photosynthetic capacity per unit leaf area rather than stomata1 factors [125, 126]. Studies have shown significant relationships of TE with carbon fixing enzyme "Rubisco" per unit leaf area [127] and chlorophyll density in leaf [128].

Some reviews concluded that the intraspecific variation in groundnut for TE is small and likely to be improved by agronomic management [129, 130]. While some reports reported limited variation for TE under well-watered environments [131]. A number of studies in groundnut reported significant genotypic variation in TE under well-watered as well as water-limited environments (Table 14.4) [19, 37, 125, 126, 131, 132].

In addition to the genotypic variability, environmental conditions and the experimental errors in making accurate measurements of crop transpiration and root dry matter have no doubt contributed to the observed inconsistencies in TE values recorded in independent studies (Table 14.4). For example, pot studies enable accurate measurements of transpiration and root dry matter; however, various parameters such as size of pots and method of imposition of drought may result in rapid changes in root zone and affect plant water status eventual differences in TE values [134, 135].

14.3.3 Surrogate Measures of TE

The discovery of the theoretical basis for the relationship between the discrimination against ^{13}C during photosynthesis (Δ) and TE in C_3 plants [124, 136] enabled possibility of using Δ as an indirect measure of TE to overcome problems involved in determination of TE. This theory has been experimentally validated in many independent studies by demonstrating genetic variability for TE as well as by identifying strong correlations between TE and Δ in a number of crop species including groundnut [19, 27, 125, 126, 131]. A review by Wright *et al.* describes various aspects of Δ vis-à-vis TE relationships, including prospects for applying Δ as a surrogate tool for TE in groundnut breeding programs [137].

While carbon isotope discrimination analysis provides an indication of the mean TE, it provides no information about the underlying physiological differences at leaf level that may have caused any differences in TE. For example, reduced leaf internal CO_2 concentrations (p_i) may arise from a reduced stomatal conductance or from an increased intrinsic leaf photosynthetic capacity at any given p_i.

Table 14.4 Transpiration efficiency in groundnut genotypes under variable moisture status.

Mean TE (g/kg) (±Std. Dev.) under well-watered condition (TE_NS)	TE (g/kg) (±Std Dev) under water deficit conditions (TE_WD)	Crop growth phase	Comments	Reference
4.99 (±0.48)	1.5 (±0.58)	Vegetative phase	17 genotypes grown in pots under rapidly declining soil water	[76]
2.03 (±0.29)	2.49 (±0.25)	Vegetative phase	11 genotypes grown posts at field capacity or 1/3 available soil water	[37]
1.70 (±0.25)	—	No WD	Two wild and seven cultivated varieties grown in pots	[125]
—	3.08 (±0.28)	33–47 Days approx	318 F3 plants derived from 12 F2 plants grown in pots (2004)	[133]
—	2.14 (±0.12)	34–66 Days approx	318 F3 plants derived from 12 F2 plants grown in pots (2005)	[133]
1.74 (+0.25)	2.12 (±0.22)	Flowering to harvest	Field trial with 13 genotypes maintained at field capacity and 1/3 available water using subsoil drip system	[128]
—	2.59 (±0.54)	Drought imposed from flowering to pod fill with one intermittent irrigation	Four genotypes grown in mini-lysimeters installed in field	[27]
—	2.70 (±0.49)	Continuous drought imposed from flowering to pod fill	Four genotypes grown in mini-lysimeters installed in field	[27]

When the goal is to identify sources of genetic variability for WUE to be utilized in crop breeding programs, it may be desirable to have better understanding of the exact traits underlying the genotypic differences in TE. This understanding may help in making a logical step forward to identify candidate genes to be used to improve drought tolerance.

Studies by Nageswara Rao *et al*. and Arunyanark *et al*. pointed out that the variation in TE is mostly driven by the photosynthetic capacity [127, 128]. However, there is limited published information where known groundnut genotype differences in TE have been fully characterized by quantifying the relative importance of

stomatal versus nonstomatal factors at the leaf and plant level. Cultivar differences in the basis of variation in TE have been identified in soybean [138].

While measurement of Δ is rapid, it may not be feasible to apply this tool in large-scale breeding programs due to economic and technical considerations. A number of surrogate measures for Δ have been reported in groundnut. Wright *et al*. and Nageswara Rao and Wright reported highly significant correlations between specific leaf area (SLA, ratio of leaf area to leaf dry weight) and Δ suggesting that SLA can be used as a surrogate measure of Δ (and hence TE) in groundnut [27, 139]. Since then significant correlations between SLA and specific leaf nitrogen (SLN) [139], SPAD cholorophyll meter reading [127], and chlorophyll density [128] have been reported in groundnut. Although these surrogate traits can be measured easily and cost-effectively, it should be noted that these are significantly influenced by crop and environmental and technical factors such as ambient temperatures [135], plant water status [131], time of sampling, leaf age [127, 140], and the accuracy of the measurement. There is a need for more research to understand the basis of $G \times E$ for TE as well as surrogate traits before embarking on using them in a large-scale breeding programs.

14.3.4
Epicuticular Wax

The epicuticular wax layer on the leaf surface acts as the first protective barrier for nonstomatal water loss. It is well established that epicuticular wax content improves water retention in leaves by reducing water loss from leaves in a number of crops such as mulberry [141], oats [142], and wheat [143]. Limited research done in groundnut suggested genotypic variations in epicuticular wax content under moisture deficit conditions, and its implication in water relation and carbon assimilation were reported [144–146]. However, more research is necessary to establish the role of epicuticular wax in drought resistance in groundnut.

14.3.5
Survival under and Recovery from Drought

The ability to survive and rapidly recover from water deficit(s) upon renewal of water supply is an integrated adaptive strategy that depends on a number of primary and secondary drought-tolerant traits. Primary drought-tolerant traits like maintenance of membrane integrity, cell viability, extent of scavenging free radicals and so on influence stress endurance at cellular level and thus survival of plants.

Decrease in chlorophyll content under water deficits has been considered as a typical symptom of oxidative stress and may be the result of pigment photooxidation leading to chlorophyll degradation and damage to chloroplast. Arunyanark *et al*. found a strong relationship between chlorophyll parameters and drought tolerance in groundnut [128]. High constitutive photochemical efficiency

and maintenance of functional photosynthetic apparatus are important components of cell level adaptation in groundnut to water deficits [62].

In a screening study among the genetically proximal short-duration groundnut lines, Clavel *et al*. [62] showed a positive relationship between *in vivo* chlorophyll fluorescence and pod yield across varying soil water conditions, indicating that the photochemical response is probably constitutive and could be a potential selection criterion to select genotypes for drought tolerance [62].

It is known that stress-induced imbalance between generation and use of electrons in chloroplast results in overproduction of reactive oxygen species (ROS) in the system leading to breaking down of membrane structure, DNA prompting the oxidation of amino acid, and proteins eventually resulting in accelerated lipid peroxidation. As part of adaptation, plants have developed ROS detoxification mechanisms that are either enzyme or nonenzyme mediated [147]. Although it is beyond the scope of the chapter to go into details of detoxification mechanisms, it is important to mention that there is growing evidence of higher ROS quenching ability and generation of antioxidants in groundnut [148, 149].

14.3.6 Acquired Thermotolerance

Plants that experience mild stress prior to a lethal stress were found to survive better than those that encountered lethal stress at the first instance. This phenomenon is often referred as "acquired tolerance." The acquired tolerance for abiotic stresses was shown to operate for a range of stresses like salinity, chilling, high temperature, and drought stress [150]. Using the principle of acquired tolerance, a selection procedure called "temperature induction response" (TIR) has been developed and successfully tested for a number of crops such as in finger millets [151], groundnut [152], peas [153], maize [154], and cotton [155]. Several studies found that new proteins are synthesized during the primary stress that might impart tolerance to subsequent and more severe stress [151, 152, 156].

As demonstrated in Table 14.5, a significant genotypic variation for "acquired thermotolerance" exists in groundnut [157]. In addition to significant genotypic variation, the lines with better acquired thermotolerance also showed higher tolerance to moisture deficits in field [156, 158].

Table 14.5 Genotypic variation in 115 groundnut genotypes in acquired thermotolerance.

S.No	Genotypic response	Susceptible	Moderately tolerant	Tolerant
1.	Range in recovery growth (%)	3–20%	21–40%	41–70%
2.	Mean recovery growth (%)	14.2%	30.5%	52.8%
3.	Number of genotypes	32	50	33

Groundnut seedlings were subjected to higher temperatures with a gradual 5 °C increase in the first 4 h (35 °C–1 h + 40 °C–1 h + 45 °C–2 h) followed by lethal temperature (53 °C for 3 h). Later the seedlings were allowed for recovery in growth at 25 °C for 72 h. Tolerance was measured using recovery of root and shoot growth following the release of high temperature stress [159].

Presence of significant variation in acquired thermotolerance in groundnut is promising, although the molecular and physiological mechanisms underlying the genotypic variation warrant further research.

Among the primary traits, the relevant constitutive traits are phenology (early duration), roots, epicuticular wax, stomatal index, and leaf traits like specific leaf area or specific leaf nitrogen are important. Similarly, osmotic adjustment, cytotoxic scavenging mechanisms, and changes in growth hormones especially ABA and protein turnover are also few traits that are relevant to drought tolerance of groundnut.

The secondary traits like leaf water status, canopy temperature, leaf folding, leaf senescence/stay green, and those associated with reproductive efficiency (described in Sections 2.3 and 2.4) are of significance.

14.4 Integration of Physiological Traits to Improve Drought Adaptation of Groundnut

Conventional breeding methods combined with extensive evaluation in multienvironment demonstrated widespread G × E interactions for most of the agronomic traits. Although breeding for yield improvement has been generally successful [160], the rate of progress has been slow and increasingly becoming cost-prohibitive [161].

There is a general consensus that further genetic enhancement of crops for improved adaptation is best achieved by trait-based breeding strategies. The relevance of trait-based breeding was first demonstrated in literature [157, 162, 163]. For instance, trait-based breeding in wheat for improving osmotic adjustment [162], ABA [163], and $\Delta^{13}C$ [157] substantially improved crop adaptation to water-limited environments.

As part of drought resistance breeding in groundnut, significant progress has been made in developing cultivars with drought "escape" mechanism. This success was possible because the phenotypic trait could be easily and accurately measured in "thermal time" units [164] in target environments (regions with short growing seasons), the trait is governed by a few genes [165], and donor lines are available from cultivated groundnut germplasm pool. In contrast, the progress in combining yield attributes with drought/temperature tolerance traits has been slow. Although physiologists and breeders recognize the importance of introgressing relevant drought adaptive traits, there has been limited research to address this issue.

The simple crop analytical model proposed by Passioura [122], $Y = T \times TE$ and HI, where Y is the pod yield, T is the transpiration, TE is the ratio of dry matter produced per unit of T, and HI is the ratio of pod weight to total dry matter, presents an attractive framework for physiologists and breeders as it allows to dissect and identify germplasm with each of the model traits to stimulate ideas for trait-based breeding.

A large-scale study was undertaken in India and Australia with an aim to compare the empirical versus physiological trait-based breeding approaches in an objective manner [58, 166]. Since this study is one of its kind in the groundnut breeding programs, the results are discussed in some detail in this chapter.

Significant genotypic variability has been reported for traits related to transpiration [58, 121, 167], transpiration efficiency [27, 37, 121, 168], and HI [58, 169, 170]. Earlier studies also reported positive correlations between TE and total dry matter [58, 171] as well as negative association between TE and HI traits in groundnut [172].

Identification of surrogates for TE such as carbon isotope discrimination [58, 125], specific leaf area [139], and SPAD chlorophyll meter reading [127, 173] enabled studies on the heritability parameters of model traits as well as on the feasibility of using TE surrogates as the indirect selection criteria in large-scale groundnut breeding programs [121, 174, 175]. Further development of a "Selection Index" enabled simultaneous selection for high levels of *T*, TE, and HI traits without compromising on yield [169, 176].

A large-scale comparative assessment of the trait-based and empirical selection approaches was made for the first time in groundnut. A set of 192 $F_{2:6}$ progenies derived from using common parents but progressed through empirical versus trait-based selection approaches were evaluated in 12 diverse environments [161, 166] to compare the efficiency of selection approaches. The study provided critical insight into utility of both direct and indirect (trait-based) selection methods to identify genotypes with up to 30% yield advantage over local checks. It further suggested that parental selection was the most critical step compared to the selection methods that followed in advancing the progenies. While the physiological trait-based selection index approach did not demonstrate an overall superiority over empirical approach [161], the study demonstrated that selection for TE was possible in the large-scale breeding programs [166].

The efficiency of trait-based approach (Tr) *vis-à-vis* empirical approach (Em) in breeding for kernel yield was estimated using the genetic concept of response to selection, computed as

$$\mathrm{RE\,Tr} = R\,\mathrm{Tr}/R\,\mathrm{Em},$$

where $R\mathrm{Tr} = i\mathrm{Tr}\ h\mathrm{Tr}\ \sigma G\mathrm{Tr}$ is the response to selection under Tr, and
$R\mathrm{Em} = i\mathrm{Em} \cdot h\mathrm{Em} \cdot \sigma G\mathrm{Em}$ is the response to selection under Em.

Then the efficiency of Tr relative to Em is given as

$$\mathrm{RE\,Tr} = \{i\,\mathrm{Tr}/i\,\mathrm{Em}\}\{h\,\mathrm{Tr}/h\,\mathrm{Em}\}\{\sigma G\,\mathrm{Tr}/\sigma G\,\mathrm{Em}\} = \{h\mathrm{Tr}/h\,\mathrm{Em}\}\{\sigma G\,\mathrm{Tr}/\sigma G\,\mathrm{Em}\}$$
$$\text{for } i\,\mathrm{Tr} = i\,\mathrm{Em}$$

where i is the selection intensity, h^2 is the heritability, and σG^2 is the genetic variance. For selection method Tr to be superior to Em, RETr value should exceed 1. The relative efficiency values for different selection environments are given in Table 14.6.

An average RE*Tr* of 1.045 (close to 1) meant that both selection methods were equally efficient in identifying genotypes with high kernel yield across 12 diverse environments (Table 14.6). However, when 12 environments were grouped into 2 major clusters based on the growing season, that is, rainy season (Rf) and post-rainy season (PR or PRM), and RE Tr was reanalyzed, it was clear that trait-based

Table 14.6 Relative efficiency (RE of trait-based (Tr) vis-á-vis empirical selection (Em) for kernel yield in F2–F6 progenies in 12 environments in India.

Environment	RE Tr
ATP_Rf	0.742
ICR_Rf_IR	0.834
ICR_Rf	1.495
JAL_Rf	1.155
JUN-Rf	1.286
TIR_Rf	0.937
UDA_Rf	0.961
ICR_PR	1.254
ICR_PRM	0.612
JAL_PR	0.961
JUNPR	1.154
TIR_PR	1.103
Average	1.045

After Ref. [161].

selection method (Tr) was more efficient (RETr = 1.227) in rainy season environments but not in post-rainy season (RETr of 0.655) [161]. Rainy season selection environments are typically characterized by unpredictable dry spells with high evaporative demands, while post-rainy environments are more predictable with managed irrigation and progressively increasing temperatures and high vapor pressure deficits as the crop progressed to maturity.

The environments were also characterized by assessing dynamic changes in the plant-extractable water during the season using the computer-simulated modeling approach [177]. In this approach, a crop simulation model (APSIM-groundnut) is used in conjunction with crop management records (planting and harvest dates, and variety), daily climate records (rainfall, solar radiation, and air temperatures), and site-specific soil properties to simulate status of plant extractable soil water on daily basis. This information obviously is site, season, and crop specific and thus enabled defining the water availability pattern experienced by the crop in a given environment. This approach has been used widely to characterize growing environments using historical climate records [178]. Using the above approach, 12 growing environments could be grouped into 4 clusters (Figure 14.2) [108].

An analysis of RE*Tr* for each of the four clusters clearly showed that the trait-based selection was consistently superior to empirical selection in those environments that experienced depleting water availability as the season progressed (RETr values ranged from 1.15 to 1.49) (Figure 14.2a–c). In contrast, the trait-based selection approach was ineffective (RETr = 0.61) (Figure 14.2d) in the environments that experienced a typical mid-season drought followed by renewed water supply to the crop.

The major outcome of the study described above is summarized below:

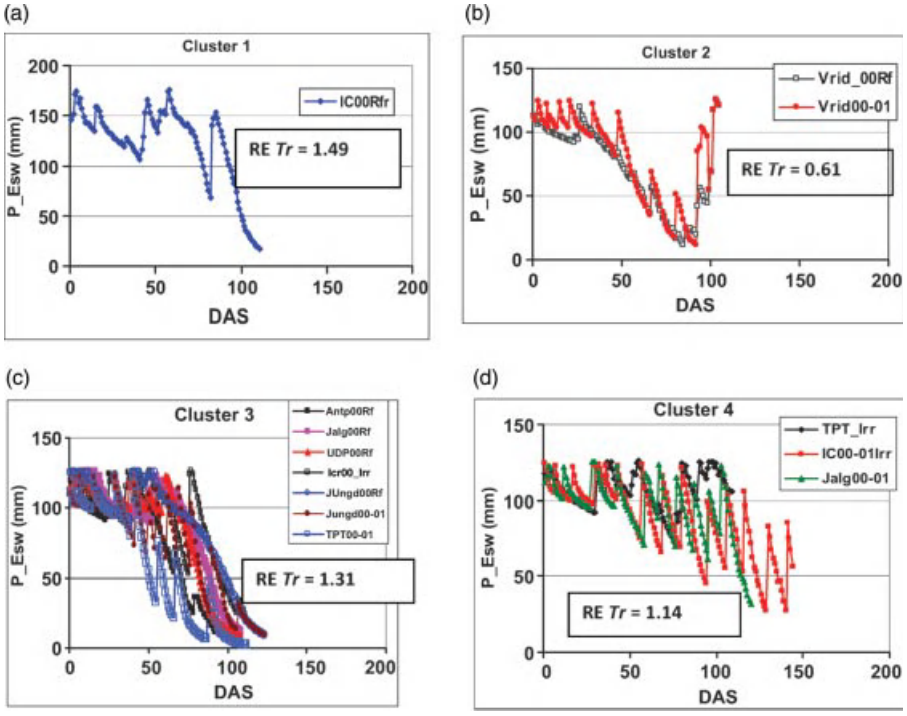

Figure 14.2 Effects of drought patterns (expressed as seasonal changes in plant extractable soil water P_Esw) on efficiency of trait-based selection approach in breeding for drought resistance in groundnut.

- There is a strong evidence for genotypic variation for the Passioura model traits in groundnut germplasm and selection tools can be used to identify parents for use in drought resistance breeding programs.
- The physiological traits, particularly TE, can be assessed rapidly using cost-effective tools, while the rapid measurement of other traits *T* and HI still remain a challenge.
- The plant's ability to maintain *T* is necessary to capitalize on high TE trait in order to produce total dry matter, and hence developing easily measurable traits for root efficiency is necessary
- It is possible to select for TE and HI simultaneously.
- Characterizing the target environment is necessary to achieve higher genetic gains in groundnut breeding programs. The environments with characteristic bimodel rainfall (resulting in mid-season drought followed by renewed water supply), require a different selection strategy (such as selecting for rapid recovery responses).

Based on the foregoing discussions, a comprehensive analysis of the other relevant drought adaptive traits besides those associated with transpiration and water use efficiency is necessary. For instance, the traits associated with water conservation and cellular level tolerance on dehydration is relevant.

Such a comprehensive conceptual framework forms the basis for a long-term strategic approach to improve groundnut productivity under drought. This necessitates generating required crop-specific genetic and genomic resources, besides developing strategies to integrate the traits.

14.5 Status of Genomic Resources in Groundnut

14.5.1 Marker Resources in Groundnut

Initial investigations on molecular diversity were based on markers like RAPD AFLP [179]. Most of the reported works using these PCR-based markers generally concluded that cultivated groundnut has little molecular diversity [177, 180–182]. Being dominant in nature, such markers were realized to have little utility in a polyploid species like groundnut. Singh *et al*. proposed that the cultivated groundnut indeed has considerable molecular diversity and emphasized the need for the development of genomic resources to capture molecular diversity [183]. Accordingly, several research groups have attempted to develop codominant marker systems such as SSRs in groundnut [184]. While generation and analysis of expressed sequence tags (EST) formed one approach, development of SSR-enriched libraries is being adopted to identify genomic SSR markers as the second approach [185, 186]. He *et al*. identified genomic SSR markers in groundnut and demonstrated the existence of polymorphism among cultivated groundnut [187]. Subsequently, several successful attempts have been made to develop genomic SSR markers [179, 181, 188–192]. These markers have been used to assess molecular diversity among cultivated groundnuts [193, 194]. The genic and genomic SSR resources developed for groundnut that is available in public data base are summarized in Table 14.7.

Table 14.7 Genic and genomic SSR marker resources for groundnut (*A. hypogaea*).

SSR type	Identification strategy	No. validated	Reference
Genic	ESTs (1350)	44	[195]
	ESTs (8785)	188	[196]
	ESTs (14 432)	290	[197]
	ESTs (24 238)	881	[198]
	ESTs (1200)	94	[197]
Genomic	Enrichment	56	[199]
	Enrichment	226	[189]
	Enrichment	123	[200]
	In silico comparison of other related species	51	[201]

Recently genomic resources from other related leguminous species such as lotus, medicago, soybean, and cowpea have also been used with significant success [194, 201].

14.5.2 Drought-Specific ESTs Libraries in Groundnut

In the absence of genome sequence information, generation of EST database representing drought-specific genes will be more rewarding. Expressed sequence tags is an effective genomic approach for rapid identification of expressed genes and has been widely used for gene discovery in various tissues, developmental stages, or under different environmental conditions. A cDNA library was constructed for groundnut experiencing water deficit by suppression subtractive hybridization approach. The transcript levels of flavonol 3-*O*-glucosyl-transferase (F30GT) gene identified in this library showed significant increase under stress [202].

A large number of ESTs have been created in peanut by different researchers for various tissues subjected to both abiotic and biotic stress (Table 14.8). One library catalogs the genes expressed in a wild species of *Arachis* (*A. stenosperma*; 6264 ESTs) under control conditions [196]. A number of ESTs have been created to understand the molecular basis of host resistance to aflatoxin contamination for developing seeds [203–207], to establish biotechnological platform for peanut germplasm [208], and to identify simple sequence repeats (SSR) for marker development [198, 209, 210].

Govind *et al*. [214] created an EST library to identify, isolate, and characterize the genes expressed during gradual drought stress acclimation in peanut [214]. Subtractive cDNA library was created and nearly 700 genes were identified. The overrepresented stress-induced clones were broadly classified into several functional categories involved in (a) metabolism: photosynthesis (4.3%), amino acid metabolism (2.3%), carbohydrate metabolism (3.4%), nucleic acid metabolism (1.4%), fatty acid metabolism (1.4%), metal handling related (2.9%), energy related (1.4%), and secondary metabolism (1.4%); (b) cellular processes: cell cycle (1.7%), protein synthesis (4.9%), posttranslational process related (4.9%), protein degradation (6.9%); (c) protecting cellular structures:biotic stress (3.1%), abiotic stress (11.7%), oxidative stress (2.0%); and (d)regulators: regulation of transcription (8.3%), hormone regulated (3.4%) (Figure 14.3).

Expression studies indicated the upregulation of auxin-induced genes (ARP), signaling cascade genes like kinases (CDPK and MAPKK), transcription factors, chaperons (LEAs and HSPs), aquaporin-related genes, ADH, praline-rich proteins, PR proteins, metallothioneins, and so on.

Some of the novel genes identified have been functionally validated by overexpression and downregulation studies. Virus-induced gene silencing (VIGS) was adopted to characterize some of the ESTs in *Nicotiana benthamiana* due to lack of efficient transformation system in groundnut [215]. Overexpression of early

Table 14.8 List of ESTs generated in peanut by different approaches.

Cultivar	Purpose and tissue used	Protocol adopted	Number of ESTs submitted and year	References
A. hypogaea (063 103)	ESTs for developing seeds		(8 seq) 2010	http://www.ncbi.nlm.nih.gov/nucest?term=peanut H.Y. Yan *et al.*, (Unpublished) *College of life Sciences, South-Central University for Nationalities]*
A. hypogaea (Luhua 14)	Full length for developing seeds	Total RNA was extracted from peanut seeds 20–60 days after pegging	678 + 3059 + 4406 (2010)	http://www.ncbi.nlm.nih.gov/nucest?term=peanut S.B. Wan *et al.* (2006) unpublished data; Xing Jun Wang, 2010 High-Tech Research Center, Shandong Academy of Agricultural Research, China
Arachis ipaensis	ESTs for developing seeds	Total RNAs were isolated from 6 to 8 weeks developing seeds of *Arachis ipaensis* and cDNA library was constructed with Smart cDNA Library Construction Kit (Clontech)	17 694	http://www.ncbi.nlm.nih.gov/nucest?term=peanut
Arachis ipaensis	ESTs for root	2 weeks root	15 093	E.D. Nagy (2010), unpublished data
Arachis duranensis (DUR25)	ESTs for developing seeds	Total RNAs were isolated from 6–8 weeks developing seeds of *Arachis ipaensis* and cDNA library was constructed with Smart cDNA Library Construction Kit (Clontech)	20 667	John Bowers (2010) University of Georgia, USA, unpublished data)
Arachis duranensis (DUR25)	ESTs for root	2 weeks root	14 624	

A. hypogaea (Georgia green cultivar)	Phorate responsive cDNA clones developed for leaf	Identified by differential display	436	http://www.ncbi.nlm.nih.gov/nucest?term=peanut M. Gallo *et al.* (2010), unpublished data Maria Gallo (2010), Department of Agronomy, University of Florida, USA
A. hypogaea	Root nodulation cDNA library developed for whole root tissue at nodulation stage	*Arachis* seeds were surface sterilized with mercuric chloride and coated with *Arachis* strain-specific bradyrhizobium cell suspension. Infected seeds were grown in sterile soil under green house conditions. Total RNA was isolated from the root tissue at nodulation stage and cDNA was synthesized by SMART cDNA library construction	439	http://www.ncbi.nlm.nih.gov/nucest?term=peanut C. Kiran *et al.*, unpublished data Khanderao, 2010 Department of Botany, Osmania University, Hyderabad
A. hypogaea (NEMATAM)	EST library for root knot nematode infection	SSH library for root infected with root knot nematode (*Meloidogyne arenaria*)	49	http://www.ncbi.nlm.nih.gov/nucest?term=peanut
A. hypogaea (Florunner)	EST library for root knot nematode infection	Root infected with *Meloidogyne arenaria*	52	Macia Galto (2009), unpublished data Agronomy Department, University of Florida [211]
A. hypogaea	Normalized cDNA for developing embryos	Total RNAs were isolated from developing embryos with Trizol (Invitrogen). The normalized cDNA libraries were constructed	20 628	http://www.ncbi.nlm.nih.gov/nucest?term=peanut

(*continued*)

Table 14.8 (*Continued*)

Cultivar	Purpose and tissue used	Protocol adopted	Number of ESTs submitted and year	References
		with Smart cDNA Library Construction Kit (Clontech)		
				V. Beilinson (2009), unpublished data Ervin D. Nagy (2009) University of Georgia, GA, USA
	Normalized cDNA for cotyledons and leaves	Total RNAs were isolated from leaves and cotyledons with Trizol (Invitrogen). The normalized cDNA libraries were constructed with Smart cDNA Library Construction Kit (Clontech)	12 327	
A. hypogaea (USDA-tifton peanut and GTC20)	cDNA library for drought and *A. parasiticus* infection	cDNA library was constructed from peanut cultivar Tifrunner (TF) or GT-C20 (C20). Tifrunner is resistant to TSMV and susceptible to *A. parasiticus*. GT-C20 is susceptible to TSMV and has reduced aflatoxin contamination. Developing seeds were challenged by drought stress and *Aspergillus*, and collected at reproduction stages R5, R6, or R7	38 856 + 1343	
A. hypogaea (Minhua 6)	Full length cDNA library for pods	The full length cDNA library was constructed from peanut cultivar Minhua 6. The peanut pod from DAP 9–50 were collected, and placed into liquid N_2 immediately and stored in −80 °C freezer	652	Guo *et al.*, 2008 (207) http://www.ncbi.nlm.nih.gov/nucest?term=peanut

				N.B. Cai, unpublished data; Zhuang, 2007 Fujian Agricultural and forestry University, Fujian, China, unpublished data
A. hypogaea (cultivar 850)	cDNA library from peanut leaves inoculated with *Cercosporidium personatum*	The cDNA forward and reverse subtractive library (SI) from peanut's leaves inoculated with *Cercosporidium personatum* was generated using suppression subtractive hybridization (SSH) methods [212], as tester cDNA from peanut leaves pooled samples colleted at 12, 24, and 72 h after inoculation with *C. personatum*, and as driver cDNA from control samples noninoculated	744	http://www.ncbi.nlm.nih.gov/nucest?term=peanut
A. hypogaea (Shanyou 523)	Peanut cotyledon cDNA library	Cotyledon, mid maturation stage	2184	Nobile *et al.* [213] http://www.ncbi.nlm.nih.gov/nucest?term=peanut
	Lambda expression library	Cotyledon, midmaturation stage	348	G. Fu *et al.* (2006), unpublished data, Department of Biotechnology, Science and Biotechnology, Guangzhou, China http://www.ncbi.nlm.nih.gov/nucest?term=peanut
Groundnut (K-134)	Subtracted cDNA library	Peanut seedlings were grown on soil:sand mixture (3: 1) and maintained at 100% field capacity by watering daily. Nineteen-day-old plants were subjected to moisture stress by withholding water. Leaf samples collected from unstressed (100% FC) and stressed (once field	116 + 6	Y.S. Yan *et al.*, unpublished data http://www.ncbi.nlm.nih.gov/nucest?term=peanut

(*continued*)

Table 14.8 (*Continued*)

Cultivar	Purpose and tissue used	Protocol adopted	Number of ESTs submitted and year	References
		capacity reached 25%) plants. The cDNA was constructed by subtractive hybridization		Sri G. Ranganayakulu *et.al.*, SK University, Ananthapur
A. hypogaea (TMV2)	Subtracted cDNA library for drought	Groundnut 15-day-old seedlings were subjected to different water stress (20%, 40%, 60%, and 100%) by withholding water till the crop plants withstands to the respective stress level. RNA isolated from leaf tissues. Subtracted cDNA library was constructed using adapter ligation technique and cloned into T/A cloning vector	845	[214]
A. hypogaea (Florunner)	ESTs for peanut root and stem	Root and stem	196	http://www.ncbi.nlm.nih.gov/nucest?term=peanut B. Yuksel, unpublished data A.H. Paterson, 2006 Plant genome mapping laboratory Georgia, USA
A. hypogaea (Jamspan90)	ESTs for peanut leaf	cDNA library was constructed from peanut cultivar Jamspan90	19	http://www.ncbi.nlm.nih.gov/nucest?term=peanut K. Matand and N. Wu, 2003 Peanut Leaf cDNA library

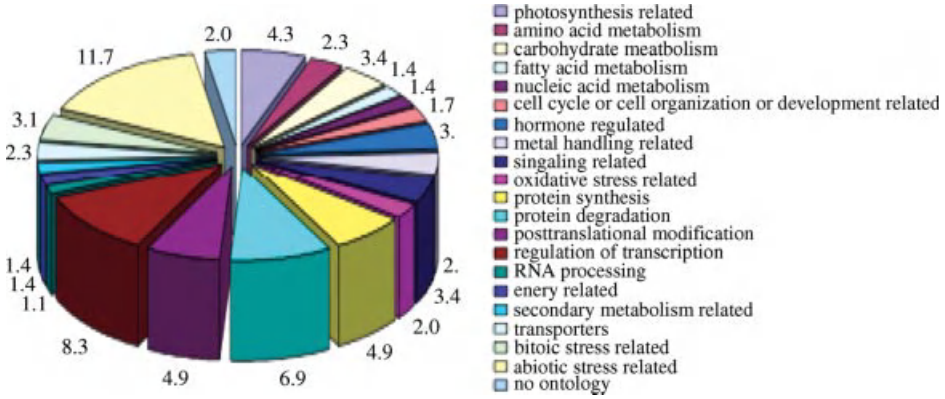

Figure 14.3 Functional classification of peanut ESTs identified from subtractive cDNA library enriched for drought response. (Reproduced with permission from Ref. [214], Springer, © 2009.)

light-induced protein (ELIP) and drought-induced protein (DIP) cloned from groundnut revealed their importance in imparting drought tolerance [196, 216]. Also, a RING box protein (AhRbx1), an important subunit of E3 ligase, showed phenomenol expression under diverse abiotic stresses, significantly its relevance in tobacco [217].

Besides this comprehensive study, there are very few reports characterizing the stress specific genes in groundnut. In another study, Luo *et al*. used a microarray of 400 unigenes to investigate the up/downregulated gene profiles in peanut cultivar A13, which is drought tolerant and resistant to preharvest aflatoxin contamination [205]. Twenty-five genes were potentially associated with drought tolerance. By adopting differential display of mRNA transcripts, nine partial cDNA clones that were upregulated and downregulated upon exposure to drought were identified [218]. Full length of two genes designated as *A. hypogaea* serine-rich protein (AhSrp) and *A. hypogaea* leucine-rich protein (AhLrp) were cloned by RLM-RACE.

14.6
Molecular Breeding and Genetic Linkage Maps in Groundnut

The cultivated groundnut (*A. hypogaea*) is an allotetratraploid ($2n = 4X = 40$) that originated from a relatively recent single hybridization event [6] between two wild diploid progenitors. While there is unequivocal agreement on the "A" genome progenitor as *A. duranensis* [219–221], there has been some controversy about the "B" genome progenitor. However, most studies indicate that *A. ipeansis* is the most probable donor of the "B" genome to the cultivated groundnut. These diploid progenitors of the cultivated groundnut are genetically quite diverse [222–224]. Although the resultant tetraploid from their hybridization may have *somehow*

developed reproductive isolation from its wild progenitors [6, 225, 226] and coupled with a single hybridization event that occurred, the cultivated groundnut represents very little molecular diversity [227].

14.6.1 Genetic Linkage Maps for Groundnut

The first genetic linkage map using microsatellite markers was constructed using an F2 population obtained from a cross between two diploid wild species with AA genome (*A. duranensis* and *A. stenosperma*) [227].

The B-genome linkage map was also constructed based on F2 population obtained from the cross between the diploid *A. ipaensis* and closely related *A. magna*, the former species being the most probable B-genome donor to the cultivated groundnut. The microsatellite markers utilized, developed for either *Arachis* species, showed high transferability (81.7%). The B-genome map compared with A-genome map using 51 common markers revealed high degree of synteny between both genomes [228].

The utilization of wild germplasm in breeding programs has received little attention due to the barriers associated with crossing wild and cultivated species. The development of synthetic amphidiploids using the diploid species (between *A. duranensis* V14167 diploid AA and *A. ipaensis* KG30076 diploid BB) of groundnut has led to the development of a SSR-based genetic map [229]. Development of genetic map will allow the synteny analysis of A and B genomes, comparison of diploid and tetraploid maps, and analysis of the introgression segments from the wild synthetic into the background of a cultivated variety. The material developed will be useful in the creation of advanced backcross and CSSL breeding populations for improvement of cultivated groundnut. Furthermore, it will be useful in the development of potential introgressed breeding lines, which can be of direct use in developing new varieties/cultivars.

Development of linkage maps in cultivated groundnut was constrained by the nonavailability of specific mapping populations besides the low level of genetic polymorphism. Hong *et al*. used a recombinant inbred population consisting of 142 progenies derived from the cross between Yueyou 13 and Zhenzhuhei as a mapping population [230]. A total of 141 SSR markers, including 127 genomic SSR and 14 EST-SSR, that distinguished the parental polymorphism were placed on the linkage map that detected 20 linkage groups covering a total map distance of 679 cM. The first mapping population for cultivated peanut was developed using over 215 recombinant inbred lines generated by crossing TAG 24 and ICGV 86031, identified for drought tolerance, Varsheny *et al*. screened the parents with 1145 SSR markers available in public domain and found 144 polymorphic markers [231]. These markers amplified 150 loci and a total of 135 markers could be mapped onto 22 linkage groups. More recently, Hong *et al*. developed three mapping populations with one genotype, Yueyou 13, a high yielding type as a common parent [209]. They screened the parents with 1044 genomic SSR markers and developed individual

linkage maps for the three populations with varied numbers of markers on the map. A composite linkage map was developed with a total of 175 markers on 22 linkage groups, of which 93 were common markers between the three populations. This study demonstrated for the first time a potential synteny, collinear order of some markers, and the conservation of collinear linkage groups. Syntennies and close match of the genome of cultivated groundnut were earlier demonstrated with the genome of Medicago and Lotus [232].

With the progress in development of marker systems and their use in constructing genetic linkage map in groundnut, efforts are now being made to discover QTL governing various traits. Khedikar *et al.* mapped the QTL governing late leaf spot and rust resistance traits using a mapping population developed by crossing TAG 24 and GPBD 4, although QTLs account for up to 50% of phenotypic variation [233]. Similarly, Ravi *et al.* mapped the QTL for several drought tolerance traits such as carbon isotope discrimination, a surrogate for water use efficiency, transpiration rate, specific leaf area, canopy conductance, total dry matter, and so on [234].

Though QTL mapping can be effectively accomplished by this strategy of characterizing a trait-specific mapping population, the identified markers often do not transfer the linked traits to other populations and often require stringent validation. Furthermore, a biparental mapping population can hardly represent one or a very few traits. These lacunae are significantly overcome by the adoption of a novel strategy based on the population genetic approach that relies on the level of allele diversity and frequency, referred to as association mapping or linkage disequilibrium mapping. This strategy involves the characterization of a diverse panel of germplasm accessions for the molecular and phenotypic diversity. Germplasm represents almost infinite meiotic recombination events and hence significantly increases the resolution of QTL discovery [235]. The other major advantage of this strategy is the possibility of screening the germplasm for a very large number of traits that confer biotic and abiotic stress tolerance besides yield and yield-attributing characters.

However, for the successful exploitation of the power of association mapping, availability of adequate molecular and trait diversity in a set of diverse germplasm is most crucial. A diverse set of reference collections of groundnut representing the global variability was accessed from ICRISAT center, Hyderabad, and extensively phenotyped for several drought tolerance traits. Significant phenotypic diversity in several traits such as root traits, water use efficiency, and so on was noticed. Desired molecular diversity was also observed using 8000 DArT markers (Table 14.9).

14.7
Transgenic Approach to Enhance Drought Tolerance

Since the functional characterization of the stress transcriptome, phenomenol progress has been made in identifying functions and regulatory genes to improve

Table 14.9 Genetic variability in several biometric parameters among the reference collection of groundnut germplasm.

Parameter	Mean	Minimum	Maximum	SD
Days to first flowering	39.1	34	46	2.3
SCMR	35.03	27	48	3.6
SLA	143	109	209	3.6
Root length (cm)	48.1	28.7	76.5	8.3
Shoot length (cm)	48.3	25.8	90.3	8.6
Leaf area (cm^2)	1749	518	4097	641
TDM (g/pl)	37.8	9.4	97.1	12.3
Root/shoot ratio	54.4	23.9	123	14.1
Root to leaf area ratio	1.16	0.47	3.29	14.06
TDM to leaf area ratio	224	105	42.2	4.9
$\Delta^{13}C$ (‰)	18.15	16.38	20.27	0.65

cellular level tolerance. These acquired stress responses are being extensively exploited to improve stress tolerance of groundnut by transgenic approach. Successful progress in this direction has been made by ICRISAT.

Groundnut transgenics have been developed by the group in collaboration with ICGEB in the cv. JL-24 with the transcription factor DREB1A under the stress-inducible promoter rd29A [236]. Increased TE was observed in many events, which is an important component of plant performance under limited moisture conditions. The DREB1A transgenics showed apparent effect on root growth under water deficit stress [237] with 20–30% increase in water uptake under water deficit conditions. When exposed to progressive water stress conditions, the transgenics appeared to show increased transpiration efficiency, which was negatively corelating with the antioxidative machinery of the plants under stress [238]. The study indicated that the changes in the antioxidative machinery in these transgenic groundnut plants under water-limited conditions played no causative role in the improved TE. Further, selected transgenic plants showed variable relation between TE and its surrogate traits, SLA, SCMR, and $\Delta^{13}C$ [239].

14.7.1
Transgenics: An Option to Pyramid Drought Adaptive Traits

Recent advances in trait-based breeding clearly demonstrated that improving productivity by enhancing drought tolerance of crops can be achieved only by bringing together diverse adaptive mechanisms while reshuffling superior alleles by conventional or molecular breeding options. Transgenic technology is emerging as potential option to introduce superior alleles from tolerant sources to pyramid traits and combat the drought in the climate change scenarios.

With the major emphasis of pyramiding drought adaptive traits by transgenic approach, a conceptual framework was elucidated. The approach has been to initially identify genotypes with superior water relations and use them as recipient genotypes to express validated upstream regulatory genes. The important steps in achieving this objective is initial identification of recipient genotypes with superior water relations, identify candidate genes to improve cellular level tolerance, develop transformation protocol to generate a large number of transgenic events, and rigorous evaluation to identify superior events that are moisture stress tolerant besides being productive under stress. The relevance of such a conceptual approach has been elucidated in recent studies (Figure 14.4) [159, 240–245].

Transgenics were developed in a groundnut variety K-134 with good root and moderate WUE by expressing validated stress genes like Helicases. Significant improvement of cellular level tolerance has been demonstrated by overexpressing these genes [235, 237–239, 246–250].

Following the *in planta* transformation protocol for groundnut [251], transgenics were developed overexpressing Helicase, PDH45, DREB1A, DREB1B, and DREB2A. Promising lines have been identified based on growth response under

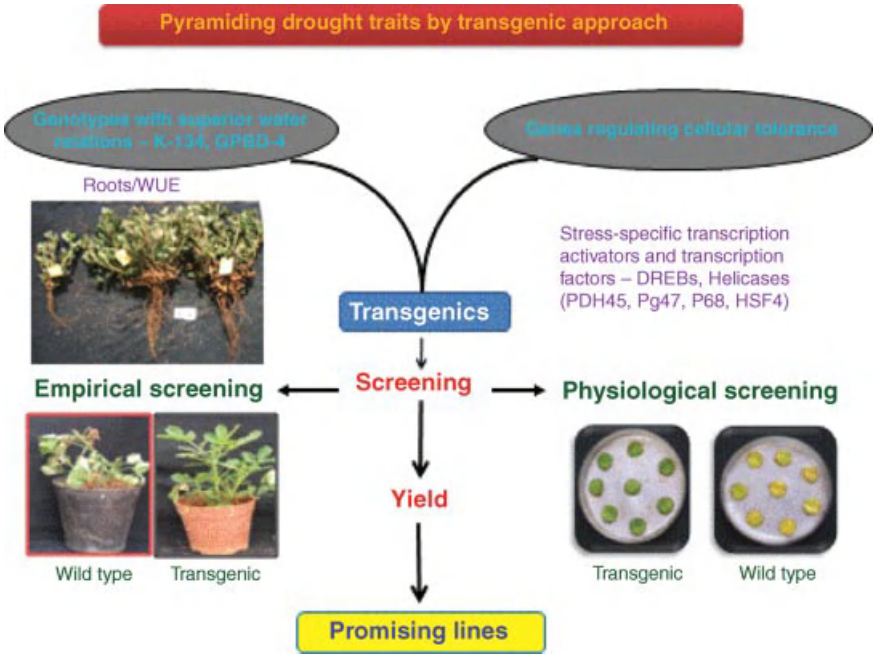

Figure 14.4 Strategy for pyramiding drought traits by transgenic approach.

drought stress, physiological stress, response screens for different biochemical processes, and productivity. Evidently, various physiological response assays suggest improved intrinsic cellular level tolerance in transgenics over wild-type plants. Transgenics overexpressing DREBs performed better than wild-type plants in PEG-induced osmotic stress. Stress-induced chlorophyll stability was observed in many transgenic plants overexpressing PDH45, which was evidenced by their stay green nature. The $\Delta^{13}C$ analysis of the transgenics (PDH45) showed increased water use efficiency.

Retardation of the stress-induced senescence at later stages of plant growth is a desirable trait at canopy level for improved pod growth. Several transgenic lines with significant reduction in leaf senescence compared to wild type at later stages of crop growth were identified. Transgenic lines showed 15–30% increase in yield under well-watered conditions and 30–40% increase in yield under stress conditions (Figure 14.5).

Transgenic approach to develop groundnut varieties with improved drought tolerance has potential (Figure 14.6). Moreover, the approach is amenable for introgression of several desirable traits into an elite genetic background.

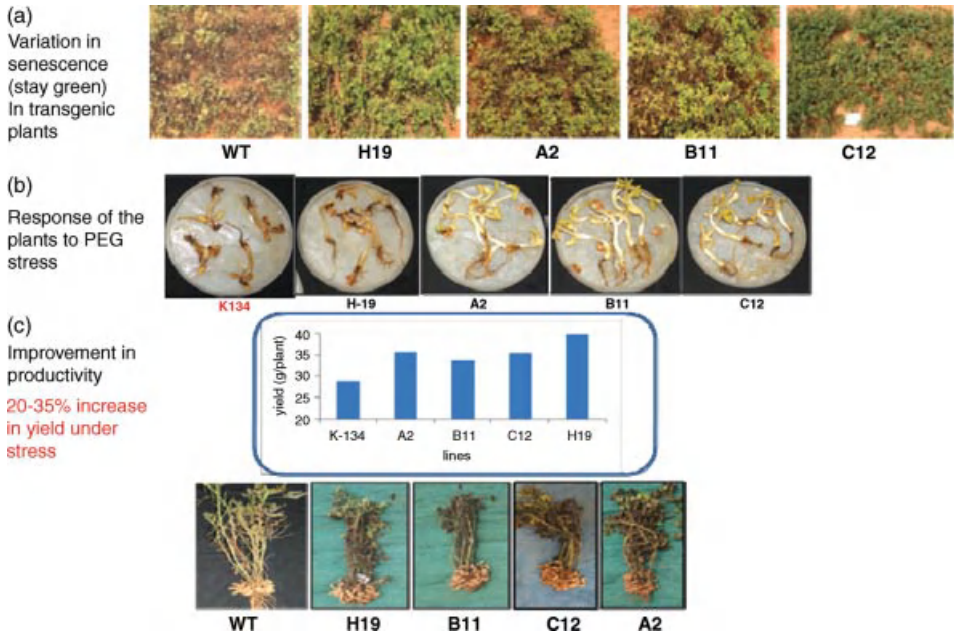

Figure 14.5 Promising transgenics overexpressing DREBs (1A, 1B, and 2A) and Helicase (PDH45) selected for evaluation under field strip trials. (a) Performance of the transgenics in response to 2000 ppm ethereal. (b) Response of the transformants to 15% PEG for 3 days followed by recovery for 3 days. (c) Improvement in the productivity of the transgenic groundnut plants under stress.

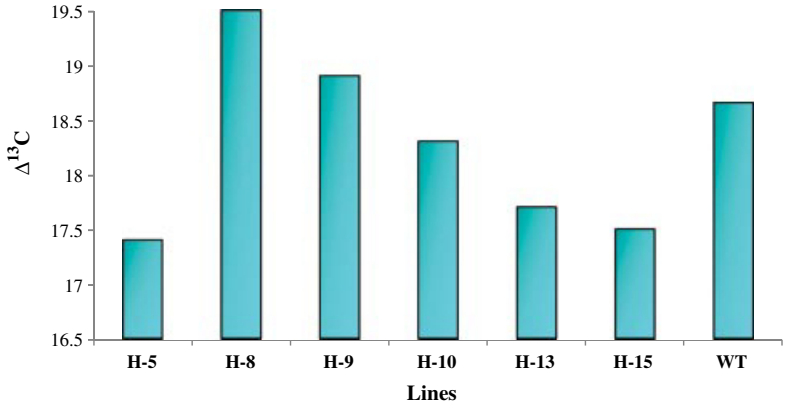

Figure 14.6 Water use efficiency in groundnut plants transformed with PDH45 Helicase gene. (a) Relation between the water use efficiency and $\Delta^{13}C$. (b) Variability in the WUE of groundnut plants with helicase gene. Leaf samples were collected from the transgenics and wild-type 90 DAS. The powdered leaf samples were then used to determine the carbon isotope composition that is used as a surrogate to WUE.

14.8 Summary and Future Perspectives

Earlier studies on the stress responses of cultivated groundnut genotypes to highly variable environmental factors, especially moisture stress and temperatures have provided required framework (a) to mathematically integrate the crop responses into crop simulation models that assisted in simulating likely crop yields under variable climatic conditions, and (b) to identify diverse and relevant stress adaptive mechanisms or traits and their subsequent validation, which in fact provided a platform to initiate trait-based breeding program.

Recently, efforts focused on characterizing the groundnut germplasm accessions for variability in drought tolerance traits, especially for traits related to phenology, transpiration efficiency and harvest index, and to limited extent root efficiency. While the diversity demonstrated in other traits relevant to drought tolerance such as cellular-level tolerance, epicuticular wax and root traits, chlorophyll content, and so on is quite encouraging; there has been limited exploitation of these traits in the breeding programs due to lack of rapid and economical selection tools.

It is clear that drought tolerance and improved productivity cannot be achieved by a single or a few simple traits. Thus, a breakthrough in improving crop productivity under water-limited conditions is possible only when diverse plant traits governing physiological mechanisms are pyramided on to a single genetic background.

A trait-based breeding program to introgress traits related to Passioura's yield model demonstrated the possibilities of improving productivity in water-limited environments in groundnut. This signifies that any approaches to bring together relevant traits for the target stress environment might improve productivity. The major focus, therefore, is to device strategies for achieving daunting task of trait

pyramiding, which is the only approach to address a complex phenomenon like drought tolerance.

14.8.1 Options and Approaches

Conventional breeding where selection for yield under drought though provided the initial genetic enhancement, for a comprehensive improvement in productivity under water-limited condition several diverse traits need to be combined into a single genetic background.

Despite the phenomenal success of breeding programs in improving crop productivity through reshuffling superior alleles, a more robust and focused breeding strategy needs to be adopted if one needs to achieve crop improvement under water-limited conditions. The first step toward this would be to identify genes and QTL that govern the variability in specific traits and/or identify crop genotypes that possess specific traits. Once these genomic and genetic resources are developed, focused molecular breeding and transgenic programs need to be evolved to improve crop productivity under water-limited conditions.

14.8.2 Molecular Breeding a Potential Option for Genetic Improvement in Groundnut

Recent advances in DNA technologies have opened up novel opportunities to enable marker-assisted selection and breeding in a number of crops. However, the implementation of molecular marker approaches in cultivated peanut has lagged behind other important crops such as soybean, rice, and maize for a number of reasons, including the complexity of the peanut genome and lack of polymorphism.

The slow progress in molecular breeding in groundnut is attributed to lack of robust QTL associated with targeted traits, which in turn was due to unavailability of a large number of codominant marker systems like SNP. Therefore, efforts should be made to initially generate large genomic resources of marker systems like SSR and SNP and subsequently to devise and standardize robust and high-throughput genotyping techniques.

Discovery of QTLs is conventionally achieved by molecular and phenotypic characterization of a trait-specific mapping population developed by crossing contrasting lines differing in a specific trait. Since diverse traits are essential for a comprehensive improvement in drought tolerance, this approach would be too expensive, cumbersome, and time-consuming. Alternatively, the characterization of a diverse panel of unrelated germplasm accessions is increasingly being adopted to identify QTL based on linkage disequilibrium mapping, also called as association mapping. Thus, the major emphasis must be on identifying a panel of diverse accessions of germplasm that is amenable to association

analysis. The panel should have sufficient molecular diversity as well as variation in traits of interest.

Another key step is to develop accurate and high-throughput technologies and phenotypic information for the targeted traits.

14.8.3
Transgenics: A Potential Future Alternative Strategy

Transgenic approach provides another potential option for improving drought tolerance and food safety of groundnut by introduction of novel alleles through a strategic transgenic program. Efficient trait pyramiding can be achieved by introducing upstream regulatory genes, and employing multigene expression cassettes with pathway engineered genes appears to be the most effective strategy. It has been demonstrated in a recent study in groundnut that trait pyramiding has advantages. The conceptual strategies to pyramid traits by transgenic approach require initial identification of genotypes with superior water relations and using them as recipient genotypes to express validated upstream regulatory genes that improve cellular-level tolerance. However, the limitations could be the issues related to biosafety.

To sum up, there are now options to initiate a successful breeding program to improve drought tolerance and water productivity. Basic understanding of the stress responses, identification of traits of relevance, progress in cloning genes, discovery of QTL, approaches for pyramiding genes and QTL, and modest progress in phenotyping have opened up new avenues to achieving the daunting task of improving drought tolerance. A new paradigm involving application of genomic tools underpinned by physiological understanding will be required to enhance the overall adaptation of groundnut to drought and high temperature stress situations.

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15
Chickpea: Crop Improvement under Changing Environment Conditions

B.K. Sarmah, S. Acharjee, and H.C. Sharma

Abstract

Chickpea, *Cicer arietinum*, is the second most important food legume in Asia after dry beans. Chickpea is an important source of protein, minerals, fiber, and vitamins in the diets of millions of people in Asia and Africa. Chickpea is also rich in essential amino acid lysine and deficient in sulfur-containing amino acids, methionine, and cysteine. Chickpea is mainly used for human consumption and only a small proportion is used as feed. It meets 80% of its N requirement from symbiotic nitrogen fixation and leaves substantial amount of residual nitrogen for the subsequent crops. It is a hardy crop well adapted to stress environments and a boon to the resource-poor marginal farmers in the tropics and subtropics. Average yields of chickpea are nearly 780 kg/ha, although farmers can harvest more than 2.5 tons/ha. The crop potential is nearly 5 tons/ha. Abiotic (drought, heat, and cold stress) and biotic (pod borers – *Helicoverpa armigera* and *Spodoptera exigua*, aphids – *Aphis craccivora*, leaf miner – *Liriomyza cicerina*, and bruchid – *Callosobruchus chinensis*) and diseases (*Fusarium* wilt, *Ascochyta* blight, *Botrytis* gray mold, and root rots) are the major stresses that constrain chickpea production in farmers fields. The major challenge is to reduce the losses due to biotic and abiotic constraints, and close the yield gap through crop improvement and crop management in future. A combination of productivity enhancement through varietal improvement, including biotechnological interventions, and integrated crop management is needed to realize the yield potential of this crop for improving food and nutritional security. Considerable progress has been made in developing high-yielding chickpea varieties to increase the productivity of this crop, while conventional breeding has been successfully used to breed disease-resistant varieties, little progress has been made in developing pod borer and drought-tolerant varieties, as the levels of resistance available in the cultivated germplasm are quite low. Wild relatives of chickpea have high levels of resistance to pod borer. Marker-assisted selection and genetic engineering of chickpea are being exploited to increase the levels of resistance/tolerance to these constraints and in future.

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15.1 Introduction

Global warming and climate change present a major challenge to the human beings as we heavily depend on natural resources, particularly agriculture, for food, feed, fodder, timber, fuel wood, and so on. Changes in climate will affect crop productivity and may degrade cultivable land [1, 2]. The natural calamities such as droughts, storms, floods, and heat waves might occur more frequently. A steady increase in temperature, decrease/increase in relative humidity, moisture stress, and increase in atmospheric carbon dioxide (CO_2) will also change the relative activity and abundance of insect pests, natural enemies, and their interaction with the host plants. Moreover, increased demand for biofuels will reduce the land available for cultivating food crops [3]. Therefore, there is need to enhance crop production by adopting modern tools of biotechnology and crop management to mitigate adverse effects of climate change.

Cereals, grain legumes, oilseeds, vegetables, and fruits are the major components of food for human beings. Among these, grain legumes play an important role in the dietary and nutritional needs of people, particularly in Asia, Africa, and Latin America. Among the many grain legumes consumed by people, chickpea is the most widely cultivated food legume in the world.

Chickpea (*Cicer arietinum* L.) is the second most important food legume in Asia after dry beans in terms of area, production, and consumption. Average annual chickpea area in the world is 10.7 million ha with a production of 8.2 million tons – Asia accounts for 90% of the area and 88% of the production of chickpea in the world. Chickpea is an important source of protein, minerals, fiber, and vitamins in the diets of millions of people in Asia and Africa. Chickpea has 23% protein, 64% total carbohydrates, 47% starch, 5% fat, 6% crude fiber, 6% soluble sugar, and 3% ash [4]. Chickpea is rich in essential amino acid lysine and deficient in sulfur-containing amino acids, methionine, and cysteine. Chickpea also contains high amounts of carotenoids such as β-carotene, cryptoxanthin, lutein, and zeaxanthin [5]. Chickpea is mainly used for human consumption and only a small proportion is used as feed. The kabuli type (white or cream seed coat) is generally used as whole grains, while desi type (colored seed coat) is used as whole seeds, dehulled splits, or flour. Chickpea is used in preparation of a wide variety of dishes, popular snacks, soups sauces, enriched breads, and baby foods. Green chickpea leaves and twigs are eaten as a cooked vegetable and contain 4–8% protein [6]. Chickpea is also used as protein-rich animal feed and the vegetable biomass is highly valued as fodder in dry areas where grazing vegetation is scarce. Chickpea also plays an important role in improving soil fertility. It meets 80% of its N requirement from symbiotic nitrogen fixation and can fix up to 140 kg N/ha from air [7]. It leaves substantial amount of residual nitrogen behind for subsequent crops and adds much needed organic matter to maintain and improve soil health, long-term fertility, and sustainability of the ecosystems. Chickpea is a hardy crop well adapted to stress environments and a boon to the resource-poor marginal farmers in the tropics and subtropics.

Chickpea is an annual, self-pollinating, diploid ($2n = 2x = 16$) pulse crop with a genome size of 931 Mb [8]. It is the third most important grain legume, which is largely cultivated in Asia, Africa (East and North), and the Mediterranean Europe [9]. In recent years, it is also being cultivated in Australia, Canada, and the United States, largely for export to India. Annual chickpea production is 9.7 million tons (mt), followed by cowpea (5.7 mt), lentil (3.6 mt), and pigeonpea (3.5 mt). The major constraints in chickpea production are biotic stresses such as pod borers, *Helicoverpa armigera*, *Spodoptera exigua*, and *Helicoverpa punctigera* (in Australia), cutworm, *Agrotis* spp., aphid, *Aphis craccivora*, leaf miner, *Liriomyza cicerina*, *Fusarium* wilt, *Ascochyta* blight, Botrytis gray mold, and the abiotic stresses such as drought, heat, cold, and high salinity [10]. The estimated yield losses due to abiotic stresses (6.4 mt) are significantly higher than that due to biotic stresses (4.8 mt) [11]. Among the abiotic stresses of chickpea, drought causes a 40–50% reduction in yield globally [8]. The advanced biotechnological approaches suitable to mitigate the major biotic and abiotic stresses are in the following sections.

15.2
Abiotic Constraints to Chickpea Production

Abiotic stresses such as drought, salinity, and high temperature affect crop growth and productivity. The crop under adverse climatic condition shows morphological, physiological, biochemical, and molecular alterations. Drought is one of the major constraints to chickpea production throughout Asia, as the crop is largely grown under rainfed conditions during the post-rainy season or residual soil moisture, and experiences end-of-season drought (terminal drought). Areas prone to drought stress are expanding quite fast [12]. With the prediction of increasing water scarcity, terminal drought will continue to be the major constraint to chickpea production in several parts of the world. Often high-temperature stress during the reproductive phase occurs along with terminal drought stress, particularly in tropical short season-growing environments and during late sown conditions in most environments. Thus, it is important to combine tolerance to both drought and heat stress.

Legumes, in general, are sensitive to salt [13], and increasing use of irrigation has often converted the arable land into saline [14]. Approximately 22% of the agricultural land is saline [15]. Salinity is also a major limiting constraint to chickpea production in many parts of Asia. Saline soils contain sufficient neutral soluble salts (mainly sodium chloride and sodium sulfate) that adversely affect plant growth and grain yield.

Chilling temperatures during early reproductive growth have been reported to cause yield losses in chickpea in many parts of Asia. The plants continue to produce flowers, but fail to set pods when the mean daily temperature falls below 15 °C. Low temperatures also adversely affect size and viability of pollen and ovules, anther dehiscence, pollen germination and pollen tube growth, and fertilization [16]. In the Mediterranean region, the change from spring to winter sowing of chickpea has enhanced yields, but tolerance to low temperature during flowering requires further improvements [17].

A steady rise in the atmospheric concentrations of carbon dioxide has been observed, from 315 ppm in 1959 to 385 ppm at present [18]. The CO_2 concentration will continue to rise to as much as 500–1000 ppm by the year 2100 [19]. This will have a profound effect on crop growth and development [20] and alter the CO_2 metabolism in the plant. Under elevated atmospheric CO_2 concentrations, the nutritional quality of crops will be reduced due to less accumulation of proteins and nitrogen content in the grain and the leaves [21, 22]. Much of the protein in leaves is involved in assimilating carbon dioxide into sugars [23]. There is some yield advantage in chickpea under elevated CO_2, but a simultaneous reduction in nitrogen concentration may decrease the protein content and negate the reduction in protein [24].

15.3
Modern Crop Breeding Approaches for Abiotic Stress Tolerance

15.3.1
Drought, Salinity, and Low Temperature

Efforts are being made to exploit traits that are expected to play an important role in drought avoidance under receding soil moisture conditions by improving water availability to the plant through more efficient extraction of available soil moisture. Drought tolerance is a complex phenomenon involving many known and unknown pathways.

To improve drought tolerance, quantitative trait loci (QTLs) have been identified for stomatal conductance [25–27], transpiration efficiency [28, 29], osmotic adjustment [30–32], relative water content [30, 25], canopy temperature [31, 30], drought sensitivity index [25], leaf ABA [29], chlorophyll content [33], water use efficiency [34], root traits [35, 36] and some yield-related traits [28, 30, 31, 34, 37–40].

The most promising drought-tolerant line, ICC 4958, has 30% higher root biomass than the popular variety Annigeri [41]. Chandra *et al.* [42] identified molecular markers for a major QTL that accounts for 33% of the variation in root weight as well as in root length. New mapping populations have been developed and are being used to identify molecular markers for additional QTLs. Moreover, understanding physiological mechanisms that regulate drought tolerance, together with the associated regulatory genes, will facilitate crop improvement for water use efficiency and tolerance to drought. The possibility of investigating the response of genes to drought and other stresses by profiling of transcriptome, proteome, and metabolome will offer more information about this complex trait. Besides, studies on functional genomics of chickpea will also help in chickpea improvement.

Earlier studies have indicated limited variability in salinity tolerance in chickpea. However, a recent screening of over 250 germplasm accessions (including 211 accessions of minicore collection) and breeding lines/cultivars revealed wide variation in salinity tolerance [7]. Some accessions gave 10–20% higher yield under salinity stress than the most tolerant variety CSG 8962 released in India. These

results have renewed interest in breeding for enhanced salinity tolerance in chickpea.

Several breeding lines (e.g., ICCV 88502, ICCV 88503, ICCV 88506, ICCV 88510, and ICCV 88516) have been developed, which are able to set pods at lower temperatures (mean daily temperature between 12 °C and 15 °C). A pollen selection method has been developed and successfully applied for transferring cold tolerance from ICCV 88516 to the popular variety amethyst in Australia [43].

15.4
Genetic Engineering of Chickpea for Tolerance to Abiotic Stresses

15.4.1
Drought and Salinity

Abiotic stress is a complex trait that may involve many genes and, therefore, expression of more than one gene is essential to have reasonable tolerance to abiotic stresses. Development of genetically modified (GM) plants by the introduction and/or overexpression of selected gene(s) appear to be quite promising for chickpea improvement. For the development of abiotic stress-resistant transgenic plants, several stress-induced genes with known or unknown enzymatic or structural functions and regulatory proteins have been used. For genetic transformation, stress-induced genes with known functions such as water channel proteins, key enzymes for osmolyte biosynthesis (proline, betaine, sugars such as trehalose, and polyamines), detoxification enzymes, and transport proteins have also been used. Enzymes involved in the metabolic pathways appear more amenable to manipulations than the structural and developmental traits. Stress-induced regulatory proteins that are involved in stress response can be used to enhance tolerance to multiple stresses, including drought, salinity, and freezing [44].

A dehydration responsive element construct, where *DREBIA* gene from *Arabidopsis thaliana* is attached to a drought-responsive promoter (rd 29A) is being used to enhance drought tolerance in chickpea [45]. This construct is known to regulate a number of genes involved in the response to drought and other stresses, such as salinity and cold temperature. In the case of tobacco, wheat, and groundnut, overexpression of *DREB1A* has been shown to improve the drought as well as low-temperature stress tolerance [46–50].

The transgenic tobacco (*Nicotiana tabacum*) developed using *CAP2* gene from chickpea (*C. arietinum*) encoding a novel AP2 family transcription factor showed increase in leaf surface area and number of lateral roots. Transgenic plants were more tolerant to dehydration and salt stress than the wild-type plants, and expressed high steady-state transcript levels of abiotic stress–response genes *NtERD10B* and *NtERD10C* and auxin-response genes *IAA4.2* and *IAA2.5* [51].

Furthermore, introduction of an osmoregulatory gene *P5CSF129A* encoding the mutagenized Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) in chickpea showed accumulation of high proline (two–sixfolds). The transgenic events showed a

decline in transpiration at lower values of the fraction of transpirable soil water (dryer soil), and extracted more water than their untransformed parents. However, the overexpression of *P5CSF129A* gene caused less increase in transpiration efficiency, thereby indicating that the enhanced proline had little bearing on the components of yield architecture that are significant in overcoming the negative effects of drought stress in chickpea [52].

15.4.2 Elevated CO_2 Concentrations

Improving C_3 carbon fixation under temperature and drought conditions can be achieved by manipulation of Rubisco enzyme. The enzyme Rubisco dominates the limitation of C_3 fixation in conditions that restrict the supply of CO_2 such as high temperature or drought. The Rubisco enzyme is higher in plants comprised of chloroplasts encoding eight large subunits (LSU), while the nuclear DNA encoded eight small subunit (SSU) proteins. The large subunit (LSU) of Rubisco contains all the structural information necessary for catalysis, while the function of the small subunit (SSU) remains elusive. Genetic screening and site-directed mutagenesis have focused on the LSU of Rubisco as the catalytic site of the enzyme is on this subunit. Amino acid substitutions in several distinct areas of the Rubisco LSU that influenced the catalytic properties of Rubisco and genetic engineering have resulted in the production of an even less-efficient Rubisco [53–55]. Another alternative approach could be conversion of C_3 pathways to C_4 since C_4 pathway evolved in hot and arid regions in response to increasing O_2 levels as a mechanism to increase the CO_2 concentration at the site of Rubisco [56]. The C_4 system uses the enzyme phosphoenol pyruvate carboxylase (PEPC) to fix CO_2 from the atmosphere into C_4 in the mesophyll cells, which results in regeneration of phosphoenol pyruvate (PEP). Plants with C_4 pathway have a number of advantages, including high photosynthetic performance and high nitrogen and water-use efficiencies (WUE), allowing this group of plants to be highly productive in subtropical regions. Conventional plant breeding approaches have been used to try and transfer C_4 traits into C_3 plants, but these were unsuccessful. In the past decade, genes encoding enzymes in the C_4 pathway have been transferred to C_3 plants resulting in the production of the introduced enzyme [57–59]. However, to achieve this objective, it is necessary to use all the genes isolated from a C_4 species such as maize [60].

15.5 Biotic Constraints in Chickpea Production

15.5.1 Insect Pests

Nearly 60 insect species are known to feed on chickpea. The important insect pests damaging chickpea in different regions are cutworms (black cutworm –*Agrotis*

ipsilon (Hfn.) and turnip moth – *Agrotis segetum* Schiff, termites (*Microtermes obesi* (Holmgr.)), leaf feeding caterpillars (beet armyworm, *S. exigua* (Hub.) and hairy caterpillars *Spilarctia obliqua* Walker), leaf miners (*L. cicerina* (Rondani)), aphids (*A. craccivora* Koch), pod borers (cotton bollworm – *H. armigera* (Hub.), native budworm – *H. punctigera* (Wallengren)), and bruchids (Chinese bruchid – *Callosobruchus chinensis* L., bean bruchid – *Acanthoscelides obtectus* (Say.), pulse weevil – *Callosobruchus analis* F., and pulse bruchid – *Callosobruchus phaseoli* (Gylh.)) [61, 62]. The pod borer *H. armigera* and the aphid *A. craccivora* are the major pests of chickpea in the Indian Subcontinent. In the Mediterranean region, the most important pest is the leaf miner *L. cicerina*. *A. craccivora* is important as a vector of the chickpea stunt disease, while *C. chinensis* is the most dominant species in storage.

A continuous search is being made to identify resistant genotypes. More than 14000 chickpea germplasm accessions have been screened for resistance to *H. armigera* at ICRISAT, India, under field conditions. Several germplasm accessions (ICC 506EB, ICC 10667, ICC 10619, ICC 4935, ICC 10243, ICCV 95992, and ICC 10817) with resistance to *H. armigera* have been identified, and varieties such as ICCV 7, ICCV 10, and ICCL 86103 with moderate levels of resistance have been released for cultivation [63]. Progress has also been made in understanding the nature of gene action, and resistance to pod borer is largely controlled by additive gene action. Good combiners for pod borer resistance have also been identified [64]. However, most of these lines are highly susceptible to *Fusarium* wilt. Therefore, concerted efforts have been made to break the linkage by raising a large population of crosses between the lines with resistance to *H. armigera* and the lines resistant to wilt.

The extent of losses to chickpea in South Asia by this pest is estimated at over US$ 400 million [11]. In the storage condition, bruchids (*C. chinensis*) cause nearly 20–30% damage.

Global warming and climate change resulting in increased temperatures and reduced humidity will impact insect–host plant interactions in several complex ways. The effects of climate change on *H. armigera* have been investigated since the larvae of *H. armigera* have a wide host range [65]. Studies conducted on *H. armigera* under various ambient CO_2 concentrations (550–750 ppm) showed that larvae developed normally under elevated CO_2, and the adult moths lived longer, but laid fewer eggs [66]. However, when the larvae were reared on milky grains of spring wheat for three generations at ambient CO_2 concentration at 750 ppm, they exhibited slow growth in the second and third instars. It has been suggested that under elevated CO_2 concentrations, net damage by the cotton bollworm will be slow due to slow development [67]. Severity of the damage caused by *H. armigera* and the population relationship between *H. armigera* and its parasitoid wasp, *Microplitis mediator*, has also been also studied [68]. The results have suggested that there are no significant changes in wheat consumptions by the larvae or in the parasitism by the wasp under elevated CO_2 at 750 ppm.

Bt cotton being resistant to *H. armigera* was also evaluated under elevated CO_2 along with conventional cotton [69]. The results suggest that damage under elevated CO_2 might be higher, but there will be less pest population. Coll and

Hughes [70] reported that the *H. armigera* reared on pea plants (*Pisum sativum*) grown under elevated CO_2 at 700 ppm were significantly smaller than those reared on plants grown under ambient conditions. Furthermore, they also reported that the omnivorous bug, which feeds on plants but also preys on the bollworm, required prey to complete its development. The bugs performed best when the larvae reared under elevated CO_2, as the larvae were smaller and thus easily overcome by the predator. Elevated CO_2 may benefit generalist predators through increased prey vulnerability, which would put pest species under higher risk of predation. However, none of the above experiments were conducted under increased temperatures, which might level off the adverse effects of elevated CO_2 on *H. armigera* [71].

15.5.2 Diseases

The occurrence of pathogens is related to temperature, rainfall, humidity, radiation, and dew [72]. The movement of pathogens to their host plants depends on several factors, including its mode of dispersal and ability to survive on sources other than its primary host [73]. As dispersal of some pathogens is influenced by rain and winds [23], changes to these factors could also affect the spread of pathogens.

Fusarium wilt, Ascochyta blight, Botrytis gray mold, and root rot are the most important chickpea diseases [74, 75]. Fusarium wilt caused by *Fusarium oxysporum Schl. f. sp. ciceri* is the most important root disease of chickpea. The susceptible varieties can have up to 100% plant mortality. Ascochyta blight caused by *Ascochyta rabiei* is a highly devastating foliar disease of chickpea in northern India, Pakistan, and Central Asia. Botrytis gray mold caused by *Botrytis cinerea* is another important foliar disease of chickpea. It is a serious constraint to chickpea production in northern India, Nepal, Bangladesh, and Pakistan. Collar rot caused by *Sclerotium rolfsii* is becoming a serious problem in several parts of India, particularly central and southern India. Dry root rot caused by *Rhizoctonia bataticola* is a serious disease whenever the crop is exposed to moisture stress and temperature above 30 °C. Resistance to Fusarium wilt is necessary for all chickpea-growing areas, and all improved varieties have Fusarium wilt resistance. The foliar diseases, Ascochyta blight and Botrytis gray mold, continue to be a big threat to chickpea production in cooler and humid areas.

Muehlbauer and Kaiser [75] reported that the resistance to Ascochyta blight is multigenic. The pathogen evolves continuously, which makes it difficult to develop lines with stable resistance to this pathogen. The wild relatives of chickpea such as *Cicer ehinospermum, Cicer pinnatifidum, Cicer bijugum,* and *Cicer judacium* possess high levels of resistance to Ascochyta blight [76, 77].

The wilt caused by the soil borne fungi *F. oxysporum f. sp. ciceri* is an economically important disease of chickpea. Haware and Nene [77] identified seven distinct races of *Fusarium* in India. Of these races, race 1 is common in central India, and race 2 in northern India. However, race 3 and race 4 appear in various pockets of Punjab and Haryana. Race, 0, 5, and 6 were identified in Spain by Jimenez-Daiz *et al.* [78].

15.5.3
Biological Nitrogen Fixation

Biological nitrogen fixation offers an alternative means to increase plant-available nitrogen [7]. Nearly 20% of all N available to the crops is due to rhizobial N fixation [79]. Herridge *et al*. [80] estimated that 50% nitrogen fixed by a chickpea crop remains underground and is available to the following crop. However, symbiotic fixation of nitrogen is sensitive to even modest soil water deficits [81]. In the case of chickpea, the high nodulating selection ICC 4948 fixed more N and yielded 31% more than its low nodulating version [82]. Sufficient numbers of compatible rhizobia are often not naturally occurring in most of the soils where grain legumes are cultivated [83], and there is need for rhizobia application to seeds [84].

15.6
Modern Molecular Breeding Approaches for Biotic Stress Tolerance

15.6.1
Pod Borers

In the field, the pod borer *H. armigera* is a major threat to chickpea cultivation. Yoshida *et al*. [85] investigated the mechanisms of resistance to pod borers and found that oxalic acid and malic acid are the major components that govern resistance to *H. armigera*. Genotypes resistant to pod borer accumulated more oxalic acid on the leaves than the susceptible genotypes. Oxalic acid showed significant growth inhibition of the pod borer larvae when included in a semiartificial diet, while malic acid had no effect on larval growth.

Development of crop cultivars with resistance to pod borer is the most cost-effective and eco-friendly option for the control of *H. armigera*, particularly under subsistence farming conditions in the developing countries [86]. Availability of stable sources of resistance is a prerequisite to develop cultivars for resistance to insect pests. Screening of more than 14 000 germplasm accessions and breeding line has resulted in the identification of several genotypes with low to moderate levels of resistance to *H. armigera* for use in breeding programs. Some of these have also been found to be resistant in different agroclimatic zones under natural infestation. Germplasm accessions of wild relatives of chickpea (*C. bijugum*, *C. judaicum*, and *C. pinnatifidum*) have shown high levels of resistance to pod borer [87].

The chickpea cultivars and wild *Cicer* species have been found to differ significantly in their ability to inhibit *H. armigera* gut proteinases [88]. But none of the species offered complete protection against the pod borer by inhibiting the gut proteinases. The wild relatives of chickpea *C. bijugum* exhibited highest larval inhibition (36%), followed by *C. echinospermum* and *C. arietinum* (cv Vijay) (33%).

Stored chickpeas are highly susceptible to attack by the bruchids *Callosobruchus maculatus* and *C. chinensis*. Germplasm with some degree of resistance to bruchids

has been identified, but it appears to be correlated with undesirable physical characteristics of the seed coat. Bruchids-resistant chickpeas are usually consist of thick, dark color seed coat with altered chemical composition, but are less desirable for human consumption.

The preliminary linkage map based on interspecific crosses of *C. arietinum* × *Cicer reticulatum* and *C. arietinum* × *Cicer echinospermum* was made available by Gaur and Slinkard [89]. The mapping population derived from a cross between a wilt-resistant kabuli variety (ICCV 2) and a wilt-susceptible desi variety (JG 62) has been used to develop the first molecular map of chickpea based on an intraspecific cross [90]. A beginning has been made to identify molecular markers for resistance to *Helicoverpa* in chickpea. Mapping genes associated with resistance to *H. armigera* has been reported by Lawlor *et al.* [91]. High levels of resistance to *H. armigera* have been identified in wild relatives of chickpea (*C. bijugum, C. judaicum, and C. reticulatum*) [63], of which *C. reticulatum* can be easily crossed with the cultivated species to develop mapping populations to identify QTL associated with resistance to *H. armigera*.

A mapping population of 126 F_{13} RILs of ICCV 2 × JG 62 has been evaluated for resistance to *H. armigera*. The overall resistance score ($1 = <10$ leaf area and/or pods damaged, and $9 = >80\%$ leaf area and/or pods damaged) varied from 1.7 to 6.0 in the RIL population compared to 1.7 in the resistant check, ICC 506EB, and 5.0 in the susceptible check, ICCV 96029. The results indicated that there is considerable variation in this mapping population for susceptibility to *H. armigera*. Another RIL mapping population from the cross between Vijay (susceptible) × ICC 506EB (resistant) has also been evaluated for resistance to *H. armigera*. Interspecific mapping populations based on the crosses between ICC 3137 (*C. arietinum*) × IG 72933 (*C. reticulatum*) and ICC 3137 × IG 72953 (*C. reticulatum*) have also been developed, and putative QTLs linked to various components of resistance to *H. armigera* have been identified [92].

Based on interspecific genetic linkage map of chickpea (ICC 4958 × PI 489777) and phenotyping for resistance to *H. armigera* and *S. exigua* under field and greenhouse conditions, QTLs associated with resistance to pod borers have been identified [93] and can be used in conjunction with biochemical markers to develop cultivars with resistance to this pest. In addition, oxalic and malic acids and protease inhibitors have been identified as biochemical markers for resistance to *H. armigera* in cultivated chickpea and are being used to identify lines with resistance to this insect [94].

15.6.2
Ascochyta and Fusarium

Ascochyta blight appears to be controlled by several genes [95]. It has also been reported that there are two major complementary recessive genes for resistance to Ascochyta blight [96, 97]. Tekeoglu *et al.* [97] identified two major QTLs and one minor QTL from the interspecific crosses between *C. arietinum* and *C. reticulum*. Morjane *et al.* [98] performed genetic characterization of various isolates of *Ascochyta* of single field using DNA fingerprinting method and 12 haplotypes were

observed with varying frequencies. Santra *et al*. [99] developed a RAPD marker specific to Indian isolate. Coram and Pang [100] studied the molecular basis of the Ascochyta blight resistance in a highly resistant chickpea accession (ICC3996) and a susceptible cultivar (Lasseter) using microarrays. After inoculation with *A. rabiei*, a time-series expression patterns of 20 defense-related ESTs were studied and found upregulation or downregulation of 10 defense-related ESTs in ICC 3996 and/or Lasseter compared to the uninoculated control. Hierarchical clustering grouped the ESTs into different clusters. Three defense-related ESTs showed differential upregulation in ICC 3996 compared to Lasseter – a leucine zipper protein, SNAKIN2 antimicrobial peptide precursor, and elicitor-induced receptor protein.

Warkentin *et al*. [101] constructed a linkage map for resistance to Ascochyta blight and identified one QTL on each of LG3, LG4, and LG6, which accounted for 13%, 29%, and 12% of the total variation, respectively. Of these, three QTLs on LG4 and LG6 were in common with the previously reported QTL for Ascochyta blight resistance, whereas the QTL on LG3 was unique to this population.

The chickpea wilt caused by *F. oxysporum f. sp. ciceris* is one of the major factors limiting production of this pulse crop. The affected plants exhibit drooping crown, xylem, and stem discoloration and root rotting. Development of resistant varieties is thought to be the most viable strategy to overcome this problem. Muehlbauer and Kaiser [75] reported that resistance to different races of Fusarium is controlled by a single gene. Evaluation of both *desi*- and *kabuli*-type chickpea accessions revealed that almost 160 accessions were resistant to the fungus [102]. The wild accessions of *C. bijugum*, *C. judaicum*, *C. reticulatum*, and *C. ehinosperum* were resistant, while accessions belonging to *C. yamashitae* were susceptible. Breeding varieties resistant to Fusarium wilt were quite successful. However, some of the cultivars do not show resistance to all the races of the Fusarium wilt [103]. Mayer *et al*. [104] identified RAPD markers, UBC-170 and CS-27, located on same side of the locus, which were linked to resistance and susceptibility, respectively. Locus specificity of the primer UBC-170 was confirmed by allele-specific associated primer s (ASAPs). ISSR marker (UBC-855) linked to the gene conferring resistance to race 4 of Fusarium has been identified and appears to cosegregate with CS-27 [105]. Later, ISSR makers UBC 825 comprising dinucleotide repeats ($[AC]_8T$) was identified, which was 5.0 cM to the wilt resistant *Foc-4* gene [106].

15.6.3
Wide Hybridization

Wild relatives of chickpea are an important source of resistance to leaf miner *L. cicerina* and the bruchid *C. chinensis* [107]. Accessions belonging to *C. bijugum* (ICC 17206, IG 70002, IG 70003, IG 70006, 70012, IG 70016, and IG 70016), *C. judaicum* (IG 69980, IG 70032, and IG 70033), *C. pinnatifidum* (IG 69948), and *C. reticulatum* (IG 70020, IG 72940, IG 72948, IG 72949, and IG 72964) [108] have shown high levels of resistance to *H. armigera*. Some of the wild relatives of chickpea have different mechanisms than those in the cultivated types, which can be used in crop improvement to diversify the bases of resistance to this pest. High

levels of antibiosis were evident when *H. armigera* larvae were fed on leaves and pods, and the mechanisms are different from those in *C. arietinum* [109].

15.7 Application of Gene Technology

15.7.1 Pod Borers

Bacteria *Bacillus thuringiensis* consists of genes that encode several insecticidal proteins during sporulation (Cry or Cyt) and vegetative growth (Vips) proteins. Crickmore *et al*. [110] described more than 140 genes that produce Cry proteins, with specificities for Lepidoptera, Coleoptera, and Diptera. The Vips also possess toxic effects toward insects [111]. Vip3 is highly toxic to *Agrotis* and *Spodoptera* [105] and *H. armigera* [113].

Globally, insect-resistant crops have been one of the successful applications of plant genetic engineering technology. The first successful genetic transformation of chickpeas was reported in 1997 using the *cry1Ac* gene [114]. Later, transgenic chickpea expressing *Cry1Ac* [115–117] and *Cry2Aa* [118] genes were also generated. Recently, chickpea lines expressing pyramided *Bt* genes, *cry1Ac* and *cry1Ab* [117], have also been developed; however, the previous reports have suggested that *Cry1Ac* is more effective against *H. armigera*, and pyramiding two or more genes with different mode of action is preferred for effective pest management.

Another strategy, known as plant-delivered RNAi or in-plant RNAi, appears to be useful for the control of various insect pests, including the lepidopterans. A cytochrome P450 gene (*CYP6AE14*), which expresses in the midgut of *H armigera*, is proven to be a suitable candidate gene to control this pest. The *H. armigera* larvae fed on transgenic tobacco (*N. tabacum*) and *A. thaliana* plants expressing *dsRNA* of this gene have shown downregulation of cytochrome P450 gene and a significant larval growth retardation [119]. However, silencing of lepidopteran genes by RNAi has been found to be difficult. There may be several factors responsible for lower efficacy of RNAi in lepidopterans such as absence of RdRP orthologues in most insects [120], barriers to uptake of dsRNA, improper sorting of dsRNA during endosome trafficking to dsRNA-processing machinery, and so on [121]. Till date, the fate of the injected or ingested dsRNA in lepidopteran has not been understood. Recently, a tobacco rattle virus vector was found to be efficient in silencing a lepidopteran (*Manduca sexta*) gene *CYP6AE14* [121].

15.8 Conclusion

Chickpea cultivars with resistance to abiotic and biotic factors will form the backbone of chickpea production in future. The development and deployment of

chickpea plants with resistance to insects would offer the advantage of allowing some degree of selection for specificity effects, so that pests, but not the beneficial organisms, are targeted. Deployment of insect-resistant chickpeas will result in decreased use of chemical pesticides and increased activity of natural enemies and thus, higher yields. For pest management programs to be effective in future, there is a need for the following:

- Utilization of wild relatives of chickpea to diversify the genetic basis and thus, increase the levels of resistance to the target insect pests.
- Identification of quantitative trait loci associated with resistance to abiotic and biotic stress factors.
- Development of insect-resistant varieties through genetic transformation using genes with diverse modes of action.
- Combining resistance to insects with resistance to important diseases, drought, and cold tolerance.
- Focusing attention on crop management and insecticide resistance management.

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16
Grain Legumes: Biotechnological Interventions in Crop Improvement for Adverse Environments

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Abstract

Grain legumes are the major food crops grown and consumed mostly by the poor farmers as an important source of protein in the drylands of the world. The ensuing climate change has posed serious potential threats to the cultivation of these crops that are important for the sustainable livelihoods of the poorest of the poor in these regions. There is evidence to suggest that the ecological dynamics and equilibriums are likely to be affected as a result of changing climate, either by making these crops susceptible to new diseases or by increasing the intensities of diseases, pests, and parasites. Despite many uncertainties, there is a growing consensus that these adversities could lead to an overall increase in the disease and pest pressure besides harsher abiotic stresses. Since most of the grain legumes have a narrow genetic base and levels of resistance to some biotic and abiotic constraints are low, making crop improvement an overarching research-for-development challenge for maximizing the benefits that grain legumes offer to smallholder farmers. Running against the headwinds, grain legume research has been immensely benefited by applications of modern biotechnological tools and approaches that have the potential to develop solutions for destructive diseases, besides making headway against the complex problems of drought. Similarly, identifying novel genes/-traits and assessing their suitability as candidate genes for genetic engineering options will be important for future breeding programs in order to achieve remarkable impacts in these grain legume crops globally. This chapter mainly provides a comprehensive picture of the different biotechnological interventions adopted for addressing various constraints in gain legume productivity and improvement, highlighting the pitfalls and possible solutions that can be taken through an integrated approach to combat the altered environmental conditions.

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16.1 Introduction

With the advent of twenty-first century, agriculture will not only be forced to compete for land and water with sprawling urban settlements but will also be required to serve on other major fronts: adapting and contributing to the mitigation of climate change, the most pronounced adverse condition on the doorstep. Climate change will affect the four dimensions of food security: availability, accessibility, utilization, and stability [1]. According to the estimation of a FAOs discussion paper, by 2050, developing countries may experience a decline of between 9% and 21% in overall potential agricultural productivity as a result of global warming [1]. Alarmingly, the poorest regions will be exposed to the highest degree of instability of food production due to substantial decline in agricultural productivity, including labor productivity, leading to increases in poverty and mortality rates.

Food and agriculture sectors will be significantly impacted by adverse climatic features that are likely to include increased occurrence of extreme heat (temperature and duration), short-term fluctuations, seasonal oscillations, sudden discontinuities, and long-term variations [2]. Despite many uncertainties and unknowns, there is a growing consensus that ecological dynamics and equilibriums are likely to be affected and there will be an overall increase in the abundance and diversity of invertebrate pests—and pest pressure—as habitats become more favorable for their establishment and development and new niches appear [1]. Furthermore, studies suggest that hosts and pathogens may be brought together in new locations and contexts, bringing new threats to crops, livestock, and aquaculture systems and new challenges, with the accompanying need for significant human and financial investments to address the challenges.

The strongest negative impact of these adversities on agriculture is expected in sub-Saharan Africa [1]. It has been estimated that climate change may reduce African potential agricultural output up to the 2080–2100 period by between 15 and 30% [1]. This denotes that SAT areas are likely to be in most vulnerable condition, the poorest and most food insecure region is also expected to suffer the largest contraction of agricultural incomes resulting in an increased dependence on food imports.

16.2 Grain Legumes: A Brief Introduction

Grain legumes or pulses are a widely adapted group of crop plants and occupy an important place in the world food and nutrition economy. The present world production of grain legumes is estimated to be 50 million metric tons and, at an average price of $400 per metric ton, its total value would amount to $2 billion [1]. They are important constituents in the diets of a very large number of people, especially in the developing countries, and are good sources of protein that help to supplement cereal diets, improving their protein nutritive value [3]. Legumes can interact

symbiotically with specific soil-borne bacteria, the rhizobia, which allow the plant to fix atmospheric nitrogen and may help to protect them against some fungal pathogens [4]. Although most legumes are consumed as dry grains, immature green pods or green seeds are also used as vegetables providing substantial quantities of minerals and vitamins to the diet. Although many species and subspecies of legumes are known, only about a dozen of them are important as commercial food crops. Beans and peas each account for about 25% of the total production of legume crops. Chickpea and broad beans rank next in importance. Some of the legumes, however, are of only regional or local importance.

Field pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), and grasspea (*Lathyrus sativus* L.) are collectively known as the cool season food legumes. These groups of legume crop plants grow vegetatively during the cool season and flower and produce seeds as day lengths become progressively longer. Carbonized remains indicate that peas, lentils, and chickpeas were domesticated in the Near East arc and were cultivated with the cereals as early as the seventh millennium BC [5]. From the presumed center of origin, peas spread to the cool-temperate areas of Central and Northern Europe and from there were introduced into the Western Hemisphere soon after Columbus [6, 7].

The major grain legumes such as soybean, peanut, pigeonpea, common bean, and cowpea are warm season legumes better adapted to the humid regions. The warm season legumes are characterized by epigeal germination, a period of rapid vegetative growth, followed by flowering when day lengths become progressively shorter during the growing season. In contrast, the cool season pulses have hypogeal germination, a period of rapid vegetative growth, followed by flowering when day length becomes progressively longer.

16.3 Major Constraints for Grain Legume Production

The adversities in growing conditions of the grain legumes pose threat and affect the adaptability and productivity that reportedly has shown an increased severity depending on the type and the specific crop location. Furthermore, crops under abiotic stress are usually more susceptible to weeds, insects, and diseases, which considerably increase the losses [8].

16.3.1 Biotic Stresses

The major biotic stresses affecting legumes include viruses, fungal, and bacterial diseases, insect pests, nematodes, and parasitic weeds that drastically decrease the grain legume production. Considerable progress has been made toward the successful management of important diseases of most legume crops through the search for host resistance. Though sources of resistance to many important diseases have been found and are being used to breed agronomically acceptable

cultivars with good levels of resistance, pathogens are highly variable, making breeding for resistance to be a long-term objective. Hence, combined disease resistance is required in most legume production systems. This has proved relatively easy to attain in some cases, but rather difficult in other cases.

16.3.1.1 Fungal Diseases

The relative importance of aerial fungal diseases and their effect on yield varies among years and cropping regions. However, some of them affect large areas in all the countries where legumes are cultivated and cause considerable losses in quality and quantity. Foliar diseases caused by biotrophic pathogens, such as rusts, downy mildews, and powdery mildews, are major limiting factors in legume production and the most important of these are present in all areas where legumes are cultivated [9]. Several rust species can infect grain and forage legumes, most of them belonging to the genus *Uromyces*, such as *Uromyces appendiculatus* on common bean, *Uromyces ciceris-arietini* on chickpea, *Uromyces pisi* on pea, *Uromyces striatus* on alfalfa, *Uromyces viciae-fabae* on faba bean, lentil, and common vetch, and *Uromyces vignae* on cowpea. Also, rust species belonging to other genera can be major problems on legumes such as *Phakopsora pachyrhizi* and *Phakopsora meibomiae* on soybean or *Puccinia arachidis* on groundnut [10]. Asian rust (*P. pachyrhizi*) is a severe disease that causes important yield losses in soybean and is spreading rapidly around the world [11–13]. Lack of natural sources of resistance makes this disease a good candidate to be solved using biotechnological tools. Normally, legume rust epidemics begin late in the season, when pod filling starts, so yield components are only slightly affected by the infection and losses are usually low. However, when the infection starts early in the season, severe epidemics can occur [14]. Similarly, powdery mildew caused by *Erysiphe pisi* is an important fungal disease in several legumes and has a worldwide distribution being particularly important in climates with warm, dry days and cool nights, adversely affecting yield and quality [9]. Severe infection may cause 25–50% yield losses. In contrast, downy mildew, caused by *Peronospora viciae* occurs in most places where the crops are grown, but is most frequent and severe in cool, maritime climates [9].

The major necrotrophic fungal disease of various grain legumes is Ascochyta blight, caused by *Ascochyta rabiei*, reportedly the most important fungi affecting pea and chickpea [15, 16]. Similarly, Botrytis gray mold caused by *Botrytis cinerea* is of lesser importance, but is also a widespread foliar disease problem in grain legumes. Besides these, there are several soil-borne diseases that are common among legume crops [17]. Most of these attack the seedling stage of the crop and are referred to as damping-off diseases, several examples include damping-off, generally caused by either *Rhizoctonia solani* or *Pythium* spp., which can result in up to 80% of plant death [18, 19] and Fusarium root-rot (caused by *Fusarium* spp.) causing severe seedling losses especially in common bean and lentils [20, 21]. In most growing areas of the world, Fusarium wilt (FW) (caused by *Fusarium oxysporum*) is a major constraint, affecting seedlings and adult plants where it causes leaf chlorosis, wilting, and death in chickpea [16, 22] and lentil in particular. Other important

soil-borne diseases such as southern stem rot (*Sclerotium rolfsii*) and the white mold (*Sclerotinia sclerotiorum*) cause both seedling and pod rots in warmer and cool weather, respectively [23].

16.3.1.2 **Viral Diseases**

During the last two decades, viruses have emerged as devastating pathogens, particularly in the tropics and subtropics, causing huge economic losses and threatening crop production to grain legumes. Viruses cause yield losses for most legume crops, for example, bean common mosaic virus (BCMV) and its close relative bean common mosaic necrotic virus (BCMNV) are the most widespread and frequent viruses of common bean leading to significant losses. Over the past two decades, bean golden mosaic virus (BGMV) has been considered the most important yield limiting disease for bean production in parts of Central America and the lowlands of the Caribbean, with yield losses between 10% and 100%. Similarly, the peanut stem necrosis disease epidemic that resulted in the death of young groundnut (*Arachis hypogaea* L.) plants occurred in the rainy season of the year 2000 in Anantapur district, Andhra Pradesh, India, where the crops were usually grown on 0.7 million ha. The disease affected nearly 225 000 ha and the crop losses were estimated to exceed Rs. 3 billion (US$65 million). Similarly, sterility mosaic disease (SMD) is the most damaging disease of pigeonpea in the Indian subcontinent, Bangladesh, Nepal, Thailand, Myanmar, Sri Lanka, and China with annual losses of over US$ 300 million. These epidemics caused by re-emerging and newly emerging viruses are becoming frequent even in regions that were earlier free from these viruses. The major contributory factors for the emergence and spread of new virus diseases are the evolution of variants of the viruses and the increase in the vector population. For example, genomic recombination in gemini viruses, not only between the variants of the same virus but also between species and even between genera, has resulted in rapid diversification.

16.3.1.3 **Insect Pests**

Insects are another important biotic stress faced by many legume crops. They cause important damages through both by direct feeding as vectors and by providing infection sites for pathogens. Examples of important insect pests in grain legumes include aphids like *Aphis glycine*, pod borers such as *Helicoverpa armigera* and *H. punctigera* in cool season legumes [24], and weevils such as *Apion godmani* and *Zabrodes subfasciatus* in warm season legumes [25, 26].

16.3.1.4 **Parasitic Weeds**

A number of parasitic plants have become weeds, posing severe constraints to major crops including grain legumes [27]. One such example is of *Orobanche foetida*, which is widely distributed in natural habitats in the Western Mediterranean area parasitizing wild herbaceous leguminous plants, also considered an important agricultural parasite in the faba bean in Beja region of Tunisia. *Orobanche minor* is of economic importance on clover that is grown for seed and has recently become a problem on red clover in Oregon [10, 28]. Similarly, *Striga gesnerioides* and *Alectra*

vogelii cause considerable yield reduction of grain legume crops, particularly cowpea, throughout semiarid areas of sub-Saharan Africa [29].

16.3.2 Abiotic Stresses: A Threat to Grain Legumes

Abiotic stress broadly include multiple stresses such as heat, chilling, excessive light, drought, water logging, wounding, ozone exposure, UV-B irradiation, osmotic shock, and salinity. Some of these stresses like drought, extreme temperature, and high salinity dramatically limit crop productivity. Drought stress is particularly important in context of the grain legumes, such as peanut (*A. hypogaea*), Brazil nuts (*Bertholletia excelsa*), and faba bean (*V. faba*). Moreover, drought–*Aspergillus* interaction results in occurrence of preharvest aflatoxin contamination in these crops [30–32]. Likewise, waterlogging due to a combination of unfavorable weather conditions and suboptimal soil and irrigation techniques can result in severe yield losses in grain legumes [33, 34]. Several of the abiotic stresses associated with legume crops can also directly affect symbiotic interactions and therefore limit legume growth [35].

Drought problems for legumes are likely to worsen with the projected rapid expansion of water-stressed areas of the world from 28 to 30 countries today to 50 countries encompassing 3 billion people by 2030 [36]. Thus, there is a crucial need to increase drought tolerance in legumes; however, increasing salinity tolerance is a parallel requirement in many areas. The more drought-tolerant legumes, such as cowpea, are deeply rooted and the reduced leaf size with thickened cuticles might be responsible for the reduced water loss under stress.

16.3.2.1 Heat Stress

Heat stress due to increased temperature is an agricultural problem in many areas in the world. Transitory or constantly high temperatures cause an array of morphoanatomical, physiological, and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. The adverse effects of heat stress can be mitigated by developing crop plants with improved thermotolerance using various genetic approaches.

16.3.2.2 Salinity

Soil salinity is one of the main abiotic stresses that plants encounter more frequently. It is expected that by 2050 more than 50% of all arable lands will be saline [37]. Soil salinity affects total nitrogen uptake and soil nitrogen contribution [38], leading to reduced yield besides limiting a constraint to their symbiotic functioning [39]. In addition, inhibition of rhizobial growth has been recognized at NaCl levels that were toxic, but not lethal to the host plants, strains visibly possessing variations in their sensitivity to salinity [39]. Nevertheless, the progress in the direct screening for grain yield under saline conditions has been hampered by the low heritability, polygenic control, epistasis, significant genotype $\times$ environment (G $\times$ E) interaction, and quantitative trait loci (QTIs) $\times$ environment (QTL $\times$ E) interaction [40].

16.4
Biotechnological Interventions in Grain Legume Improvement

Breeding for resistance or traditional breeding program has served for a long time as the only solution to the problem regarding the adverse environmental condition. However, lack of knowledge regarding genetic variation and in-depth study on molecular mechanism behind stress tolerance largely affected the progress of the crop improvement programs. Recent advancements on different biotechnological approaches opened a new era of stress biology and widened the scenario of traditional breeding programs using molecular breeding approaches. Pure line breeding, population breeding, mutation breeding, and wide hybridization have been used for development of new varieties of legume crops and have led to incremental improvements in the yield potential of these crops.

Most of the grain legumes have a narrow genetic base [41], and levels of resistance to some biotic and abiotic constraints are low. Since the overarching research-for-development challenge in grain legumes is to apply crop improvement to maximize the benefits that grain legumes offer to smallholder farmers, running against the headwinds grain legume researchers have nonetheless achieved remarkable impacts globally by increasing yields and developing solutions for destructive diseases besides making headway against the complex problems of drought. Applications of the developed genomic tools and modern breeding approaches potentially will shorten the breeding cycles and eventually lead to development of superior cultivars. Similarly, identifying novel genes/traits and assessing their suitability as candidate genes for genetic engineering options will be important for future grain legume breeding programs.

Keeping in view the adversities faced by the grain legumes, this chapter deals with the novel and efficient modern breeding methods/biotechnological tools for accelerating the grain legume improvement programs. Various biotechnological approaches for crop improvement of the major grain legumes such as groundnut, chickpea, pigeonpea, soybean, common bean, cowpea, and lentil are being discussed in detail in the following sections.

16.4.1
Groundnut

Groundnut (*A. hypogaea*) (also known as peanut, earthnut, and monkey nut or goobers) is an important oilseed cash crop containing 36–54% oil, 16–36% protein, and 10–20% carbohydrates, cultivated in over 100 tropical and subtropical countries of the world [1]. About 70% of the world's peanut is produced in the SAT and India has the largest peanut growing area with 6.7 million ha (27.3%) and stands second in the production at 6.5 million tons (18.2%). Since 80% of the crop is grown under rainfed conditions by resource-poor farmers, the rainfall pattern during the presowing months and the availability of substitute high-value oilseed crops like soybean and sunflower of short durations requiring less water have had a significant negative impact on acreage allocation decisions of the farmers. Moreover, a

big gap exists between the realized yield and potential yield of peanut at both subsistence and commercial systems of production in Asia and Africa due to both abiotic and biotic factors. The major abiotic factors affecting peanut production include drought, high temperature, low soil fertility, low soil pH, and iron chlorosis. Among the biotic factors, fungal diseases, virus diseases, bacterial wilt disease, aflatoxin contamination, nematodes, foliar insect pests, and soil insect pests are important [42].

Groundnut crop improvement by conventional breeding has been struggling to meet the demands of increasing population, especially in seed quality improvement and developing virus- and insect-resistant varieties. Several advanced research institutes or groups are working to apply modern biotechnology for groundnut improvement in developing countries. These include marker-assisted selection (MAS), tissue culture, embryo rescue, and genetic modification techniques.

16.4.1.1 Biotechnology for Tolerance to Abiotic Stresses

Drought is the major cause for low and erratic pod yield in peanut that contributes to over 6.7 million tons loss in annual world peanut production [43], resulting in estimated monetary losses of over US$ 520 million annually [44]. Yield losses in peanut due to water deficits vary depending on timing, intensity, and duration of the deficit, coupled with other location-specific environmental stress factors such as high irradiance and temperature [45]. Due to the scarcity of available water in SAT regions, drought management strategies, whether agronomic or genetic, therefore, need to focus on maximizing extraction of available soil moisture and the efficiency of its use in crop establishment: growth, biomass, and grain yield [46].

The first genetic map for cultivated groundnut (*A. hypogaea*), an amphidiploid (4X) species was developed at ICRISAT that demonstrated its utility in molecular mapping of QTLs controlling drought tolerance-related traits as well as in establishing relationships with diploid AA genome of groundnut and model legume genome species [47, 124].

In order to develop a genetic linkage map for tetraploid cultivated groundnut, a total of 1145 microsatellite or simple sequence repeat (SSR) markers available in public domain as well as unpublished markers from several sources were screened on two genotypes, TAG 24 and ICGV 86031 that are parents of a recombinant inbred line mapping population. As a result, 144 (12.6%) polymorphic markers were identified that amplified 150 loci. A total of 135 SSR loci could be mapped into 22 linkage groups [47, 124]. More recently, nearly 700 genes were identified in subtractive cDNA library from gradual process of drought stress adaptation in groundnut [48]. A high-density oligonucleotide microarray for groundnut has also been developed using 49 205 publicly available ESTs and tested the utility of this array for expression profiling in a variety of groundnut tissues [49].

Recently, a straightforward laboratory protocol used acquired thermotolerance (ATT) in groundnut seedlings as a measure of one mechanism of heat stress tolerance. Sixteen genotypes were evaluated for acquired themotolerance in two independent experiments. A change in the temperature sensitivity of chlorophyll

accumulation was used as an indicator of acquired thermotolerance [50]. Interestingly, another study indicated that lipoxygenase and 1L-myo-inositol-1-phosphate synthase, which aid in inter- and intracellular stress signaling, were more abundant in tolerant genotypes of groundnut under water deficit stress. Here, the acetyl-CoA carboxylase, a key enzyme of lipid biosynthesis, increased in relative abundance along with a corresponding increase in epicuticular wax content in the tolerant genotypes suggesting an additional mechanism for water conservation and stress tolerance. In addition, there was a marked decrease in the abundance of several photosynthetic proteins in the tolerant genotypes along with a concomitant decrease in net photosynthesis in response to water deficit stress [51].

Drought is a very complex trait, involving the concerted action of many genes and gene families, a feature that renders engineering drought tolerance very challenging. There have been very few efforts on developing groundnut transgenics for abiotic stresses. Transgenic groundnut plants transformed with AtNHX1 gene were reported to be more resistant to high concentration of salt and water deprivation than their wild-type counterparts. Salt and proline level in the leaves of the transgenic plants were also much higher than that in the wild-type plants [52]. At ICRISAT, groundnut was transformed using a single regulatory gene (DREB1A transcription factor), which in turn regulates the expression of downstream genes leading to the activation of many functional genes [53, 54]. Preliminary results of these transgenics showed that several events acquired the capacity to extract more water from the soil profile or had altered leaf conductance. O the 50 independent transgenic events thus produced, 6 transgenic events have single transgene insert and variable transpiration efficiencies (TE) and desirable root traits were selected for further evaluation of pod yield under both drought and fully irrigated conditions, in a series of greenhouse and field environments. These trials were also the basis of a comprehensive study of the component traits leading to drought adaptation. The transgenic peanuts were evaluated based on their effectiveness in (i) capturing the water; (ii) effective usage of captured water for producing biomass via photosynthesis; and (iii) converting assimilate into a harvestable form based on the equation $Y = T^{*}TE^{*}HI$. Nevertheless, outputs of these study have the potential to draw us closer to a transgenic solution to this global problem by reconciling both molecular and agronomic approaches toward a common focus of groundnut breeding for drought tolerance.

16.4.1.2 **Biotechnology for Resistance to Biotic Stresses**

The major biotic stresses for groundnut include the foliar fungal diseases, leaf spot (early and late), and rusts. Besides, seed and soil-borne diseases like collar rot, stem rot, and dry root rot have also been identified as important in groundnut. Among viral diseases, bud necrosis (BND), stem necrosis (PSND), peanut mottle (PMV), and peanut clump (PCV) are considered to be of economic importance in groundnut. With regard to the insect pests, a wide range of pests like leaf miner, tobacco caterpillar, white grub, jassids, thrips, aphids, red hairy caterpillar, and termite are known to cause serious damage to groundnut crop [55].

A considerable number of SSR sequences for resistance to various diseases have been identified from peanut genome by several research groups [56–65] The SSR markers have been identified and characterized for association with resistance traits such as rust and late leaf spot resistance [66, 67] and resistance to *Ralstonia solanacearum* [68] and *Sclerotinia minor* [69]. Genetic linkage maps with SSR markers have been constructed for diploid AA genome [59], BB genome [70], tetraploid AABB genome derived from a cross of cultivated with amphidiploids [71], and tetraploid AABB genome in the cultivated peanut [72].

For virus resistance, the most common gene, which has been targeted at early stages of virus multiplication, is the coat protein gene of the target virus. The first ever-transgenic groundnuts, resistant to the peanut clump virus (IPCV) wide spread in West Africa and pockets of Asia, have been developed [73]. Besides, exploitation of pathogen-derived resistance (PDR) to groundnut rosette disease (GRD) by using GRAV-*cp* gene to induce host plant resistance to GRD has been carried out at ICRISAT and is undergoing testing in Africa. Transgenic plants of groundnut varieties, Gajah and NC 7, have been developed using one of the two forms of PStV coat protein (*cp*) gene that exhibited high levels of resistance to peanut stripe potyvirus (PStV), wide spread in East and Southeast Asia [74].

Similarly, transgenic options to combat peanut bud necrosis disease (PBND) and PSND include development of transgenic groundnut plants expressing either the nuleocapsid gene or the coat protein gene in groundnut. Biotechnological interventions to address the problem of spotted wilt of groundnut caused by tomato spotted wilt virus (TSWV) have been underway by various groups. Milla *et al*. [75] used AFLP markers to establish marker trait association for TSWV resistance in groundnut. These technologies are in different stages of development and have a potential to be incorporated in peanut breeding programs for improvement following their regulatory approvals. Since protection of transgenic plants against many of these viruses is under both RNA- and protein-mediated control [76], efforts on harnessing antisense and RNAi technology for resistance to these viruses are being pursued by various groups [77, 78].

Besides viruses, fungal diseases in groundnut are the most significant limiting factor causing more than 50% yield losses throughout the world. Early leaf spot caused by *Cercospora arachidicola* S. Hori (*Mycosphaerella arachidis* Deighton), late leaf spot caused by *Phaeoisariopsis personata* Berk. & M.A. Curtis (*Mycosphaerella berkeleyi*), rust (*P. arachidis*), crown rot (*Aspergillus niger* Teigh.), collar rot caused by *Aspergillus* spp., root rot caused by *Macrophomina phaseolina*, stem rot caused by *S. rolfsii*, and yellow mold (*Aspergillus flavus* and *Aspergillus parasiticus*) causing aflatoxin contamination are the major fungal diseases affecting groundnut crop. Aflatoxin contamination in top groundnut-producing states of the United States caused average annual losses of US$ 26 million to its southeastern groundnut industry [79]. Chemical control and conventional breeding have yielded only limited success. Although wild relatives of groundnut possess resistance to foliar diseases to the level of even immunity [80–82], the interspecific hybridization has not been highly successful in introgression of the desirable traits where desired due to complexity of inheritance and several inherent breeding barriers [83, 84]. This

narrow genetic basis of the cultivated groundnut *A. hypogaea* L. hampers the development of improved varieties through conventional breeding.

To overcome these bottlenecks, transgenic options for groundnut crop improvement for various diseases have been carried out by various groups. Transgenic groundnut expressing a tobacco chitinase gene II [85] and rice chitinase and an alfalfa glucanase gene [86] have been shown to possess enhanced resistance to the late leaf spot and *Sclerotinia* blight, respectively. Glucanase gene from tobacco introduced into groundnut (PR protein from heterologous source) showed enhanced disease resistance to *C. arachidicola* and *A. flavus* [87]. Transgenic plants of groundnut carrying mustard defensin gene showed increased disease resistance to *C. arachidicola* Hori. and *P. personata* [88]. Similarly, overexpression of barley oxalate oxidase gene in transgenic groundnut showed enhanced resistance to oxalic acid producing fungi, *S. minor* [89].

Since aflatoxin contamination of groundnut not only causes significant economic hardship for producers but also poses a serious health threat to humans. Transgenic interventions for enhancing host plant resistance to *A. flavus* infection have been carried out in groundnut. There have been various studies carried out for developing transgenic groundnut plants either overexpressing and/or downregulating different genes for resistance to *A. flavus* and aflatoxin [90–92].

So far, groundnut is virtually unexplored at the genomic level because of the large genome size (2800 Mb/1C) and complication and hence expressed sequenced tags (EST) have been considered to be a quick and economical approach to identify important groundnut genes involved in defense response against fungal infections, also providing data on gene expression and regulation [93, 94]. Efforts have been made to identify and characterize the peanut EST regulated during interaction with the fungus *Cercosporidium personatum* (causing late leaf spot) using suppressive subtractive hybridization (SSH) to prepare the subtracted cDNA libraries [95]. Utilizing the genomic and proteomic tools, genes and proteins associated with *A. parasiticus* and drought stress were identified in groundnut [96–98]. Such genes have a potential to be used for enhanced fungal disease resistance in groundnut through marker-assisted selection in breeding or by direct upregulation or downregulation of the target gene using genetic engineering.

16.4.2 Chickpea

Chickpea is the most important food legume of semiarid tropics (SAT) and taxonomically one of the closest crops to the model legume *Medicago*. Chickpea, from their region of domestication in the Near East, quickly spread to the Indian subcontinent where it became a principal pulse crop and a dietary mainstay [99]. Chickpea was successfully introduced to Central and South America and to the western United States [99]. Chickpea is the major pulse crop in the Indian subcontinent where it is produced on nearly 7 million ha [99]. Chickpea's yield potential is limited by a series of biotic and abiotic stresses, including Ascochyta blight, Fusarium wilt, drought, cold, and salinity. It is mostly grown under

rainfed conditions in arid and semiarid areas around the world. Despite growing demand and high-yield potential, chickpea yield is unstable and productivity is stagnant at unacceptably low levels. Together with low temperatures and water stress, high salinity is responsible for crop losses of millions of tons of various legume (and other) crops and continuously deteriorating environmental conditions combined with salinity stress to further compromise chickpea yields. Hence, major yield increases in chickpea could be achieved by development and use of cultivars that resist/tolerate abiotic and biotic stresses. To accelerate molecular breeding efforts for the discovery and introgression of stress tolerance genes into cultivated chickpea, functional genomics approaches and/or transgenics are rapidly growing. Recently, a series of genetic tools for chickpea have become available that have allowed high-powered functional genomics studies to proceed, including a dense genetic map, large insert genome libraries, expressed sequence tag libraries, microarrays, serial analysis of gene expression, and transgenics and reverse genetics.

16.4.2.1 **Biotechnology for Tolerance to Abiotic Stresses**

Chickpea (*C. arietinum* L.), a deep-rooted legume grown in semiarid regions of the world, is liable to terminal drought, which impacts plant growth and descends global annual yield stability. Chickpea varieties susceptible/tolerant to abiotic stress have been characterized under abiotic stress conditions (especially drought stress), although very little is known about the genes involved in abiotic stress responses. Nevertheless, the characterization of genes involved in the differential behavior of these cultivars may constitute a good basis to extrapolate these results to other grain legumes. In order to identify and understand the molecular mechanisms of tolerance to abiotic stress such as drought, a total of 10 996 ESTs were generated from 10 cDNA libraries derived from root tissues of drought-tolerant (ICC 4958) and drought-sensitive (ICC 1882) chickpea genotypes grown under control and drought stress conditions. The drought-responsive ESTs along with 7439 salinity-responsive ESTs generated from salt stress challenged tissues of JG 11 and ICCV 2 genotypes of chickpea, and 7097 public domain chickpea ESTs were comprehended to derive 9569 unigenes (2431 contigs and 7138 singletons) and were used to identify and design EST-based SSR and SNP markers. The availability of such large number of ESTs derived from different resources provided an added advantage for computational SNP discovery, first by assembling ESTs derived from different individuals and then scanning the multiple sequence alignments (MSA) of the contig to find sequence differences that correlate with the source of the EST, providing opportunities to understand the genetic mechanisms underlying stress responses in this legume. (http://www.icrisat.org/bt-gene-discovery.htm).

Similarly, a transcriptional profiling study in chickpea was carried out using cDNA microarray approach under drought, cold, and high salinity to look at the gene expression in the leaf, root, and/or flower tissues in tolerant and susceptible genotypes [100]. Besides, superSAGE analysis for gene expression in chickpea roots in response to drought resulted in sequencing of a total of 80 238 of 26 bp tags [101] (Among these tags, a total of 7532 (43%) UniTags were more than 2.7-fold

differentially expressed, and 880 (5.0%) were regulated more than 8-fold upon stress resulting in unambiguous annotation of 22% (3858) of these tags. This is the first study to prove the potential of SuperSAGE technology for molecular breeding in the nonmodel crops. Interestingly, in this year the same group has done an excellent modification of this technique as deepSuperSAGE, and raised a huge amount of data against abiotic stress [101].

In sharp contrast to the importance of chickpeas as staple food and industrial raw material, the salt responses at the transcriptome and proteome levels had been dealt with at only very low throughput until some years ago, that is, tens or at the most hundreds of genes had been considered [100]. Molina *et al.* [102] applied deepSuperSAGE to detect early global transcriptome changes under salt-stressed chickpea. The salt stress responses of 86 919 transcripts representing 17 918 unique 26 bp deepSuperSAGE tags (UniTags) from roots of the salt-tolerant variety INRAT-93 2 h after treatment with 25 mM NaCl were characterized. Of the total 144 200 analyzed 26 bp tags in roots and nodules together, 21 401 unique transcripts were identified. Of these, only 363 and 106 specific transcripts were commonly upregulated or downregulated (>3.0-fold), respectively, under salt stress in both organs, witnessing a differential organ-specific response to stress. These results demonstrated that ROS-scavenging and ROS-generating pathways undergo strong global transcriptome changes in chickpea roots and nodules 2 h after the onset of moderate salt stress (25 mM NaCl). These newly identified transcript isoforms are potential targets for breeding novel cultivars with high salinity tolerance [102].

Most of the earlier understanding of dehydration-responsive cellular adaptation in chickpea has evolved from transcriptome analyses and the comparative analysis of dehydration-responsive proteins, particularly proteins in the subcellular fraction, is limiting. Bhushan *et al.* [103] have initiated a proteomics approach to identify dehydration-responsive extracellular matrix (ECM) proteins in JG-62, a drought-tolerant variety of chickpea where the dehydration-responsive temporal changes of ECM proteins revealed 186 proteins with variance at a 95% significance level. This study, for the first time, demonstrated that over a hundred ECM proteins are presumably involved in a variety of cellular functions, namely, cell wall modification, signal transduction, metabolism, and cell defense and rescue, and impinge on the molecular mechanism of dehydration tolerance in plants. Another study provided insights into the complex metabolic network operating in the nucleus during dehydration in chickpea [104]. Approximately 205 protein spots were found to be differentially regulated under dehydration. Mass spectrometry analysis allowed the identification of 147 differentially expressed proteins, presumably involved in a variety of functions including gene transcription and replication, molecular chaperones, cell signaling, and chromatin remodeling. The dehydration-responsive nuclear proteome of chickpea revealed a coordinated response, which involves both the regulatory and the functional proteins [104].

Efforts on enhancing the drought tolerance using transgenic interventions have also been carried out in chickpea, where transgenics carrying *P5CSF129A* gene encoding 1-*pyrroline-5-carboxylate synthetase* were constitutively expressed

for overproduction of an osmolyte proline, which is known to have a role in osmotic adjustment and cell protection under water deficits [105]. In an another effort, *DREB1A* cDNA from *Arabidopsis thaliana*, capable of transactivating DRE-dependent transcription in plant cells under the control of stress-inducible *rd29* promoter, was introduced to a popular chickpea cultivar for improving drought and salinity tolerance in this important pulse crop (K.K. Sharma, unpublished results).

16.4.2.2 **Biotechnology for Resistance to Biotic Stresses**

In chickpea, genetics of resistance to Ascochyta blight [106–109] and Fusarium wilt [110–114] have been extensively analyzed. A comprehensive overview of previous genetic mapping efforts in chickpea is available [115]. The advent of sequence-tagged microsatellite sites (STMS) markers [116, 117], however, provided the opportunity to integrate the different available maps.

Cool and wet weather conditions are typical for Mediterranean winters, favoring the development of Ascochyta blight that is caused by the necrotrophic fungus *A. rabiei* (Pass.) that affects all aerial parts of chickpea. Although sources of resistance have been identified [106], the development of stable blight-resistant lines would allow a shift to sowing into the rainy season. The genetics of resistance to Ascochyta blight has been extensively analyzed because the disease is of great agronomic and economic importance. Nevertheless, more durable resistance could probably be achieved by pyramiding of resistance genes via MAS and is currently a major challenge for chickpea breeders [118].

Among the many insect pests, the legume pod borer, *H. armigera*, is the most devastating pest damaging chickpea in Asia, Africa, and Australia. Despite the efforts made over the past four decades to breed crops for resistance to insects, the progress has been less than satisfactory in many cases. The resistance to *Helicoverpa* in chickpea has so far been found to be low to moderate. Therefore, it is imperative to evaluate the use of biotechnology to provide alternative and sustainable levels of resistance to this insect pest. Genetic transformation has been an important strategy to introduce the insecticidal protein genes from the bacterium *Bacillus thuringiensis* (*Bt*) and other heterologous sources for improving the resistance of chickpea. Although extensive work has been carried out in developing transgenic plants with *Bt* and other insecticidal genes to combat the insect pest *H. armigera*, there has not been a major breakthrough in controlling this devastating pest in this important pulse [119]. Since there has not been a major breakthrough in identifying events showing a much higher expression of the insecticidal protein sufficient to cause a 90–95% insect mortality under natural conditions, efforts are also focused on using several promoter–gene combinations for efficient expression of the *Bt* genes. Efforts are being made to use SARs (scaffold attachment regions) and MARs (matrix attachment regions) for high gene expression and exploring possibilities of using suppressor genes such as *AC2* to enhance the *Bt* gene expression in this crop. There is a need to produce larger numbers of transgenic events of chickpea using various *Cry1* and *Cry2* gene constructs to obtain one or more agronomically acceptable insect-resistant chickpea lines.

Aphis craccivora, the homopteran group of sucking pest, causes major damage to chickpea by extracting nutrients from the phloem [120]. Apart from the direct damage, they transmit different viruses that cause damaging diseases to the crop. Expression of ASAL driven by a phloem-specific *rolC* promoter was carried out in chickpea transgenics followed by their evaluation by binding analyses of its respective *cis*-elements with host nuclear transcription factors. The novel in planta bioassay conducted on *A. craccivora* revealed that insect survival and fecundity decreased significantly in T1 plants in comparison to the untransformed control chickpea [120]. Apart from pod borer and aphids, bruchids cause substantial loss during storage in chickpea [121]. The cowpea weevil (*Callosobruchus maculatus*) and azuki bean weevil (*Callosobruchus chinensis*) infest chickpea seeds heavily. To address this, α-amylase inhibitor gene isolated from *Phaseolus vulgaris* was introduced into chickpea cultivar K850 through *Agrobacterium*-mediated transformation. Results of bioassays revealed a significant reduction in the survival rate of bruchid weevil *C. maculatus* reared on these transgenic chickpea seeds [122].

While transcriptomics studies including microarrays have been used extensively for transcriptional profiling of plant responses to biotic and abiotic stresses, most of these are focused on either biotic or abiotic stresses, making it difficult to construe the genes that may be common to both biotic and abiotic stress responses. Such information may help molecular breeders to develop cultivars with broad-spectrum resistances to these stresses. A 768-featured boutique microarray was employed to compare the genes expressed by chickpea in response to drought, cold, and high salinity and the fungal pathogen *A. rabiei* and 46, 54, 266, and 51 differentially expressed transcripts were identified, respectively [123]. The expression of common genes indicated crosstalk in the genetic pathways involved in responses to these stress conditions. The response of ICC 3996 to *A. rabiei* was more similar to that of high-salinity stress than to drought or cold stress conditions [123].

16.4.3 Pigeonpea

Pigeonpea (*Cajanus cajan*), an important food legume crop in the semiarid regions of the world and the second most important pulse crop in India, has an average crop productivity of 780 kg/ha that is relatively lower than many other legumes. The low yields may be attributed to nonavailability of improved cultivars, poor crop husbandry, and exposure to a number of biotic and abiotic stresses in pigeonpea growing regions. Narrow genetic diversity in cultivated germplasm has further hampered the effective utilization of conventional breeding as well as development and utilization of genomic tools, resulting in pigeonpea being often referred to as an "orphan crop legume."

Conventional breeding efforts in pigeonpea crop improvement have been successful in producing improved seed quality and reduction of crop maturity duration. Nevertheless, genetic improvement of pigeonpea has been restricted due to the nonavailability of better genetic resources and strong sexual barriers between

the cultivated and wild species. The recent developments in plant biotechnology have provided immense potential in overcoming some of these constraints, thereby offering opportunities for its successful integration with conventional crop improvement strategies. The breakthrough in pigeonpea genome sequencing [124, 125] has resulted in identification of newer genes related to biotic and abiotic stresses in this legume crop, which can effectively be utilized to find stress-responsive genes in other legume crops through comparative studies. Pigeonpea genome analysis predicted 48 680 genes and also showed the potential role that certain gene families, for example, drought tolerance-related genes, have played throughout the domestication of pigeonpea and the evolution of its ancestors [124].

16.4.3.1 Biotechnology for Tolerance to Abiotic Stresses

Drought, cold, heat, and salinity are the abiotic stresses that affect the pigeonpea yield. Besides, waterlogging, heavy rains, and frost are very harmful for the crop. Hence, improvement of pigeonpea for tolerance to these abiotic stresses is very important for obtaining increases in the harvest index and ultimately the yield. Pigeonpea has remarkable drought tolerance traits, which have been used for the isolation of stress-responsive genes. A recent study identified 75 ESTs obtained from the cDNA libraries of drought-stressed plants, 20 ESTs proved to be unique to the pigeonpea [126]. Expression profiles of selected genes revealed increased levels of m-RNA transcripts in pigeonpea plants subjected to different abiotic stresses. Transgenic *Arabidopsis* lines, expressing C. cajan hybrid proline-rich protein (CcHyPRP), *C. cajan* cyclophilin (CcCYP) and *C. cajan* cold and drought regulatory (CcCDR) genes, exhibited marked tolerance, increased plant biomass, and enhanced photosynthetic rates under PEG/NaCl/cold/heat stress conditions [126]. These genes, as such, hold promise for engineering crop plants bestowed with tolerance to major abiotic stresses [126]. A recent study identified 5692 unique candidate single feature polymorphisms (SFPs) that are microarray-based molecular markers detected by hybridization of DNA or cDNA to oligonucleotide probes, extending the marker repertoire with functional marker systems in pigeonpea [127] With the pigeonpea genome sequence now available, genes need to be confirmed experimentally, but are, nonetheless, candidates that can be used to gain insight into the genetic architecture of pigeonpea's drought tolerance and for screens to identify superior haplotypes for improvement. This will facilitate the assembly and alignment of the sequence of other legume crops as well as the unique genes found in pigeonpea may be exploited to improve other legumes for various traits. These candidate genes are useful resource for undertaking the gene expression analysis as well as development of functional markers for both basic and applied research, especially for drought tolerance in pigeonpea improvement [127].

Although relatively tolerant to drought, pigeonpea is sensitive to photoperiod and temperature. While low temperatures affect the short-duration varieties of pigeonpea, high temperature and photoperiod affect the yield of medium and long-duration varieties rendering them to terminal drought. Likewise, in cool areas, maturity in long-duration pigeonpea is accelerated and severe competition occurs between intercropped maize whose maturity is delayed and pigeonpeas resulting in yield

reduction of both crops. It is proven that membrane lipids hold the key for improvement of photosynthesis under low-temperature and high-temperature stress conditions [128]. Not much progress has been made using transgenic solutions for various abiotic stresses in pigeonpea, which need to be improved for getting high yield varieties for this important grain legume. Moreover, pigeonpea is classified as moderately sensitive to salinity [129], development of salt-tolerant variety could be thus useful for Indian farmers as it is grown predominantly in the states where more than 51% of the saline soils in India are located [130].

16.4.3.2 **Biotechnology for Resistance to Biotic Stresses**

Modern genomic tools such as molecular markers and candidate genes associated with resistance to biotic stresses such as Fusarium wilt, sterility mosaic disease, and pod borer (*H. armigera*) offer the possibility of facilitating pigeonpea breeding for improving biotic stress resistance. Availability of limited genomic resources, however, is a serious bottleneck to undertake molecular breeding in pigeonpea to develop superior genotypes with enhanced resistance to above-mentioned biotic stresses. FW- and SMD-responsive ESTs in pigeonpea have been analyzed and this information can be used for the development of novel SSR and SNP markers in pigeonpea [131]. For enhancing the genomic resources of pigeonpea against biotic constraints, ICRISAT has developed large-scale SSR markers from BAC (bacterial artificial chromosome)-end sequences (BESs) and their subsequent use for genetic mapping and hybridity testing in pigeonpea [132]. Besides, genomic studies on pigeon pea have been conducted with emphasis on genetic mapping and evaluation of polymorphism using RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), microsatellites, and simple sequence repeats (SSR) for *H. armigera* resistance genes [133].

Bulk segregant analysis (BSA) has been employed to identify simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) markers associated with sterility mosaic disease that is considered to be an important disease of pigeonpea causing substantial loss in yield [134]. From 13 polymorphic AFLP primer combinations between the parents, 2 AFLP primer pairs generated 4 markers. O these, two were reported to be linked in coupling phase to the susceptible dominant allele amplifying only in susceptible individuals that can be effectively used for marker-assisted selection [134]. With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, there have been efforts on using a rice chitinase gene for transformation in pigeonpea [135].

Since conventional breeding methods have not been very successful in producing pest-resistant genotypes of pigeonpea due to the limited genetic variation in cultivated germplasm, ICRISAT has developed an efficient method to produce transgenic plants of pigeonpea through *Agrobacterium tumefaciens*-mediated genetic transformation [136]. Lawrence and Koundal [137] transformed pigeonpea with a cowpea protease inhibitor gene for developing transgenic pigeon pea resistant to chewing insects, mainly pod borers. Similarly, Surekha *et al.* [138] successfully transformed pigeon pea with synthetic *cry* I E-C gene. Transgenic pigeon pea events carrying the *Bacillus thuringiensis cry1Ab* and soybean trypsin inhibitor

(SBTI) genes are being developed and evaluated for resistance to *H. armigera* under greenhouse and field conditions in Patancheru, Andhra Pradesh, India.

16.4.4 Soybean

Soybean is the most valuable legume crop, with numerous nutritional and industrial uses due to its unique chemical composition. With its high protein (40%) and moderately high oil (20%) contents, soybean is the world's main source of vegetable protein and oil, accounting for 55% of all oilseeds produced. The enormous agronomic importance of soybean, coupled with the development of modern molecular biology, has led to an increasing level of activity to develop soybean genomics. The efforts in the soybean research community have led to substantial progress in the areas of molecular marker development, expressed sequenced tag databases, BAC end sequences, microarrays, and efficient genetic engineering capabilities. A physical map for cv. Forrest was completed with NSF support (and a physical map of cv. Williams 82, developed with USB funding, is nearing completion (unpublished)). Thus, soybean is also positioned as a key model for translational genomics in grain legumes.

16.4.4.1 Biotechnology for Tolerance to Abiotic Stresses

Depending on hybrid characteristics, soybeans use about 450–700 mm of water during the growing season [139] with drought reducing its yield by about 40% [140]. The most critical period for water stress in soybean has been reported to be during the flowering stage and the period following flowering. However, despite the large resources committed to soybean breeding, progress in improving drought resistance has been slow for a number of reasons [141]. A large number of QTLs have been identified in soybean for traits related to agronomic, physiological, seed composition, and both biotic and abiotic stress parameters (www.soybase.ncgr.org). However, to date only a few QTLs associated with drought resistance traits have been identified.

In the last decade, considerable progress has been made in developing genomic resources for soybean, including the sequencing of the entire soybean genome of approximately 975 Mb (http://www.phytozome.net/soybean#C). Various types of physical maps have also been using RFLP, RAPD, SSR, and AFLP markers [142]. A high-density genetic linkage map of soybean using EST-derived microsatellite markers was generated using a hybrid between the Japanese cultivar "Misuzudaizu" and the Chinese line "Moshidou Gong 503" by Japanese researchers [143, 144]. Also, the possibility of segmental duplications in the previously suggested regions of the soybean genome was confirmed by inspecting the colinearity between the genomes of soybean and *Lotus japonicus* [144]. Detailed genetic and physical maps of the soybean genome, which together cover the soybean genome by more than 35-fold, have also been created using microsatellite markers from BAC libraries [145]. The Williams 82 *Bsty*I library containing 92 160 clones with an average insert size of 150 kb covering approximately 12 genome

equivalents has been constructed. A genetic map was then integrated to this physical map by anchoring approximately 1000 SSR and STS markers, developed from expressed sequence tags, which are associated with drought responses, disease resistance, seed development, and composition traits [146]. In addition, a six-dimensional pool array has been recently developed from 49 152 *Bsty*I soybean clones (~6× genome equivalents), comprising 208 BAC pools [146]. The integrated genetic and physical map will be useful for comparative genetic analysis, map-based cloning of QTLs of desired traits, and genomic sequencing. Recently, the entire soybean genomic sequence has been released with 66 153 protein-coding loci (http://www.phytozome.net/soybean#C). Besides, a spotted cDNA microarray is available containing 36 000 elements constructed from soybean cDNAs, which were derived from a variety of EST libraries representing a wide source of tissues and organs, developmental stages and stress, and pathogen-infected plants (Vodkin *et al.*, 2004). The ESTs isolated from the subtracted library of drought-stressed soybean root tips and submitted to GenBank will greatly contribute to the stress-specific unigenes for further functional genomics work aimed at better understanding of the drought stress response of the soybean root system [147]. So far QTLs in soybean under water deficit conditions have been reported only for WUE and leaf ash [148]. More studies are needed to identify QTLs that influence root architecture and shoot turgor maintenance. Mapping for new QTL(s)/gene(s) and determination of gene action under drought will likely provide key resources to improve tolerance to drought stress in soybean. There exists a substantial genetic variation in soybean for salt response and QTLs associated with salt tolerance in soybean have been identified [149]. With the availability of mutant populations and major achievements in marker-assisted selection and soybean transformation, it is now possible to study and characterize the genes related to drought resistance, leading ultimately to better soybean productivity.

Besides these efforts on functional genomics, there have been enormous efforts on transgenic interventions for abiotic stress tolerance in soybean. Transgenic soybean plants overexpressing the *Arabidopsis* Δ1-pyrroline-5-carboxylate synthase gene, *P5CS*, showed greater tolerance to drought stress due to an increased free proline level and relative water content and a reduced level of reactive oxygen species, particularly hydrogen peroxide [150–152]. Since this report, much effort has been directed toward isolating drought stress-related genes in all physiological and biochemical aspects of drought stress, TFs, and their respective promoters, which will in turn provide novel tools and resources for the development of engineered soybean with improved drought resistance. The identified soybean candidate genes are usually tested for their ability to enhance drought tolerance in *Arabidopsis* before pursuing their engineering into soybean. Chen *et al.* [153] isolated an *AtDREB* homologous gene *GmDREB2* from soybean, constitutive expression of which increased the survival rate of transgenic plants without growth retardation under water deficit and salinity stress conditions. In addition, a few basic leucine zipper genes encoding bZIP TFs were cloned from soybean and found to be induced by drought and high salt treatments. By overexpressing some of these genes in *Arabidopsis*, the researchers found that transgenic plants have increased

freezing and salt tolerance, but no significant difference in drought tolerance [154, 155]. In two other recent studies, it was found that overexpression of a GmERF TF family member, the *GmERF089* gene, and a chilling inducible *GMCHI* gene promoted enhanced tolerance to drought stress in tobacco and *Arabidopsis*, respectively [156, 157]. Recently, 31 *GmNAC* genes, including the six genes previously identified by Meng *et al.* [158], were identified and cloned from soybean. Systematic expression analysis of these 31 *GmNAC* genes demonstrated that nine genes are dehydration inducible [159]. These nine *GmNAC* genes and their respective promoters are promising tools for genetic engineering to improve drought resistance of soybean, as the NAC family was reported to be a major group of TFs that play a role in root development and stress tolerance in plants [160–163].

Recent reports on transgenic soybean for abiotic stress tolerance include transformation with coding sequence for cyanamide hydratase (Cah), an enzyme that converts toxic cyanamide to urea, from the soil fungus *Myrothecium* [164]. Another report on the constitutive expression of *nectarin1* (*NTR1*) gene from *Brassica campestris* in transgenic soybean resulted in enhanced accumulation of methyl jasmonate (MeJA). *NTR1* gene encodes jasmonic acid carboxyl methyl transferase, which is involved in plant development as it regulates the expression of plant defense genes in response to various stresses such as wounding, drought, and pathogens. The higher levels of MeJA in the transgenic soybean plants conferred tolerance to dehydration during seed germination and seedling growth as reflected by the percentage of the fresh weight of seedlings.

These recent advances in soybean research, ranging from breeding programs to genome sequencing and genomics technologies, provide unprecedented opportunities to understand global patterns of gene expression and their association with the development of specific phenotypes, as well as promising tools for the genetic improvement of closely related species grown in adverse environments by molecular breeding or transgenic approaches. Profiling soybean transcriptome, proteome and metabolites will lay the foundation for a systems biology approach to understand key processes such as growth characteristics, stress responses, and yield. Identification of several root-related and stress-specific candidates could help understand the biochemical networks involved in stress responses. Characterization of these candidates, engineering of selected genes through translational genomics pipeline, and field testing of the transgenics are in the pipelines in different research laboratories.

16.4.4.2 **Biotechnology for Resistance to Biotic Stresses**

As soybean crop acreage increased over the years, the crop "matured" and is now host to numerous pathogens and pests. Soybean cyst nematode and *Phytophthora sojae* are the primary causes of yield loss in most of the soybean production regions followed by charcoal rot, virus diseases, sudden death syndrome, seedling diseases and a mix of leaf blights, stem rots, and other nematodes. In addition to these soybean diseases, eight major insect pests also contribute to yield losses due to feeding and damage to seed quality. This damage is more consistent in the southern United States and sporadic in the Midwest. A new insect threat has emerged with the

newly introduced soybean aphid in the Midwest, where insecticide applications are now becoming a routine practice. Asian soybean rust, a disease that causes serious losses in many parts of the world, was first detected in the continental United States in November 2004 and is caused by the fungus *P. pachyrhizi*. Long known to occur in Asia, the fungus spread to Zimbabwe, South Africa, Paraguay, Brazil, Columbia, and now the United States during the past 10 years. Yield losses in other parts of the world due to soybean rust are reported to range from 10% to 90%.

Soybean has several R-gene and QTL clusters to biotic pathogens already identified and mapped in the soybean genome. It is highly likely, based on other host pathogen systems, which QTLs for the slow rusting (partial resistance) phenotype for soybean rust may map to these regions as well. There have been numerous efforts in the past 10 years to identify new genes and sources of resistance to many of soybean's biotic pests. Biotechnological approaches for the development of genetically engineered soybean lines, which express insecticidal molecules, are being widely studied. Genetic transformation of soybean to induce resistance to lepidopterans using the insertion of Bt toxins dates back to early 1990s [165, 166], where these transgenic soybeans showed resistance toward *Helicoverpa zea*, soybean looper Pseudoplusia, tobacco burworm (*Heliothis virescens*), and velvet bean caterpillar (*Anticarsia gemmatalis*). Moreover, the occurrence of proteinase inhibitors (PIs) as defense-related proteins in soybean have also been reported to inhibit the growth of insect pests larvae, including the coleopteran *Tribolium confusum* (Haq) [167, 168] and other insect species, such as *Anagasta kuehniella*, *Hypera postica*, and *Anthonomus grandi* [169, 170]. Recently, soybean biotechnology has been extended to expressing double-strand RNAs (dsRNA) in order to drive gene silencing in nematodes. The posttranscriptional gene silencing using dsRNA RNAi constructs decreased cyst nematodes in transformed soybean roots [171–173].

The glyphosate-tolerant GM soybean alone corresponds to 52% of all biotech crops planted in world area. Indeed, considering soybean, herbicide tolerance has still been the major aimed trait, with around 10 novel varieties showing tolerance to different chemical compounds in their final steps of R & D pipeline to commercial events. However, there is an obvious need and seed market demand for insect pests and plant pathogens resistance traits in soybean. In a very near future, the first GM soybean resistant to insect pests and nematode will be available as single traits or together with herbicide tolerance (stacked traits). Hence, it is expected that in near future, the production of soybean will be possible with less or none agrotoxic residues or mycotoxins enhancing the soybean quality and crop production.

16.4.5 Cowpea

Cowpea, *Vigna unguiculata* L. Walp., is one of the most important food and forage legumes in the semiarid tropics as well as a valuable and dependable commodity for farmers and grain traders. Of the ~21 million acres grown worldwide, 80% of cowpea production takes place in the dry savannah of tropical West and Central Africa, mostly by poor subsistence farmers in developing countries. Apart from

improvement of agronomical traits, the biotechnological interventions in cowpea breeding and improvement programs aim toward combating abiotic stress (drought, salinity, and heat) tolerance, photoperiod sensitivity, plant growth type, and seed quality with resistances to the numerous bacterial, fungal, and viral diseases and insect, invertebrate (nematode), and herbivorous pests. However, cowpea being adapted to different environmental conditions could potentially be used as an alternative crop for salt-affected soils [174].

16.4.5.1 Biotechnology for Tolerance to Abiotic Stresses

Cowpea is one of the important food legumes cultivated by poor farmers in Sub-Saharan Africa and Asia known to have a better tolerance to drought and high temperature compared to other legumes [175]. Preserving membrane integrity by avoiding membrane proteins degradation is essential for plants to survive in drought stress and hence gives cowpea an edge over other legumes [176]. Transcriptomic studies suggest that several coping mechanisms exist in cowpea for preventing lipid and proteins degradation and for generating reaction oxygen species (ROS) (superoxide radicals, O_2-; hydrogen peroxide, H_2O_2; and hydroxyl radicals, OH). Several reports indicate that drought-tolerant cowpea cultivars adopt these strategies by maintaining the level of expression of certain genes such as cystatin and aspartic protease and by promoting membrane integrity [176]. The transcripts coding these proteins were isolated in drought-tolerant cowpea cultivars subjected to water deficit and their expression localized in different organs [177, 178]. Interestingly, while phospholipase D is a major lipid-degrading enzyme in cowpea cultivars sensitive to drought, the expression of the gene encoding phospholipase D1 (VuPLD1) moderately increased in the drought-tolerant cowpea cultivars [179]. Moreover, the cowpea-tolerant cultivars have been reported to overexpress the gene that encodes ascorbate peroxidase in the chloroplast, while this enzyme is activated in the cytoplasm, peroxisome, and chloroplast [180]. The isolation and characterization of nine drought-inducible genes (CPRD) from 4-week-old cowpea plants by differential screening have been carried out to elucidate the molecular response of cowpea plants to drought stress [181]. In another study, two novel cDNAs (one of them VuNCED1 gene encodes the 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme involved in ABA biosynthesis) have been isolated by a series of differential screening under drought stress. Moreover, several transcripts known as CPRD (cowpea clones responsive to dehydration), CPRD8, CPRD14, CPRD22, and VuNCED1 encode a 9-*cis*-epoxycarotenoid dioxygenase responsible for ABA (abscisic acid) biosynthesis which is highly expressed in cowpea during drought, high salinity, and heat stresses [181, 182]. To understand the molecular bases of thermotolerance, differentially expressed transcripts from cowpea nodules have been identified following subjection to heat shock treatment [183]. These transcripts showed homologies with low molecular weight heat shock proteins, wound-induced proteins, disease-resistant protein, xylan endohydrolase isoenzyme, and different housekeeping genes.

Although much has been studied about the drought tolerance mechanisms in cowpea, a very little perusal of literature is available regarding the biotechnological

approaches exclusively meant for salinity tolerance in cowpea. Often the genes identified as thermotolerant or drought resistant are found to be involved with salinity stress. Salt stress affects cowpea (*V. unguiculata* L. Walp) varieties at different growing stages and according to a recent study in Cuba, *Vigna* genotypes show significant differences in their tolerance to salinity [184].

The recent influx of molecular markers has enhanced our understanding of cowpea's genome structure and organization. Studies based on RAPD, DAF, and SSR markers revealed a low genetic diversity among cowpea varieties and molecular polymorphism between drought tolerance and sensitive varieties, and also between the higher and lower nitrogen fixing cowpea accessions [185–188]. The first attempt to build a genetic map of cowpea was performed by Fatokun *et al*. [189] by using a population resulting from a cross between an improved genotype and its wild progenitor *V. unguiculata* ssp. *dekindtiana*. Despite the disadvantage of this type of cross, which may be related to the identification of the loci that may be polymorphic only between more divergent genotypes, but not between more closely related genotypes, especially the ones of interest, the authors located a quantitative trait loci for seed weight that was conserved between cowpea and *V. radiata* ssp. *sublobota*. Subsequently, the second linkage genetic map developed on cowpea consisted of 181 loci, including 3 morphological markers and a biochemical marker (dehydrin) that allowed mapping of genes involved in earliness and seed weight, respectively, in linkage groups 2 and 5 [190].

16.4.5.2 Biotechnology for Resistance to Biotic Stresses

Several fungal, bacterial, and viral diseases affect cowpea at different stages of growth. The major and common diseases are anthracnose, Sclerotium stem, root, and crown rots, damping-off, Cercospora leaf spot, Septoria leaf spot, Fusarium wilt, and scab. Storage pests, *Callosobruchus maculatus* and *Calloso chinensis* cause severe damage to the cowpea seeds during storage. Transgenic cowpea has been developed for insect resistance using the bean (*P. vulgaris*) α-amylase inhibitor-1 (αAI-1) gene. Expression of αAI-1 gene under bean phytohemagglutinin promoter resulted in accumulation of αAI-1 in transgenic seeds and the recombinant protein was active as an inhibitor of porcine α-amylase *in vitro*. Transgenic cowpeas expressing αAI-1 strongly inhibited the development of *C. maculatus* and *C. chinensis* in insect bioassays [191].

16.4.6 Common Beans

16.4.6.1 Biotechnology for Tolerance to Abiotic Stresses

Abiotic stresses (climatic and edaphic) probably represent total loss in yield in common beans [192]. Estimates of area subject to phosphorus (P) deficiency suggest that 50% of bean production suffers from P deficiency, 40% may suffer from aluminum toxicity, and 73% suffers from drought. Moreover, higher temperatures and greater evapotranspiration, combined with lower rainfall, are expected to exacerbate drought in important bean-producing areas of northern Central America, Mexico,

Brazil, and southern Africa. Predictions have been made that higher temperatures in these regions will affect the altitudinal range of adaptation of bean genotypes, reducing the root growth and accelerating the decomposition of soil organic matter (mineralization) making problems of stress even more acute. However, since other bean-growing areas such as East–Central Africa and the Northern Andes will receive greater precipitation, excess rainfall, waterlogging, and associated root rots will be a problem [193].

Breeding for drought resistance in beans has a long history, in the program of EMBRAPA in Brazil [194]. Improved drought resistance has resulted from combining germplasm adapted to the dry highlands of Mexico with small seeded types from lowland Central America, through recurrent selection within each genepool. In the case of drought, roots have long been recognized as playing an important role in beans. A drought-resistant line, BAT 477, presented deep rooting under drought stress, permitting access to soil moisture at greater depths [195, 196]. However, deep rooting alone does not ensure drought resistance, and data on root density at various levels of the soil profile suggest that deep rooting genotypes are not always the best yielding materials (CIAT, 2007; CIAT, 2008). The response to drought at the physiological and molecular levels has been studied in two common bean varieties with contrasting susceptibility to drought stress (Zhu *et al.*, 2002). A number of genes were found to be upregulated (both ABA dependent and independent) in the tolerant variety Pinto Villa relative to the susceptible cultivar, Carioca, with the former displaying a more developed root vasculature in drought conditions than the latter. The International Centre for Tropical Agriculture has taken initiative for improving drought tolerance in common bean (Broughton *et al.*, 2003). The physiological studies done so far linked the intermediate drought tolerance trait in breeding line BAT 477 to greater root growth under water deficit conditions [195] and further showed that genetic control of this trait was expressed in roots and not shoots [196]. Additional genetic sources for drought tolerance were also identified in the 1980s, especially from Mexican varieties. During the 1990s, a combination of genetic sources was used to develop varieties with higher tolerance and additional mechanisms (e.g., photosynthate mobilization from leaves and stems to developing seeds) for drought–stress tolerance were identified [197]. By 2002, these drought-tolerant varieties were being tested in the field and attempts to integrate genomic techniques with traditional breeding were initiated in parallel.

16.4.6.2 **Biotechnology for Resistance to Biotic Stresses**

One of the most significant biotic stresses to bean crops is caused by nematodes, especially the genus *Meloidogyne*. Its occurrence is widespread and the damage is particularly striking in the case of continuous cultivation, as it occurs in center pivot-irrigated areas [198]. It is mainly controlled by the use of resistant cultivars associated with management and crop practices. The identification of resistant lines has been frequently reported in the literature [199, 200]. Common bean is exposed to *Fusarium oxysporium f sp. phaseoli* (Fop) causing wilting and early death of the plants that has been tried controlling through use of resistant cultivars [201].

The main biotechnological intervention for disease management in common bean is based on MAS. A set of microsatellites is being put together to efficiently map other populations. Linked markers were identified for the bean golden mosaic virus, anthracnose, bacterial blight, and angular leaf spot and are being used as part of a marker-assisted selection program. Other mapping populations have been developed and are being used to tag quantitative trait loci for disease and pest resistance (http://webapp.ciat.cgiar.org/biotechnology/genomics.htm).

Although a very limited work has been done using transgenic approach in common beans, a system to obtain so-called transgenic composite plants from *P. vulgaris* is available. These plants have a transgenic root system, obtained through *Agrobacterium rhizogenes* transformation of derooted seedlings [202].

16.4.7 Lentils

Lentil is a relatively small crop compared to wheat, rice, maize and soybean, ranking third among the cool season food legumes, in an harvested area of 4.08 million hectares annually, behind pea and chickpea (http://apps.fao.org/faostat). Lentils were successfully introduced to the Western Hemisphere and are now grown extensively in the United States, Canada, Chile, and Argentina, becoming an important crop and a dietary mainstay in the drier areas of the Near East and North Africa. Although mostly discontinued, lentil was a widely grown crop in southern and central Europe.

The major abiotic stresses that affect lentil are cold, drought, heat, salinity, nutrient deficiency, and nutrient toxicity. Of these stresses, drought and heat are considered the most important world wide. While the cold stress is considered important in the West Asia–North Africa (WANA) region, salinity is an important stress factor in the Indian subcontinent and to some extent in WANA. To date, progress has been made in mapping the lentil genome and several genetic maps are available that eventually will lead to the development of a consensus map for lentil. Nevertheless, marker density has been limited in the published genetic maps and there is a distinct lack of codominant markers that would facilitate comparisons of the available genetic maps and efficient identification of markers closely linked to genes of interest [203]. Comparative genomics and synteny analyses with closely related legumes promise to further advance the knowledge of the lentil genome and provide lentil breeders with additional genes and selectable markers for use in marker-assisted selection. Genomic tools such as macro- and microarrays, reverse genetics, and genetic transformation are emerging technologies that may eventually be available for use in lentil crop improvement.

16.4.7.1 Biotechnology for Tolerance to Abiotic Stresses

Lentil is able to produce something of value in many of the semiarid regions primarily through drought avoidance [204]. Early senescence and crop maturity forced by drought conditions are often more severe due to the usually associated high

temperatures. Molecular approaches such as marker-assisted selection may have merit; however, considerable work is needed to identify the important regions of the genome, most likely through a QTL analysis and validation of associated molecular markers.

Heat stress often accompanies drought causing difficulties in separating the two stresses and their effects on lentil growth and yield. There is general agreement that heat affects the distribution of dry matter to reproductive growth and that high temperatures have an adverse effect on lentil yields. Evaluation of the world collection of lentil germplasm has indicated that useful genetic variation is available for improving adaptation to environmental extremes [205].

Susceptibility to cold temperatures has limited production of lentil in cold highland areas of the world. However, germplasm is available that has useful degree of tolerance to cold temperatures, which makes it possible to breed winter hardy cultivars that can be planted in the fall with a reasonable expectation of surviving the winter [206]. Winter hardiness in lentil is conferred by several genes, with the combined effects of several quantitative trait loci accounting for 42% of the variation in winter survival [207]. Molecular markers associated with these QTLs, and postvalidation have potential use in a marker-assisted selection program for winter hardiness in lentil.

Although salinity problems with lentil are not widespread, it can be acute in certain regions of South Asia, the Nile Delta of Egypt, and in some areas of Turkey. Canada also has some difficulty in high salinity areas of Saskatchewan. Of the legumes, lentil is more salt sensitive compared to faba bean and soybean. Salt stress can adversely affect nodulation and N_2 fixation presumably by restricting growth of the root hairs and the potential sites of infection by Rhizobium. Some germplasm with tolerance to salt stress has been identified [208]. Future direction of lentil genomics can be summarized: (i) new marker development and fine mapping, (ii) development of new genetic materials applicable to advanced genomics, and (iii) application of advanced genomic tools for lentil genomics [209].

16.4.7.2 **Biotechnology for Resistance to Biotic Stresses**

As the case with many other legumes, rust and Ascochyta blight are the two most important foliar diseases of lentils in major lentil-producing countries. However, breeding for resistance to Ascochyta blight in lentil has been initiated only recently and is still at a very preliminary stage of large-scale screening of collections of germplasm to identify resistant resources. Multilocation testing of promising cultivars from germplasm screening coordinated by ICARDA has led to the registration of several resistant cultivars in many countries [210]. However, the recent identification of resistance genes and their relationships in several cultivated lines [211] and the confirmation of the presence of different pathotypes [212] have provided the basis to design a breeding program aimed at transferring and combining these genes. A combined bulk population and pedigree selection has been used successfully in lentil breeding at ICARDA. Nevertheless, with the development of novel techniques and the increasing understanding of the host–pathogen system, more efficient breeding methods will be applicable in breeding for resistance to Ascochyta blight in lentil [213].

Combining genes conferring resistance to different pathotypes into a single cultivar (gene pyramiding) makes it useful for diversified environments where different pathotypes are likely to be dominant. Since multiple resistance genes may have additive effect, and even if it is not, the presence of more genes implies that pathotypes have to be virulent to all the genes before a resistant cultivar loses resistance [214]. This procedure of combined resistance has been adopted at ICARDA for improving resistance to Ascochyta blight of chickpea [215]. This method can also be used in breeding for lentil Ascochyta blight resistance as different pathotypes and pathotype-specific resistance have been identified [216]. Genes for Ascochyta blight resistance have been identified in wild lentil species, and hence transferring these from wild species into elite cultivars will be an important approach in breeding for resistance. Ford *et al*. [217] identified seven RAPD markers linked to the resistance locus conferring Ascochyta blight resistance in "ILL 5588" using bulk segregation analysis. Five of the seven RAPD markers were within 30 cM of the resistance locus and the closest flanking markers were approximately 6 cM and 14 cM away from the resistance locus.

Although not a routine, genetic transformation in lentil has been feasible in last few years [218]. Therefore, production of transgenic lentil plants and consequently the application of transgenic techniques in lentil breeding may soon become important for the genetic improvement of this legume.

16.5
Future Prospects

The exploitation of the genetic and genomics resources and biotechnological interventions already employed in model legumes can be a well adopted biotechnological approach for grain legume improvement. Since *Medicago truncatula* is already being studied to unravel resistance to a large number of pathogens, from parasitic plants, bacterial pathogens, and nematodes to fungal and oomycete pathogens. Hence, the transcriptomic and proteomic approaches developed for this model legume can be used to understand the molecular components and identify candidate genes involved in defense against these pathogens for the cool season legumes [219]. Besides, soybean can serve as model for the many crop species in the Phaseoleae due to its vigorous studies being undertaken for nodulation, mycorrhization, and plant-symbiont signaling [220].

16.6
Integration of Technologies

Although significant efforts were made in the past to adapt the plant to the environment, the emerging concept is to genetically tailor the crops for maximizing resource-capture efficiency, yield, and yield stability. The genomics-assisted crop breeding offers unprecedented opportunities to identify major loci influencing

the target traits and to select for plants with the desirable combination of alleles via marker-assisted selection, marker-assisted backcrossing (MABC), or marker-assisted recurrent selection (MARS). Similarly, the current and fast emerging technologies such as RNAi technology, targeted gene replacement using zinc finger nucleases, chromosome engineering, MARS and GWS, NGS, and nanobiotechnology should be utilized in an integrated way to combat the adverse conditions in legume crops. Although the advances in biotechnology greatly facilitate grain legume improvement, a more comprehensive knowledge of resistance or tolerance mechanisms is required to direct breeding in these crops. Indeed, only a better understanding of the underlying mechanisms activated in response to stresses will allow an efficient application of biotechnology in sustainable agriculture. The advent of the "omic" technologies together with the functional genomic tools is a promising approach to achieve this. Similarly, genetic engineering options for targeting of transgene expression to particular conditions (e.g., using stress-responsive or tissue-specific promoters) can be potentially integrated with proteomic and metabolomic approaches for monitoring the effect of the transgene in order to be able to take advantage of the knowledge being gathered from "omic" technologies. Overall, for biotechnology to fulfill its potential for grain legume breeding, there need to be a synergy between classical breeders and biotechnologists to first ensure that the tools of biotechnology are applied to the most pressing and appropriate problems and, second, to ensure that pathways for delivery/uptake into breeding programs are in place. However, the ultimate objective of effective utilization of the genomic resources, identification of suitable genes for candidate gene-based association mapping and/or to be used as transgenes could only be possible through an integrative approach taking the classical breeding and biotechnological approaches together.

16.7 Conclusion

Genetic diversity is critical for any successful breeding program and genetic resources are important sources of such diversity as well as of traits that permit continued yield increases under climate change scenarios as materials may have evolved under some of the harshest conditions [221]. Despite the known fact that the wild germplasms for different crops have found to be a rich source of resistance to these constraints, introgression of genes for resistance or tolerance into cultivars are a cumbersome jobs due to bottlenecks in crossability. Hence, the present-day agricultural research programs require focus on an integrated genetics and genomics approach to dissect molecular processes from transcriptome to phenome. Where diversity is lacking for critical traits in cultivated species, tapping wild relatives can be employed through wide hybridization to create novel diversity in polyploidy species [222]. Moreover, with the improvisation through conventional breeding approaches, different molecular breeding approaches are also being used to accelerate utilization of the substantial variability among the grain legume

landraces and germplasm lines for various morphological, physiological, and agronomic traits.

However, challenges such as nonavailability of linkage data, low levels of DNA polymorphism within the primary (cultivated) gene pool, and availability of lesser number of molecular markers pose considerable restrictions and hence genome sequencing initiatives would raise newer genetic and genomic resources in grain legumes.

Moreover, transgenic technology is ought to be a possible solution negating constraints to grain legume productivity and improvement, widening the chance of introducing the transgenes for resistance or tolerance to cultivated varieties without compromising their yield potential. Identification of novel promoter and enhancer elements will also be critical to achieving efficacious expression of antifungal/ antimycotoxin genes in grain legumes. In addition to nuclear transformation, development of plastid transformation protocols will enable high-level expression of multiple resistance genes in the transgenic crop development reducing the chances of out crossing.

Grain legume crops are genetically related and therefore exhibit synteny at the genetic and genomic levels and consequently functional similarities at physiological and phenotypic levels. Hence, cross-crop learning has tremendous potential for understanding of genetic and physiological mechanisms and control points for disease and pest resistance, drought and other stress adaptation, nutritional quality, biological nitrogen fixation, and other key traits. Clearly, modern biotechnological interventions have the potential to contribute to more productive and stable grain legume farming systems with increased productivity and income and improved health and resilience to climate change.

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17
Pulse Crops: Biotechnological Strategies to Enhance Abiotic Stress Tolerance

S. Ganeshan, P.M. Gaur, and R.N. Chibbar

Abstract

Pulse crops are leguminous plants whose grains are used exclusively for food. In Asia, Africa, and many developing countries, pulses constitute a major source of dietary protein and extensive efforts are being undertaken to improve pulse production. However, due to global climate change, abiotic stresses are increasingly impeding crop production. Conventional plant breeding has contributed tremendously to the development of improved crop varieties, but other biotechnological tools are needed to complement breeding efforts to accelerate development of pulse crop varieties tolerant to abiotic stresses such as drought, salinity, and high and low temperatures. Genomics resources such as molecular markers have started to expedite marker-assisted breeding and quantitative trait loci (QTL) introgression in chickpea for drought tolerance. Similarly, transcriptomic resources such as expressed sequence tags and expression profiling such as microarrays also contribute to further understand abiotic stress tolerance in pulses and for the development of genic markers. In pulse crops, development of *in vitro* regeneration techniques and transgenics has been slow and more resources need to be allocated to expedite their development. *In vitro* regeneration techniques are also useful for embryo rescue of wide hybrids. Transgenics, although controversial, offer a faster means to develop abiotic stress-tolerant pulse crops. While enhancement of abiotic stress tolerance in pulse crops implies higher returns in the developed countries, in developing countries it will contribute to food and nutritional security and sustainable production. It is therefore encouraging that ICARDA, ICRISAT, and CGIAR (Generation Challenge Programme) invest extensively into using new technologies for improvement of pulse crops in these regions of low-input farming.

17.1
Pulse Crops: Definition and Major and Minor Pulse Crops

A pulse is a leguminous crop harvested solely for the dry seed, excluding the crops that are mainly grown for oil extraction (e.g., soybean and peanut). Being

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leguminous crops, pulses play an important role in crop rotation due to their ability to fix nitrogen. The major pulses include common bean or kidney bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), chickpea, bengal gram or garbanzo (*Cicer arietinum*), cowpea (*Vigna unguiculata*), lentil (*Lens culinaris*), pigeonpea or red gram (*Cajanus cajan*), mung bean or green gram (*Vigna radiata*), urad bean or black gram (*Vigna mungo*), lupins (*Lupinus* spp.), faba bean or broad bean (*Vicia faba*), bambara bean or bambara groundnut (*Vigna subterranea*), vetch (*Vicia sativa*), rice bean (*Vigna umbellata*), moth bean (*Vigna acontifolia*), tepary bean (*Phaseolus acutifolius*), adzuki bean (*Vigna angularis*), lima bean (*Phaseolus lunatus*), and runner bean (*Phaseolus coccineus*). The minor pulses include lablab or hyacinth bean (*Lablab purpureus),* jack bean (*Canavalia ensiformis*), sword bean (*Canavalia gladiata*), winged bean (*Psophocarpus teragonolobus*), guar bean (*Cyamopsis tetragonoloba*), velvet bean (*Mucuna pruriens* var. *utilis*), and yam bean (*Pachyrrizus erosus*).

17.2
Pulse Production: Global and Different Countries from FAOStat

During 2008, the pulses were grown in 71.8 million ha, producing 61.5 million tons of dry seeds with an average yield of 856 kg/ha [1]. The FAOStat provides individual crop statistics only for few pulses and clubs remaining pulses in groups. The dry bean group of pulses (includes common bean, lima bean, adzuki bean, mung bean, urad bean, scarlet runner bean, rice bean, moth bean, and tepary bean) accounts for one-third of the total pulse production. In the remaining production, 16.3% is contributed by pea, 13.8% by chickpea, 9.3% by cowpea, 7.0% by faba bean, 6.7% by pigeonpea, 4.6% by lentil, 1.6% by lupins, 1.6% by vetches, and 5.7% by minor pulses. During 2008, 170 countries grew pulses, but about 79% of the pulse area was in Asia (48.4%) and Africa (30.5%). Americas, Europe, and Oceania accounted for 15.2%, 3.7%, and 2.1% of the area, respectively. The major pulses-producing countries include India (23.2%), Canada (8.1%), China (8.0%), Myanmar (5.8%), Brazil (5.7%), Nigeria (4.8%), the United States (3.1%), Russian Federation (3.0%), Ethiopia (2.9%), and Australia (2.8%). Most of the pulses are consumed within the producing countries and the international trade is about 16% of the total production. The major pulse-importing countries are India (26.3%), Egypt (4.4%), China (4.0%), Pakistan (3.4%), UAE (3.2%), the United States (2.8%), the United Kingdom (2.8%), Italy (2.8%), Turkey (2.8%), and Brazil (2.7%), while the major pulse-exporting countries are Canada (32.3%), the United States (11.5%), China (10.7%), Myanmar (7.8%), Australia (6.3%), and France (5.1%).

17.3
Abiotic Stresses Affecting Pulse Crops

The improvement of crop production in the face of acute global climate changes has become a challenging endeavor. Besides the environmental impact, in many

regions of the world, crop production has been limited due to socioeconomic and political instability. However, advances in genomics research are expected to contribute greatly to alleviate crop production limitations in many of these regions, where, unfortunately, hunger, malnutrition, and poverty are widespread. In the Western Hemisphere, environment and climate change are likely to have the most negative consequences on crop production. Abiotic stress challenges faced by plants include drought, salinity, flooding, metal toxicity (heavy metals), mineral nutrient deficiency, high temperature, low temperature, freezing temperature, UV stress, photoinhibition, and anaerobiosis. Often several of these may affect plants simultaneously, leading breeders to rethink selection strategies for abiotic stress tolerance by considering selection under multiple stresses. While this recognized strategy is likely to be valuable, the complexity of selection for a number of stress tolerance traits can be resource-demanding and time-consuming for a breeding program. Therefore, numerous studies have been undertaken to understand the abiotic stress challenges and how they affect plant performance.

Among the abiotic stresses affecting pulse crops, drought is probably one of the major concerns, especially in the semiarid tropic (SAT) regions such as Asia and Africa, where it is considered one of the most detrimental stresses for pulse crop production [2]. Drought tolerance and water use efficiency are intricately related. Drought refers to the insufficient availability of soil moisture, leading to limitation in the supply of water to a growing plant. In arid and semiarid areas, rainfall patterns tend to be inconsistent, and when combined with high temperatures, moisture for crop growth becomes limiting [3]. Therefore, water use efficiency by crops is a viable strategy to surmount such conditions and breeding and genomics strategies are being developed to maximize this potential [4]. As a result of water deficit and drought, plants are also adversely affected by increase in soil salinity, which leads to disruption of plant water status [5]. Salinity and drought combined pose a major problem to normal plant growth in arid and semiarid regions [6]. While growing salinity-tolerant varieties is useful, it is however important to note that tolerance leads to further retention of salinity levels in the soils, affecting subsequently planted crops [7]. Salinity tolerance is conferred by plant's ability to exclude, as opposed to partitioning of, ions within the plant, without affecting its performance significantly [8, 9]. Proper management practices are therefore necessary to sustain agricultural production under such adverse soil conditions. Cereals are generally more tolerant to saline conditions than legumes [10] and could therefore be used in crop rotations to minimize buildup of salt in soils. In addition, it has also been suggested that evaluation for salinity tolerance be performed at vegetative as well as reproductive stages of growth, since in crops like chickpea salinity affects both these stages and sensitivity is more pronounced at podding stage [11].

Soil fertility can also be limiting for crop production and is due to depletion and degradation of soil nutrients [12]. Therefore, alternative methods for supplementing soils need to be undertaken. Use of fertilizers can be prohibitive for resource-deprived farmers in developing countries and organic supplements are likely to be the best solutions in this situation [13, 14]. When availability of nutrients is low, varieties capable of maximum nutrient use efficiency would be valuable. However,

in recent years, nutrient uptake with relevance to the symbiotic arbuscular mycorrhiza (AM) and its association with abiotic stresses have become important [15]. There have been extensive studies on the beneficial effects of AM:plant associations for sustainable cropping of temperate crops (for details, see Refs [16, 17]) and research applications for tropical agriculture are being undertaken. For example, mycorrhization has been shown to help in the uptake of phosphorus and other nutrients (for details, see Refs [13, 15]) and also allows plants to tolerate abiotic stresses such as heavy metals [18] and salinity [19]. In *C. cajan*, it has been shown that the AM: root association led to accelerated acquisition of phosphorus by a plant [20]. Similarly, in *Glycine max* [21] and *C. cajan* [22], it has been shown that nutrient uptake and growth are improved under salinity stress due to associations with AM. Radiant frost is another common abiotic stress experienced under more temperate climatic regimen for cool season pulse crops such as chickpea, lentil, faba bean, and field pea [23].

17.4
Mechanisms Underlying Stress Tolerance: A Generalized Picture

Plants, being sessile in nature, need to perceive and adjust as needed to abiotic stress challenges. However, plants can withstand only a certain level of stress and once the optimum threshold is surpassed, cellular and metabolic functions become perturbed leading to suboptimal performance. For crops, this suboptimal performance essentially leads to reduced yield. Plants have nonetheless adapted to or have been selected for adaptation to abiotic stresses over time, but further tolerance is required to meet the ever-increasing abiotic stress challenges. Abiotic stresses include those adversities perceived by plants when exposed to drought, salinity, cold, heat, anaerobiosis, heavy metals, light intensity/UV, and nutrient limitations and essentially limit crop productivity. These abiotic stresses in essence disturb the homeostatic equilibrium within the plant (for details, see Ref. [24]). Prior to the availability of genomics tools, a one-gene approach was used to attempt to explain abiotic stress response in a "cause and effect" strategy. However, tolerance to abiotic stresses is complex and in spite of the identification of numerous genes with potential roles in abiotic stress responses, further understanding and dissection of the cascades of events that lead to the ability to withstand such stresses are required. Genome-wide expression profiling approaches have enabled the elucidation of the roles of many of the genes induced in response to abiotic stresses (for details, see Ref. [25]). Interestingly, in leguminous crops, genome-wide studies for abiotic stress tolerance are very few and done on model legumes such as alfalfa and soybean [26–28]. Such studies are important to identify and establish network(s) involved in stress response pathways, which could eventually be manipulated to minimize crop losses due to abiotic stresses [29], especially for pulse crops such as lentil, chickpea, pigeon pea, and common bean. Nonetheless, there have been some recent genomics studies on some of the major pulse crops for abiotic stress tolerance.

Generalized stress responses in plants begin with sensing of a stress by the primary sensor, followed by a signaling cascade of events, with calcium being the second messenger. The occurrence of a multiplicity of physiological, biochemical, and molecular events in response to abiotic stresses is well known, including accumulation of intracellular compounds such as nucleic acids, proteins, carbohydrates, and amino acids. The series of events from the perception of stress to the signal transduction to phosphoprotein cascades and transcription factor activation/suppression leading to induction of stress-responsive genes and allowing the plant to respond to perceived stress either as a defensive or a protective reaction are also well elucidated [30]. However, the initial perception and perturbation of cellular function may vary, depending on the abiotic stresses, although some of the stress response pathways are common to various types of abiotic stress challenges such as drought, salinity, osmotic stress, and cold [31–33]. For example, drought, salinity, and low, high, and freezing temperatures lead to membrane integrity disruption, generation of reactive oxygen species (ROS), accumulation of toxic by-products, photosynthetic process dampening, and nutrient uptake reduction [34]. At molecular levels, cross-talks and specificities of signaling pathways also exist in response to abiotic stresses (for details, see Ref. [35]). In *Arabidopsis*, for example, a histidine kinase was induced in response to salt and considered to be a osmosensor [36]. As a generalized response, elevated levels of calcium in response to a number of abiotic stresses as an early signal or second messenger have also been well established (for details, see Refs [37, 38]). A number of sensors related to calcium-regulated proteins such as calmodulin [39–41], calcium-dependent protein kinases (CDPKs) [42], and calcium-regulated phosphatases [43, 44] have also been associated with signal transduction pathways in response to different abiotic stresses. The CDPKs, however, have been shown to be stress-specific due to the occurrence of many isoforms [45]. Similarly, among the Ca^{2+}/phosphatase interactions, the occurrence of phosphatase isoforms points to stress-specific roles [43]. Thus, due to the multiple roles of calcium sensors by way of different isoforms conferring specificity, they are also central to cross-talks among various abiotic stresses [35].

Another important component of the signal transduction pathways in response to biotic and abiotic stresses and plant hormone signaling and cell division involves the mitogen-activated protein kinase (MAPK) cascades [46–49]. The MAPK cascades have been extensively studied and characterized in higher plants for their roles as signaling molecules [50]. In sequence genome survey of 20 MAPKs in *Arabidopsis*, 10 MAPK kinases and 60 MAPK kinase kinases were identified [51]. The regulation of activity of proteins by MAPKs can occur in two possible scenarios; in the first scenario, an activated MAPK phosphorylates nuclear transcription factors, cytoskeletal components, and/or other kinases; in the second scenario, other regulatory proteins and MAPK components interact physically, irrespective of phosphorylation state [49]. Once the signal transduction pathway has served its purpose and the downstream response has been elicited, inactivation of MAPKs needs to occur and phosphatases reset the signaling pathways by dephosphorylating the MAPKs [52]. Furthermore, phosphatases are able to hold the MAPKs in the cytoplasm or nucleus [53], effectively terminating the signal [54].

Downstream to the signal transduction pathways, induction of transcription factors is invoked, some of which integrate with another level of complexity in the cross-talks among different abiotic stresses. A well-elucidated example of this cross-talk is provided by low temperature, drought, and abscisic acid induction of genes by these transcription factors [55, 56]. The promoters of some of these cold- or drought-induced genes contain CRT (C-repeat element) or DRE (drought-responsive element), respectively, and as characterized in *Arabidopsis* [56–58], are targeted by the *DREB1* or *CBF* transcription factors under low-temperature exposure and by *DREB2* under salt or drought exposure. The cross-talk among the low temperature, drought, and salinity signaling pathways has been the subject of many studies using the *RD29A* gene of *Arabidopsis*, whose promoter contains the C-repeat element [59]. *RD29A* promoter::*GUS* fusion constructs in *Arabidopsis* leaf protoplasts were shown to be induced by low temperature and drought as a result of overexpression of *DREB1* and *DREB2*, respectively, indicating integration of two different signal transduction pathways, leading to the expression of one gene [57]. With the use of global transcriptomic profiling approaches mostly in *Arabidopsis*, it has been clearly demonstrated that under any type of stress, up to 25% of the genome is differentially regulated and that irrespective of the stress applied, a group of 59 genes, 21 of which were transcription factors, were induced [60].

17.5
Strategies to Enhance Abiotic Stress Tolerance: Conventional

There has been tremendous improvement in the productivity of pulse crops over the years due to the availability of genetic resources and breeding knowledge [61]. Although the combination of breeding and diverse germplasms has contributed to yield increments, enhancement of abiotic stresses has proven to be more challenging using conventional approaches. This is mainly due to the quantitative nature of inheritance to tolerance against abiotic stresses. Nonetheless, plant breeders have been able to develop varieties tolerant to some extent to abiotic stress challenges by using various strategies from conventional breeding methodologies to germplasm collections to mutagenic approaches.

17.5.1
Breeding

Plant breeding as an expedited form of evolution has contributed tremendously to the development of new improved crop varieties. It has been viewed as an art at many instances due to the breeder's ability to sometime recognize intuitively certain attributes in breeding lines that would make a variety more suitable. Over time, as emphasis was placed on nutrition, adaptation to environments, quality, and economic return, selection became primordial in initiating plant breeding programs [62]. It can therefore be stated that early gatherers were in effect the first breeders since they intentionally selected for desirable traits such as palatability of

plant products and ease of harvest during the domestication process of plants, without having much of an impact on the general structure of crop plants [63]. Prior to the rediscovery of Mendel's laws of genetics, breeders were deemed to be successful based on their ability to carefully observe and identify variations showing improved qualities for further advancing as varieties [62]. However, despite advances in areas of genomics, plant breeding will continue to be the essence of all crop improvement strategies. Plant breeding essentially involves identification of genetic variability and combining this variability to generate plant types with desirable attributes. However, due to the erosion of genetic diversity, there has been a need to mine for variability from other sources. Thus, from traditional breeding from the use of germplasm resources from wild species, land races, and distant relatives, plant breeders have created new variability by chemical and physical mutagenesis. The phenomenon of somaclonal variation recognized in plant cell cultures [64] also became potential sources of variation.

Application of scientific principles to classical breeding approaches occurred with the rediscovery of the laws of inheritance [65]. Increasing adaptation of cultivars to specific environments was sought by assessing increase in productivity through identification of sources of variability in local germplasm resources or through introduced land races, or through breeding lines from other breeding programs, wild species, or genera. If further variability was needed, plant breeders screened for spontaneous mutations or induced mutations physically or chemically. Such variant genotypes subsequently became parental lines in hybridization experiments and used for qualitative or quantitative trait selections. Depending on the traits under selection, the breeder sets realistic objectives to maximize identification of desired traits in progeny of the crosses. The qualitative and quantitative nature of the traits as well as their heritability needs to be taken into consideration. Thus, plant breeders set objectives in order to determine selection criteria, aid in the choice of breeding method and choice of parents, and indicate when selection must begin. The objectives of the plant breeder should also reflect the end user preferences such as the consumer and the producer so that quality-enhanced and marketable varieties are produced. The general scheme of breeding outlined above is no different in pulse crops [61]. Furthermore, breeding programs have drawn from knowledge gained from model legumes such as *Medicago truncatula*, *Lotus japonicus*, *G. max*, and *P. sativum* to breed for quality and well as for other agronomic traits and biotic and abiotic stresses in more complex and less well-understood pulse crops such as faba bean [66].

It is estimated that 60% of the worldwide common bean (*P. vulgaris* L.) production is drought-prone [67]. At the International Center of Tropical Agriculture (CIAT), a breeding program was initiated for the development of drought-resistant breeding lines. Drought-resistant breeding lines had significantly higher yields than commercial varieties under drought [67]. Similarly, in chickpea (*C. arietinum* L.), which is usually grown under rainfed conditions, drought accompanied by heat stress is limiting to growth and a study was initiated to select genotypes resistant to drought/heat with particular reference to selection criteria [68]. The study found that seed weight was least affected by the drought/heat stress

conditions and had the highest heritability and could possibly be used in early breeding selections [68]. The study also indicated that days to the first flowering and maturity to escape terminal drought and heat stresses should be evaluated before many other phenological traits [68]. Similar to these studies, deeper rooting systems in chickpea have been associated with better coping against drought [69, 70]. Root traits such as rooting depth and root biomass have also been shown as ideal traits to breed for terminal drought stress tolerance in chickpea [71]. In faba bean (*V. faba*), deprivation of water led to increased root growth and root density and both were suggested to be adaptive mechanisms to cope with the limited water supply [72].

17.5.2
Mining Germplasm Resources

Although breeding efforts have generated numerous improved varieties, erosion of genetic diversity as well as limited availability of germplasm resources has led to a need for the identification of new sources of variability in wild species and land races or for *de novo* generation. Therefore, plant breeders started to screen for mutations for incorporation into breeding programs. This practice led to coining of the term "mutation breeding" and has been practiced for almost a century. Naturally occurring mutants have indeed heralded the Green Revolution due to the use of dwarfing genes found in a Japanese wheat cv. Norin-10. High-yielding dwarf rice varieties possessing strong straw were derived from spontaneous semidwarf mutation phenotype in a rice cv. Dee-geo-woo-gen from Taiwan.

In leguminous crops, breeders have sought for variation in land races, wild species, and wild progenitors. However, this has led to a narrow genetic base for improvement of legumes as these belong to the primary gene pool (GP-1) [73]. The secondary gene pool (GP-2), which consists of species that can potentially be crossed with GP-1, has been used for common bean improvement, but has been challenging for lentil improvement [74]. The availability of large collections of pulse crops germplasm resources around the world such as ICRISAT (India), ICARDA (Syria), CIAT (Colombia), and IITA (Nigeria) now offers the possibility of screening for many traits of interest [74] from collections within these resources. While mostly agronomic and quality aspects have been the focus of breeding employing germplasm resources from these centers, mining for abiotic stress tolerance genes has now become an important component. In an attempt to contribute functional markers for allele mining in chickpea germplasm resources, a root expressed sequence tagged (EST) resource was developed and was suggested to be potentially useful for candidate gene identification for abiotic stress tolerance [75].

17.5.3
Variation Creation: Traditional Mutagenesis and TILLING

A significant addition to the tool belt of the plant breeder in the early part of the last century was inducible mutagenesis. Although naturally occurring mutations were

identified as early as the 1900s by de Vries, with their potential for use in breeding (cited in Ref. [76]), it was only when physical mutagenesis by X-rays in *Drosophila* [77, 78] and in barley [79] were generated that the new field of induced mutagenesis research started and quickly became part of the field of plant breeding. To date, 3124 mutant varieties are listed with the FAO/IAEA Mutant Varieties Database [80]. Almost 70% of these mutants were advanced over generations and released as new varieties, while the remaining were used in crosses with other varieties [81]. The first variety to be derived from mutagenic X-ray irradiation was the tobacco cultivar, Chlorina, which was commercially released in 1934 (Tollenaar, 1934, cited in Ref. [76]). About two decades later, a commercial variety of mustard, cv. Primex, was released from the X-ray-induced mutation treatments. Of the direct mutant varieties released, radiation was used in about 89% of these and 75% of the overall mutants in the database were in crops [81]. Mutation breeding for grain legumes has also been widely applied, especially in conjunction with the Joint FAO/IAEA Division [82, 83]. The FAO/IAEA Mutant Varieties Database lists 202 mutant varieties of pulse crops [80] and were generated for mostly agronomic improvement, with a few for abiotic stress tolerance such as drought tolerance in *C. cajan*, cold resistance in *C. arietinum*, and salinity tolerance in *L. culinaris*.

Induced mutations gained new impetus in the genomics era, with the specific targeting of known genes. The TILLING (Targeting-Induced Local Lesions IN Genomes) [84] strategy has become widely applicable for variation mining in crop plants. The TILLING method enables identification of single base pair changes in genes of interest [85]. Seeds are generally mutagenized with ethlylmethane sulfonate (EMS). M1 plants grown from these seeds are selfed, planting individual M2 seeds for DNA extraction and cataloging M3 seeds. PCR amplification for the gene of interest is done on pooled DNA from the M2 plants. PCR products are denatured and upon reannealing heteroduplexes are formed. Denaturing HPLC was originally used to analyze heteroduplexes for mutations [84, 86]. Subsequently, treatment of the heteroduplexes with an endonuclease, CELI, which specifically cleaves mismatches between mutated and nonmutated variant heteroduplexes, was used [87]. CELI, which was extracted from celery, recognizes single base mismatches and cleaves on the 3′-side of the mismatch [88]. Electrophoretic separation of cleaved heteroduplexes allows identification of mutations. Mutant plant is then identified by screening DNA from individual samples constituting the pool. EMS generally causes G/C to A/T transitions and the randomly distributed G/C to A/T transitions in *Arabidopsis thaliana* account for up to 99.5% of mutations [89]. TILLING populations are available for *A. thaliana* [85], *Lotus japonica* [90], maize [91], barley [92], wheat [93, 93–96], and oat [97]. In legume crops, TILLING populations have been slow to be created and have been restricted to the model legume *L. japonica* [90] and to soybean [98]. However, in recent years, TILLING populations have been produced for pulse crops including common bean [99, 100] and pea [101]. In order to extend our capabilities to mine for abiotic stress-tolerant genes in other major pulse crops such as lentil, chickpeas, cowpea, and pigeonpea, TILLING populations need to be developed. For example, in the *Medicago* TILLING population, a leucine-rich repeat, *Srlk* mutant gene, was found not to limit root

growth in response to salt stress [102]. Therefore, TILLING populations for pulse crops will be very valuable to mine for abiotic stress genes.

17.6 Strategies to Enhance Abiotic Stress Tolerance: Biotechnology and Genomics

Plant breeding approaches have resulted in the successful development of a number of different commercial varieties of crops. Even today, plant breeding is pivotal in the development of improved varieties. However, erosion of naturally occurring genetic variability has limited the development of newer cultivars with improved qualities by plant breeders [103]. The toolbox of plant breeders has therefore expanded with the use of novel germplasm resources, made available from both *de novo* as well as from induced sources. More recently, the availability of TILLING populations in many crops has further generated sources of variability for traits of interest, although still rudimentary for pulse crops. The TILLING approach is likely to expedite breeding programs due to the variability of a number of defined mutants for traits of interest. Breeding for abiotic stress-tolerant crops is a major initiative around the world. However, the quantitative nature of inheritance of abiotic stress tolerance renders this task challenging. Genetic mapping strategies are valuable tools for locating genomic regions associated with abiotic stress tolerance and quantitative trait loci (QTL) mapping has become an integral part of breeding efforts for such complex traits. Other technology-driven tools for pulse crop improvement include *in vitro* culture systems and the phenomenon of somaclonal variation and transgene technology. Besides these, the functional analysis of genes involved in abiotic stress tolerance, including EST resources, is important for developing breeding strategies to enhance abiotic stress tolerance.

17.6.1 Genetic Mapping and QTL Analysis

The complex genetic nature of inheritance to abiotic stress tolerance traits such as drought, salinity, and low temperature and the difficulty of phenotyping for such traits under field conditions along with high genotype $\times$ environment interactions were initially daunting [2]. However, with the advent of molecular markers and marker-assisted selection (MAS) tools, there has been an enhanced ability to more effectively select for traits for incorporation into new varieties. More recently, the ability to identify quantitative trait loci markers for polygenic traits has expanded marker-assisted breeding. The repertoire of molecular markers has grown considerably over the years from the tedious RFLP types to the more versatile RAPD- and AFLP-type markers. However, nowadays SSR, SNP, and DArT markers have become widely accepted for use. In leguminous crop breeding, these varieties of markers have been employed with varying combinations for biotic as well as abiotic stress tolerance selection, more success being achieved for biotic stress MAS as in

soybean for resistance against cyst nematode [104] or for resistance against common bacterial blight in pinto bean [105]. Nonetheless, there have been successful reports of MAS for abiotic stress tolerance or toward achieving that goal by way of identification of QTL. For example, selection for drought-tolerant common bean genotypes has been shown using five RAPD markers with improved performance under stressed conditions [106, 107]. In cowpea, QTL associated with drought response phenotypes were identified [108, 109]. Similarly, root trait QTL for drought avoidance were identified in chickpea and will likely contribute to expedite development of varieties for enhanced drought avoidance [71]. Frost tolerance QTL have been identified for faba bean and suggested to be valuable in future for efficient screening of large populations [110]. Several winter hardiness QTL have also been identified in lentil, with one QTL being found to be common to all environments tested [111]. In pea, the flowering locus Hr was found to colocalize with a major winter-frost tolerance QTL, thereby making it ideal for MAS [112]. Similar to drought MAS, limited information is also available for MAS for salinity tolerance in pulse crops compared to other legumes such as soybean. Salt tolerance QTL identified in wild and cultivated soybean were found to be conserved and accounted for a large dominant effect [113]. SSR marker alleles flanking a major salt tolerance QTL were also identified in soybean that could be used for MAS for salinity tolerance [114], including seedling growth [115].

One powerful use of MAS has been suggested for gene pyramiding in the development of crop plants with tolerance to multiple stresses [116] or for introgression of multiple QTLs for a specific abiotic stress [117]. While gene pyramiding and QTL introgression have been shown for cereals for both biotic and abiotic stresses, efforts are currently underway for achieving the same in pulse crops, particularly for abiotic stress tolerance. This was mainly due to lack of abiotic stress-related QTL in pulse crops. In common bean, resistance to rust and anthracnose was developed by using marker-assisted backcrossing (MABC) [118]. Introgression of multiple QTL for root morphological characteristics associated with drought tolerance was shown in rice [119] and it is likely that similar approaches will be successful in pulse crops in the near future as suggested from chickpea root trait QTL studies for drought tolerance [71]. It is foreseen that in the next 5 years with major QTL being identified for abiotic stresses in pulse crops, the relevance of pyramiding and QTL introgression will increase dramatically. In chickpea, a hot spot region that affects several traits (root length density, root biomass, and shoot biomass) contributing to drought tolerance has been identified from two mapping populations (ICC 4958 $\times$ ICC 1882; ICC 283 $\times$ ICC 8261) segregating for root traits (Gaur *et al.* unpublished). This region contributes up to 36% of phenotypic variation in both root and shoot biomass and root length density. Terminal drought is the major constraint to chickpea productivity, particularly in the semiarid tropics where it is generally grown rainfed on residual soil moisture after the rainy season. Root traits, particularly rooting depth and root biomass, are known to play an important role in drought avoidance through more efficient extraction of available soil moisture. This genomic region flanked by SSR markers TAA 170 and ICCM 0249 has been introgressed into three cultivars (JG 11, KAK 2, and Chefe) using marker-assisted

backcrossing. While TAA 170 and ICCM 0249 markers were used for foreground selection, eight AFLP primer combinations were used for background selection. BC3F4 progenies are available and will be evaluated along with donor and recipient parents in both irrigated and rainfed conditions. Marker-assisted breeding for root traits is expected to improve precision and efficiency of breeding for drought tolerance in chickpea.

17.6.2 Transcriptomic Resources

Gene expression profiling approaches have had tremendous impact on obtaining global snapshots of genes under any particular condition of plant growth, be it spatial, temporal, developmental, or environmental. While such expression profiling approaches have been routinely conducted for all major crops for abiotic stress challenges, in pulse crops examples are now only emerging. In chickpea, 2800 root trait and drought-responsive ESTs were developed and annotated [120]. More recently, a set of 20 162 drought- and salinity-responsive chickpea ESTs and gene-based markers have been developed [121]. Similarly, in pigeonpea 9888, Fusarium wilt and sterility mosaic disease-responsive ESTs were developed [122] and the pigeonpea genomics initiative is already starting to provide more resources for pigeonpea improvement [123].

Other transcriptomic approaches have included differential display PCR (DDRT-PCR), cDNA-AFLP, and microarrays, although the latter has been mostly restricted to model legumes for abiotic stress transcriptome profiling. Using DDRT-PCR, drought-modulated gene(s) in common bean were examined and 8.7% of the 1200 DDRT bands were found to be regulated by drought [124]. Similarly, cDNA-AFLP profiling in cowpea during biological nitrogen fixation under heat stress revealed 55 transcripts that were upregulated and nine transcripts that were down-regulated by heat stress [125]. Subsequently, using the transcript-derived fragments as probes against a cowpea heat-stressed root nodule cDNA library, it was shown that two of the full length clones isolated coded for a small heat shock protein gene and a nodulin gene [126]. Studies such as the cDNA-AFLP transcriptome analyses are valuable since they allow gene discovery under abiotic stress challenges, as recently reported in wheat, wherein temporal and spatial specificity of induced transcripts under low-temperature exposure occurred [127]. The cDNA-AFLP profiling is a low-cost alternative for gene discovery, especially in laboratories with limited resources [103] and will be valuable for pulse crop transcript profiling under abiotic stress challenges.

Compared to other crops, microarray-based studies in pulse crops have also been lagging behind. Most microarray studies relating to abiotic stresses have been done in model legumes such as *M. truncatula* or soybean. For example, in *M. truncatula*, a time-course experiment was conducted for salt stress in roots using the Affymetrix Medicago GeneChip and MtED (*Medicago truncatula* Expression Database) was suggested to be a useful resource for studying abiotic stress in other legumes [128]. A similar experiment for root apex responses to salt compared to whole roots

response to salt using a 16 K+ microarray chip showed that there was a 30-fold expression difference in transcription factor genes, suggesting different spatial adaptive response within the roots to soil environments [26]. Microarray analysis of aluminum-stressed root tips of *M. truncatula* revealed novel genes involved in resistance or tolerance to Al [129]. Such studies need to be extended to pulse crops, especially since EST resources are becoming increasingly available.

Next-generation sequencing (NGS) is the most recent technological addition to expediting genome sequencing. Genomic sequencing information is valuable for different purposes such as gene identification and molecular marker development in varieties of interest. With the availability of a reference genome as in *Medicago* [130], single nucleotide polymorphism (SNP) markers can be developed for other varieties. Even if a reference genome is not available, NGS can be performed. For example, in chickpea, using Solexa tags of root tissues of drought-tolerant and drought-sensitive genotypes, 5.2 and 3.6 million reads, respectively, were generated, with the identification of about 500 SNPs [131]. Chickpea transcriptome has also been sequenced with short reads on Illumina Genome Analyzer platform and will be valuable for marker development and gene identification [132].

The role of small RNAs in posttranscriptional regulation of gene expression is now well established, including their roles in abiotic stress tolerance (for details, see Refs [133–135]). A few encouraging reports have recently shown the roles of miRNAs in abiotic stress tolerance in pulse crops. For example, cowpea miRNAs have been identified and their potential roles in salinity stress tolerance due to differential expression in roots have been shown [136]. Stress-responsive miRNAs were also identified in common beans subjected to nutrient deficiency stress and manganese toxicity [137]. Another study has identified and validated miRNAs in different organs of common bean and under growth conditions such as drought, abscisic acid treatment, and *Rhizobium* infection [138]. Recently, eight potential miRNAs from horsegram (*Macrotyloma uniflorum*) were identified by computational mining of EST database at the NCBI and shown to be novel plant miRNAs involved in a variety of responses including environmental stress [139].

17.6.3
Transgenic Approaches

Transgenic approaches, as controversial as they may be, offer perhaps one of our fastest means for the development of abiotic stress-tolerant pulse crops. However, the transgenic technology can also complement functional genomics studies to validate expression of cloned genes related to abiotic stress tolerance. In pulse crops, both *in vitro* culture and genetic transformation were slow to be developed. This was mostly because of heightened interest in cereals such as rice and wheat, because of their important contributions to the energy supply of human beings. Although cereals were generally considered recalcitrant to *in vitro* culture and genetic transformation, successes were achieved due to tremendous resources that were allocated for the production of transgenic cereals. Similar resources and

efforts need to be directed toward pulse crop transformation to increase the efficiency and reproducibility of transformation systems for pulse crops.

Since the first report of *Agrobacterium*-mediated delivery of genes to produce transgenic plants and Mendelian transmission of the transgene in 1983 [140], a number of other gene delivery methods have been reported. Although *Agrobacterium*- and particle gun-mediated delivery are the most popular methods, other methods have also been successfully used to produce transgenic plants for commercial applications and/or basic studies to understand plant growth and development. The availability and versatility of different DNA delivery methods are becoming important for pulse crop improvement, since transcriptomic resources are becoming increasingly available. Furthermore, although sequences of known functions in the databases can be used for homology-based prediction of gene function of unknown sequences, more precise functions of the genes of interest are often difficult to ascertain, except by a transgenic approach [141]. Therefore, the availability of high-throughput gene transfer systems for economically important pulse crops has become highly necessary for rapidly assessing gene function. Such transformation systems are routine in model systems such as *Arabidopsis*, but are still lacking for many economically important crop plants. This is particularly critical for pulse crops that have been relatively recalcitrant to transformation. Of particular potential for pulse crop transformation is the *in planta* transformation system. Such nontissue culture transformation approaches by *Agrobacterium* have been reported in some leguminous crops and are promising for pulse crops transformation. For example, pricked peanut embryo axes were transformed by inoculating them in *Agrobacterium* suspension [142]. Seedlings or flowering plants of *M. truncatula* were also transformed by vacuum infiltration of *Agrobacterium* [143]. The electroporation-mediated transformation of nodal axillary buds of pea, cowpea, and lentil and production of transformed plants are encouraging for further refinement of this strategy for transforming pulse crops [144].

17.6.4
In Vitro Regeneration and Transformation

Notwithstanding the recalcitrance of pulse crops to tissue culture and transformation, there are many successful reports (for details, see Ref. [145]). *In vitro* culture as such, prior to being a target for use in transformation, was used for embryo rescue in wide hybridizations and for *in vitro* selection. For example, interspecific hybrids of lentil were rescued by embryo culture [146, 147]. Similarly, hybrid plants from a cross between *P. vulgaris* L. and *P. lunatus* L. were obtained by embryo rescue and confirmed by rDNA analysis [148]. In chickpea, although limited success for interspecific hybridization and embryo rescue was initially encountered [149], some success has been obtained [150]. Embryo rescue was also used to produce hybrids between *Cajanus platycarpus* × *C. cajan* [151, 152]. Considering that tolerance to abiotic stresses exists in many wild species of pulse crops, hybridization between cultivated species and their wild counterparts, followed by embryo rescue,

is likely to be a strategy worth exploring. Several wild species of *Cicer* have been shown to be tolerant to cold or drought [149, 153, 154]. One such species, *C. pinnatifidum*, tolerant to cold and belonging to the tertiary gene pool, was crossed with *C. arietinum* and the hybrid was rescued by embryo culture [150]. Successful hybrids have also been produced by embryo rescue in cultivated lentil × wild lentil sp. [155, 156]. Hybridization among several *Vigna* sp. and mung bean cultivars, followed by embryo rescue, gave successful hybrids with potential for use in improving these *Vigna* sp. for abiotic stress tolerance [157].

In vitro selection for tolerance to biotic and abiotic stresses was also an area of extensive research in the 1970s and 1980s. Combined with the phenomenon of somaclonal variation [64], *in vitro* selection was deemed to contribute new variation to breeding programs. The idea that plant cells can be treated as microorganisms and subjected to selection pressure existed for a long time and essentially breeders could perform selection on cultured cells and tissues under appropriate selective agent to identify and regenerate plants from tolerant or resistant cell lines. While this approach was widely explored for disease-resistance selection by employing fungal toxins or crude culture filtrates from fungal cultures shown to contain fungal toxins in tissue culture medium, advances for abiotic stress tolerance selection have also been attempted [158]. Abiotic stress was generally applied as NaCl or mixture of salts for salt tolerance selection and as polyethylene glycol (PEG) or mannitol for drought tolerance selection. For example, salt-tolerant *V. radiata* plants were regenerated by selective NaCl pressure [159, 160]. As with transgenic studies, *in vitro* selection studies in pulse crops have equally lagged behind and need to be revisited. Studies on *in vitro* selection for salt tolerance in other leguminous crops such as soybean [161] and alfalfa [162] have been done. Some studies in *Vigna* have used callus for selection to drought by employing PEG in the culture medium [163], but no drought tolerant regenerated plants have been reported to date.

As with other biotechnological approaches for pulse crop improvement, genetic transformation strategies have likewise been slow to be implemented. ICRISAT has taken a leading role in recent years in improving mandated pulse crops for abiotic stress tolerance, especially drought tolerance, and include use of genetic transformation technology [164]. Transgenic chickpea lines overexpressing a mutagenized *pyrroline-5-carboxylate synthetase* (*P5CS*) gene led to elevated proline levels under water deficit in the greenhouse, but no significant effect on yield was observed, although transpiration efficiency was modestly improved [165]. A similar strategy in soybean, but using the *L-Δ^1-pyrroline-5-carboxylate reductase* (*P5CR*) gene, showed elevated accumulation of proline under stress and the better ability to metabolize proline after rewatering [166]. Interestingly, a *P5CS* gene from *Vigna aconitifolia*, altered by site-directed mutagenesis to prevent feedback inhibition of proline [167], was used to produce transgenic tobacco plants with increased drought tolerance [168]. Such studies are encouraging and especially since the *P5CS* gene was cloned from a pulse crop. Other studies have targeted transcription factors regulating expression of many genes upon abiotic stress challenges. The most extensively studied transcription factors are the *dehydration-responsive element-binding/C-repeat-binding* (*DREB/CBF*) from *Arabidopsis* and their involvement in

multiple abiotic stresses [57, 169]. Transgenic peanut plants expressing *DREB1A* from *Arabidopsis* showed increased transpiration efficiency under limiting water availability [170]. Production of transgenic pulse crops tolerant to abiotic stresses is thus possible, but resources need to be allocated to realize this possibility.

17.7 Concluding Remarks

Pulse crop improvement for tolerance to abiotic stresses still needs tremendous resources to be invested to cater to world food security and nutrition. While in the developed countries pulse crop improvement for abiotic stresses will lead to profitable returns on production, in developing countries it will improve food security, nutrition, and sustainable production. Furthermore, due to low-input farming in many developing countries and the occurrence of drought and low soil fertility, the need to develop improved pulse crop varieties is urgent. The Generation Challenge Programme (GCP) (http://www.generationcp.org) under the umbrella of the Consultative Group on International Agricultural Research (CGIAR) is precisely aiming to provide molecular biology-based resources for crop improvement in developing countries. Some of the pulse crops targeted for development of molecular markers for stress tolerance include cowpeas, beans, and chickpeas for the sub-Saharan African and South Asian regions. Such programs and those undertaken by ICARDA and ICRISAT are likely to provide much required impetus to pulse crop improvement for abiotic stress tolerance. However, there is a need for more international partnership, especially from developed countries already extensively researching pulse crop improvement. The accessibility of next-generation sequencing is opening up genomics resources hitherto not completely utilized and such resources will be valuable to expedite pulse crop improvement. In the short term, transgene technology needs to be explored and adopted to deliver transgenic pulse crops with abiotic stress tolerance.

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Section IIIC
Rosaceae

Improving Crop Productivity in Sustainable Agriculture, First Edition.
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18
Improving Crop Productivity and Abiotic Stress Tolerance in Cultivated *Fragaria* Using Omics and Systems Biology Approach

Jens Rohloff, Pankaj Barah, and Atle M. Bones

Abstract

Strawberries, especially the hybrid *Fragaria* × *ananassa*, are a popular high- value crop due to the pleasant taste and aroma as well as the content of health-promoting/beneficial phytochemicals. World production of strawberry in 2008 was approximately 4130 metric tons cultivated on more than 250 000 hectares and the production is increasing. Strawberries are cultivated in a broad range of climatic zones from subtropical to temperate and boreal regions, although most commercial production is limited to areas with seasonal temperate temperatures. *Fragaria* is relatively tolerant to drought, but production is prone to losses during storage and transport and environmental conditions like wet weather, low temperatures, and pathogen attacks.

The draft genome of wild strawberry (*Fragaria vesca*) was published in 2011. Together with other sequence data, this opens up for studies at functional and comparative genomics level using global techniques like transcript, protein, and metabolite profiling. Using case examples, we present possibilities for genome-wide approaches to obtain a deeper mechanistic understanding of abiotic tolerance in *Fragaria*. Furthermore, we discuss how genome-wide studies have opened up possibilities for studies of stress responses and identification of potential targets for breeding.

These rapidly evolving genome-wide techniques generate large data sets, and data processing and modeling depend on corresponding computational methods to integrate, analyze, extract, and visualize data and results. We discuss the potential of *Fragaria* crop systems biology and how these techniques and methods can be used for improvement of *Fragaria* crop productivity and abiotic stress tolerance.

18.1
Introduction

The cultivated strawberry *Fragaria* × *ananassa* Duch. belongs to the agriculturally and economically important plant family of Rosaceae and is regarded as a significant berry crop worldwide because of its popular taste, flavor, and abundance of nutrients. World

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production increased by 30% between 1999 and 2009 [1]. Total production in the USA, the largest strawberry producer, accounted for more than $2 billion in 2008. However, recent data presented at the 7th International Strawberry Symposium in Beijing February 2012 showed that China now is the biggest producer of strawberries. In comparison to other Rosaceae crops, strawberry yields quite high producer prices (USA 2008: $1858/t), only approached by the more valuable sweet cherries, almonds, and raspberries. Moreover, under optimized conditions, area yield surpasses even the high-productive apples, pears, and peaches, which realize only one-third of the price of strawberries; hence, the economic significance and sales potential of strawberries in many countries.

Marketed freshly, strawberries show a high degree of perishability in the postharvest period due to the berries' soft fruit character and thus economic losses are often recorded during field production as a result of suboptimal growth parameters and plant stress related to abiotic and biotic factors. While the latter might be addressed by agricultural practice, the influence of unfavorable environmental conditions with regard to light, temperature (UV, heat, and cold), water, and soil (drought and salinity) is less predictable and controllable. Plants respond toward abiotic stress through initialization of signaling processes, which trigger transcriptional regulation and gene activation, followed by the induction of tolerance and/or resistance mechanisms. Growth performance and berry yield therefore strongly rely on the plant's plasticity and evolved homeostatic control systems to maintain essential biological functions in order to circumvent stress and so eventually survive. Cold response and freezing tolerance in perennial *Fragaria* species might serve here as an example of an abiotic factor being especially addressed by breeders in temperate and cold-temperate zones due to short growing seasons and harsh climatic conditions [2]. Though *Fragaria* varieties have even been developed for production under colder climates, the freezing tolerance of strawberry plants is still rather limited. For the facilitation of knowledge transfer and improvement of the *Fragaria* breeding progress, the diploid woodland strawberry (*Fragaria vesca* L.) has been adopted as a plant model species [2, 3], since vast genomic information based on cDNA libraries from cold-, heat-, drought, and salt-stressed *F. vesca* has been made available in recent years [4]. Moreover, its comparatively small genome [5], plant size, vegetative and seed propagation, and fruit production further establishes *F. vesca* as an attractive model. Woodland strawberry is widely spread across the Northern Hemisphere from subtropical to subarctic zones, and can be found at altitudinal levels up to 3000 m. Thus, a huge variety of environmentally adapted phenotypes is available for utilization in functional, structural, and comparative genomic studies on *F.* × *ananassa*. Diploid and octoploid *Fragaria* are characterized by a high degree of genome colinearity [6]. Processes and mechanisms induced upon plant stress, such as cold, are highly evolutionarily conserved throughout the plant kingdom [7], and biological information gathered from either *F.* × *ananassa* or *F. vesca* is likely to be resembled by both species.

Modern high-throughput and high-resolution systems provide advanced analytical instrumentation for the dissection of the complexity of plant responses to abiotic stress through generation of vast genomic, proteomic, and metabolic information. Such information from sequencing and profiling platforms needs to

be subsequently processed regarding statistical treatment, mapping, integration and modeling, and finally, data storage and inquiry. The interdisciplinary field of systems biology has established the necessary tools and methods in order to build the bridges between various data resources and to make comprehensive biological information accessible – also in terms of crop biology [8].

Here, we present a recent update on strawberry production and breeding with special emphasis on the impact of abiotic factors on plant performance and yield. Omics approaches and the use of systems biology for potential crop improvement in strawberry are particularly emphasized, and recent developments and innovations in *Fragaria* breeding have also been presented.

18.2
Abiotic Factors and Agronomic Aspects

18.2.1
Botany and Agricultural History

18.2.1.1 Botany and Distribution

The Rosaceae family contains almost 100 genera [9]. It includes quite significant fruit and nut crops worldwide, namely, raspberry and blackberry (genus *Rubus* L.), cherry, peach, apricot, almond (*Prunus* L.), pear (*Pyrus* L.), apple (*Malus* Mill.), and others. The most recent taxonomy of the strawberry genus (*Fragaria* L.) comprises 20 species, in addition to 3 wild hybrids and 2 cultivated hybrids [10]. As an evolutionary quite young genus that evolved about 3 Mio years ago [11], *Fragaria* shows a modest diversity compared to the much older and closely related genus *Rubus* that has more than 300 species. Though strawberries have been popular throughout the agricultural history due to their nutritionally notable and highly flavorful soft fruits, their utilization and cultivation for food purposes are restricted to a few species of economic importance (Table 18.1). The most prominent member is the cultivated octoploid strawberry (*Fragaria* × *ananassa* Duch.), which evolved through interspecific hybridization of the Chilean strawberry (*Fragaria chiloensis* (L.) Mill.) and Virginia strawberry (*Fragaria virginiana* Mill.) in Brest, France, in the late eighteenth century.

Due to improved berry size, plant vigor, and taste, the new hybrid *F.* × *ananassa* quickly spread to other parts of Europe (England and Germany) and also to the United States. Selection and breeding led to the first varieties in the beginning of the nineteenth century, and breeding programs were established in agriculturally leading countries of the Northern Hemisphere after 1900. Though other species such as *F. chiloensis*, *F. virginiana*, *F. moschata*, and also *F. vesca* had been domesticated and grown for centuries before the arrival of the new hybrid, and are still cultivated, they were eventually displaced. The cultivated strawberry carries an advantageous combination of traits due to heterotic effects, which were inherited from their parental species. Besides superior plant growth and berry yield, cold tolerance and disease resistance have been inherited from the genetic background of

Table 18.1 Economically important strawberry species worldwide.

Plant name	Botanical name	Ploidy	Distribution	Characteristics
Woodland strawberry	*F. vesca* L.	2	Europe, North Africa, temperate Asia, and North America	Small berries with characteristic, intense aroma; varieties used in small scale for jam production and culinary purposes
Musk strawberry	*Fragaria moschata* Duch.	6	Temperate regions of Asia and Central, South, and Northern Europe	Varieties used for culinary purposes; medium-sized berries are very flavorful with intense strawberry and fruit aromas
Chilean strawberry	*F. chiloensis* (L.) Mill.	8	Pacific coastal regions of North and South America, Argentina, and Hawaii	Wild parental of cultivated strawberry, glossy leafs, medium-sized less tasteful berries, whitish flesh, still grown in South America
Virginia strawberry	*F. virginiana* Mill.	8	North America	Wild parental of cultivated strawberry, medium-sized sweet and tasteful berries
Cultivated strawberry	*Fragaria* × *ananassa* Duch.	8	Main cultivation areas in the Northern Hemisphere, in warm and cold-temperate climates (see Table 18.2)	Hybrid between the octoploids *F. chiloensis* and *F. virginiana*, vigorously growing plants, large and tasty berries
Vescana hybrid	*Fragaria* × *vescana* Rud.Bauer and A. Bauer	10	Small-scale cultivation and culinary purposes in Central and Northern Europe	Hybrid cross between diploid *F. vesca* and octoploid *F.* × *ananassa*, small but very flavorful berries

F. chiloensis and *F. virginiana*. These diverse species have today advanced as prime genetic resources for the reassembling of *F.* × *ananassa* and the potential development of new, tolerant varieties, as is later discussed.

18.2.1.2 Nutritionals and Phytochemicals

For both consumers and industry, not only the sugar and acid content but also the characteristic aroma and flavor composition of strawberries are regarded as valuable fruit traits. In addition, strawberries are a significant source of so-called

antioxidants exhibiting functional roles in plant growth, metabolism, defense, and stress response and showing biological and health-related activity in human nutrition [12]. The beneficial quality of strawberries is mainly based on the abundance of phenolics [13, 14], comprising anthocyanins, hydroxycinnamic, hydroxybenzoic, and ellagic acid structures, and ascorbic acid. The biosynthesis of core metabolites within the phenylpropanoid pathway of *F.* × *ananassa* has been intensively researched in recent years [15]. Generally, the significance and abundance of bioactive molecules are expressed by their summarized antioxidant activity, since these compounds are supposed to play an important role in the chemopreventive activity against several human diseases [16]. Abiotic factors such as light intensity and quality [17] and temperature [18] directly affect flavonoids and other phenols in strawberry fruit. Several studies point out the overall determining effect of the genotype on phenol content and composition compared to the environmental and agricultural influences [19, 20]. This is also true for the composition of strawberry aroma compounds that are highly variety dependent, although environmental factors such as weather conditions and agricultural practice strongly influence aroma patterns in berry fruit [21].

18.2.1.3 Economic Aspects of Production and Environment

Major regions of strawberry production worldwide are restricted to the subtropical, humid temperate, and humid cold climates of the Northern Hemisphere (Table 18.2). *Fragaria* breeding has advanced throughout the past century allowing effective development of varieties being optimalized for climatic conditions as shown, for example, by the US breeding efforts leading to regional selections for the coastal plains, Mid Atlantic and Midwest, and California [22]. This also implies that high variation in yield, when comparing country data, cannot be necessarily deduced from climatic factors alone, and it should be seen in the background of regional strawberry production systems –since farmers generally have access to highly productive genotypes, and superior varieties are often grown in several countries or even different continents. However, distinct parameters such as annual sunshine hours and mean temperature have to be considered main impact factors, even though correlations with yield data appear rather weak (corr. = 0.4; data not shown).

Unpredictable hard winters and rainy seasons in boreal regions might severely impair strawberry performance in one or even several seasons, while negative effects of, for example, drought in warmer climates can still be compensated by appropriate irrigation management. In other words, the yield potential of the same variety grown in different geographic and climatic locations can be fully exploited only if the variability of critical abiotic factors is minimized – that is, under conditions that are rather fairly fulfilled in the Nordic regions of Europe and North America. This aspect was also highlighted by recent studies under the EU-COST network "EUROBERRY No. 863," showing a latitudinal gradient from Northern to Southern Europe with increasing berry yield [23]. Thus, economically significant regions of efficient large-scale production are mainly located in the United States and Mexico, Central and Southern Europe, North Africa, and East Asia.

Table 18.2 Production, economy, and climatic parameters in strawberry production.

Country	Production (Mt)	Area (ha)	Yield t/ha	US$/t (2008)	Latitude (range)	Σ Light[a] (kWh/m^2)	Day length[a] (h)[b]	Mean Temperature[a] (annual °C)	Climate (Köppen–Geiger)
Europe	1 338.1	161 987	8.3	2 819					
Spain	263.7	7 100	37.1	2 288	26–45N	1500–1900	14–15	5–20	BSk, Csa, Csb
Poland	198.9	53 551	3.7	1 080	49–55N	1000–1100	16–17	5–10	Cfb
Russia	158.0	23 000	6.9	2 011	41–65N	1000–1300	15–19	0–15	Dfb
Germany	150.1	12 800	11.7	3 625	47–55N	1000–1300	15–17	5–10	Cfb
United Kingdom[c]	87.2	4 656	18.7	3 851	49–61N	900–1100	16–18	5–15	Cfb, Cfc
Ukraine	57.9	8 200	7.1	1 496	44–53N	1100–1400	15–17	5–10	Dfb
Italy	56.4	3 100	18.2	2547[d]	35–48N	1300–1800	14–16	5–20	Cfb, Cfc, Csa
Belarus	50.4	7 800	6.5	2 348	51–57N	1000–1200	16–18	0–10	Dfb
France	45.0	3 000	15.0	3 198	41–51N	1100–1600	15–16	5–15	Cfb, Csc
The Netherlands[c]	41.0	1 700	24.1	5 098	50–54N	1000–1100	16–17	5–10	Cfb
Belgium[c]	40.0	1 067	37.5	3 271	49–53N	1000–1100	16–17	5–10	Cfb
Serbia	35.8	7 916	4.5	1 050	41–47N	1400–1600	15–16	5–15	Cfb
Romania	22.0	2 507	8.8	1 413	43–49N	1300–1500	15–16	5–15	Cfb
Austria	17.1	1 253	13.7	2 950	46–50N	1100–1300	15–16	5–10	Cfb
Czech Republic[c]	12.5	2 467	5.1	2 751	48–51N	1100–1200	15–16	5–10	Cfb
Sweden	11.8	2 000	5.9	2 918	55–70N	800–1000	17–19	0–10	Cfb, Dfb
Finland	11.6	3 270	3.5	5 707	59–71N	800–1000	18–19	0–5	Dfb, Dfc
Norway	9.8	1 624	6.0	4 904	57–81N	800–1000	17–19	0–10	Cfb, Dfc
North America	1 289.9	26 673	48.4	2 338					
USA (contiguous)	1 270.7	23 504	54.1	1 858	27–48N	1500–2000	14–16	5–25	Cfa, Csa, Csb
Canada	19.2	3 169	6.1	2 817	41–84N	1400–1600	15–19	0–10	Dfb

Asia	771.0	32 456	23.8	2 073					
Turkey	292.0	12 150	24.0	1 360	35–43N	1500–2000	14–15	5–20	Cfa, Csa, Csb
South Korea[c]	203.2	6 665	30.5	1 911	33–39N	1300–1500	14–15	5–15	Cfa, Cwa
Japan	185.0	6 000	30.8	8 137	24–46N	1300–1600	13–15	0–15	Cfa, Dfb
Iran[c]	28.5	2 233	12.7	688	24–40N	1600–2200	13–15	10–25	BSh, BWh
Israel	23.6	800	29.5	2 787	29–34N	2000–2100	14	15–25	BWh, Csa
China	13.1	1 091	12.0	1 295	23–55N	1000–2000	13–17	5–20	Cfa, Cwa
Central and South America	355.7	15 345	23.2	1 578					
Mexico[c]	207.5	6 176	33.6	744	14–33N	1700–2000	13–14	15–25	Aw, BSh, BWk, Cfa
Colombia	43.1	1 249	34.5	1 857	5S–14N	1400–1900	12–13	15–25	Af, Am, Aw
Chile[c]	40.0	1 546	25.9	n.a.	17–56S	1100–2000	13–17	5–20	BWk, Cfb, Csb
Peru	20.0	1 180	16.9	321	4–18S	1700–2200	12–13	5–20	BWh
Venezuela[c]	16.4	2 622	6.3	2 311	0–13N	1600–2100	12–13	20–25	Am, Aw, BSh
Africa	344.5	15 999	21.5	1 011					
Egypt	200.0	12 000	16.7	278	22–32N	2100–2400	13–14	15–25	BWh
Morocco	130.0	3 000	43.3	1 041	27–36N	1800–2000	14–15	10–25	BSh, Csa
Australia/NZ[c]	33.2	1 440	23.1	4 250	9–53S	1300–2200	13–17	5–25	BSh, Cfa, Cfb
Total	4 132.4	253 900	148.3	14 069					

Climate – A: humid equatorial, B: arid, C: humid temperate, D: humid cold, W: desert, S: steppe; Precipitation – f: fully humid, s: summer dry, w: winter dry, m: monsoonal; Temperature – h: hot arid, k: cold arid, a: hot summer, b: warm summer, c: cool summer; n.a. recent data not available.

a) Cultivation areas

b) Day length June or December.

c) Production 2008.

d) Producer price 2007.

18.2.2
Abiotic Factors in Strawberry Production

While natural factors such as light, temperature, rain, and O_3 and CO_2 levels in open-field cultivation are controllable only to a limited extent, if at all, cultivation factors such as fertilization, organic matter, and soil pH can be easily modified due to current agricultural practice. The development of new strawberry varieties with enhanced tolerance to abiotic stress mainly strives to improve the plant's adaptability to unfavorable conditions and extend the geographical range of cultivation and thus the profitability of strawberry production. For this reason, the next sections will mainly focus on abiotic stress, tolerance mechanisms, and deduced breeding goals in *Fragaria* with regard to natural environmental factors and their impact on strawberry plant growth, production, and survival. The abiotic aspects related to postharvest and shelf life have not been dealt with, and the reader is referred to relevant reviews [21, 24]. However, the basic effects of the abiotic environment will be presented later, and the next section will address biological processes and mechanisms in more detail in the context of *Fragaria* breeding.

18.2.2.1 **Light**

Due to the photosynthetic activity of higher plants, light has to be considered the most important abiotic factor in strawberry production. Light directly affects plant growth with regard to the production of large amounts of biomass and berry fruit, containing both nutritional compounds (sugars and acids) and phytochemicals (flavonoids and other phenolic structures and ascorbic acid). In crop biology, the photosynthetically active radiation (PAR) in the spectral range of 400–700 nm and the photosynthetic photon flux density (PPFD) (expressed as $\mu mol/(m^2\ s)$) constitute essential production measures. In combination with temperature, the elevated solar radiation and the sunshine hours positively affect not only the strawberry production but also the biosynthesis and levels of fruit phytochemicals [25]. Increasing PPFD *per se* does not necessarily constrain plant growth and cultivation; however, short-day conditions (12 h daylight) as well as high temperatures and transpiration rates under tropical climates practically limit sustainable strawberry production to subtropical, temperate, and boreal regions. On the other hand, extreme long-day conditions during the summer season with up to 24 h of light in regions above the Arctic Circle in the Northern Hemisphere do not restrict berry production [26]. Due to *Fragaria*'s environmental plasticity and the development of suitable *F.* × *ananassa* varieties, strawberries generally show a high degree of adaptability to different light and temperature conditions. Based on the genetic variation of the parental species, *F. chiloensis* and *F. virginiana*, the so-called June-bearing, ever-bearing, and day-neutral strawberries have been developed. The short-day response in June-bearers has recently been shown to be strongly inherited from *F. chiloensis* [27], whereas the contribution of the parental species to the long-day response in ever-bearing varieties is yet to be determined [28]. Although natural light quality with respect to spectral composition is considered a minor factor, field experiments have shown that spectral color shifts when using red-colored mulch can improve berry quality

(size, sugar, and aroma), presumably due to altered phytochrome-mediated gene expression [29]. In contrast, enhanced levels of UV-B and UV-A light might negatively impact the photosynthesis and thus plant growth [30] and hence have to be addressed in the background of climate change.

18.2.2.2 **Temperature**

Though *Fragaria* can be considered a tolerant genus toward climate extremes, the range of successful strawberry production is restricted to geographical zones with seasonal temperate temperatures. On the other hand, both diploid *F. vesca* and the octoploid parentals *F. chiloensis* and *F. virginiana* show a high degree of adaptability to cold, temperate, and warm climates. Unfavorably high temperatures favor photorespiration in *F.* × *ananassa*, a C_3 plant, and increase plant water loss. Chilling stress, as known from many subtropical and tropical plant species, plays a minor role in strawberry cultivation. However, one has to consider the growth-enhancing effect of chill temperatures especially on day-neutral and June-bearers, which hardens plants toward temperature fluctuations in the growing season and thus improves fitness. This is particularly true for cultivation in moderate to warm regions, where night temperatures might still drop below zero degrees in spring time and potentially lead to flower damage during blooming. In contrast, in strawberry production in Central and Northern Europe, plants remain dormant throughout the winter season and partly experience longer periods with mild to severe frost temperatures. In order to survive, these plants are naturally hardened by a gradual temperature decrease (cold acclimation) during autumn, in combination with altered light conditions switching from long- to short-day conditions. Although frost-tolerant varieties have been developed, both the meristematic tissue of overwintering crowns and the roots might be irreversibly damaged. Thus, winter-frost, snow cover periods in combination with short vegetation periods are the limiting factors for strawberry cultivation in the Northern Hemisphere.

18.2.2.3 **Water**

In natural environments, *Fragaria* species show a remarkable tolerance toward drought and can be found not only in the open sunny locations with water deficit but also in locations with excess of soil water and, simultaneously, conditions of O_2 deprivation. When the available soil moisture drops below a critical point, plants respond by closing their stomata upon stress-induced signaling of the phytohormone abscisic acid (ABA) and thus decrease transpiration and water loss. Though strawberry tends to lose cell turgor pressure quite rapidly compared to other plant species, showing obvious signs of wilting, it is capable of recovering from extended periods of dehydration without irreversible cell death. In addition, water utilization by strawberry plants might be improved under elevated CO_2 levels, as shown in Ref. [31]. Water might be considered a less important stress factor in many regions, since large-scale strawberry cultivation relies on automated drip irrigation systems, often in combination with tunnel production and sprinklers, to prevent plants from severe drought. As a consequence of climate change with temperature increase and variation of atmospheric gases, however, today's crop

production is challenged. Water deficiency is particularly critical during flowering and fruit set when plants rely on sufficient nutrient supply from the soil or growth substrate, and large amounts of assimilates are translocated in the plant from the leaves to the fruits. Thus, dehydration might lead to a decrease in berry yield [32], but the effect highly depends on the genotype as shown for *F.* × *ananassa* [33].

18.2.2.4 **Soil**

Fragaria species are generally adapted to grow even in soils with natural high acidity far below pH 5 or in calciferous habitats with high pH. However, availability and root uptake of nutrients either as cation or anion depend highly on soil pH (and other edaphic factors). In terms of strawberry cultivation, the soil optimum ranges between pH 5–6.5 [34], depending on the chosen variety, in order to maintain optimal plant growth and to fully utilize the plant's yield potential. Increased soil salinity, another soil-related stress factor, plays a significant role in warm temperate and subtropical regions, especially in coastal belts and under the use of irrigation water, and might negatively affect strawberry plant growth, yield, and taste. Though *F.* × *ananassa* can be considered a Na^+ excluder, that is, not accumulating sodium under elevated salt levels, plant performance is clearly reduced [35]. Heavy metal concentrations in soils might also be regarded as an abiotic factor influencing plant growth of *Fragaria* species. Highly contaminated locations favor the uptake of heavy metal ions and potentially lead to unacceptable concentrations of, for example, Cd in berry fruit [36], though *in planta* distribution and levels in edible plant parts do not necessarily correlate with levels in the soil due to potential atmospheric pollution [37]. However, other health-related aspects rather than plant stress constrain the cultivation of strawberry or other food crops in such locations, and leave the issue to the potential breeding of low heavy metal accumulating *F.* × *ananassa* varieties and the selection of suitable field locations and organic fertilizer application methods.

18.2.2.5 **Atmospheric Gases and Airborne Contamination**

Enhanced CO_2 levels due to environmental change or agricultural practice (tunnel and greenhouse production and use of organic mulching) do not negatively impact the *Fragaria* growth and yield or even improve the plants' water utilization, as already mentioned. The interested reader is referred to the excellent life cycle study of strawberry production systems in the United Kingdom [38], showing, among other aspects, the enhancing effect of organic production on plant canopy CO_2 levels. Octoploid hybrids show a relatively high photosynthetic capacity, compared to wild species of lower ploidy level [39], whose species are naturally adapted to lower light levels when growing in mixed plant communities. Elevated ozone levels, on the other hand, might impair plant growth severely through a reduction of leaf area, potentially decreased berry yield, and indirectly, by affecting the following seasons' berry production [40]. The same group had earlier shown that *F.* × *ananassa* varieties differentially compensate for ozone stress due to enhanced Ca concentrations in leaves [41]. Ozone levels in industrialized regions of the Northern Hemisphere in particular have been increasing since the past decades, reaching levels of

up to 60 ppb during summer seasons [42], and thus pose a real threat to crop production in the future. Furthermore, anthropogenic entries of SO_2 and NO_x into the atmosphere have been drastically diminished in the past 30 years, and thus, fortunately, almost abolished the problem of acid rain in crop production and forestry.

18.2.2.6 Abiotic Stress Alleviation through Agricultural Practice

Though agricultural practice will not be discussed in detail here, it is worthwhile mentioning several topics in the context of plant abiotic stress, since practically applied treatments are rooted in the study of basic plant molecular and physiological processes. One important aspect is the prehardening of waiting bed and plug plants through periods of chill temperature, not only for flower induction but also for improved cold tolerance. More recently, so-called *priming* methods have been investigated in order to increase the plants' tolerance toward biotic and abiotic stress, including the treatment with synthetic or natural chemicals – ABA against cold stress [43], BTH against *Phytophthora* [44], betaines for plant vigor [21], and the potential of root inoculation with mycorrhizal fungi against drought stress [45]. Moreover, positive effects of externally applied plant hormones have also been reported for the alleviation of water stress by methyl jasmonate [46], and salicylic acid [47] and calcium and potassium nitrate treatment against salt stress [48].

18.2.3 *Fragaria* Breeding toward Abiotic Factors

In the background of the above-mentioned abiotic stress-related issues in the genus *Fragaria*, the interest in relevant breeding goals related to abiotic stress tolerance might differ from that in other significant crop plants. Though strawberries have to be considered an economically and health-related interesting agricultural product, a vast extension of existing cultivation regions seems not to be realistic, not least due to the agronomical competition with necessary staple crops for human nutrition. However, market-oriented reasoning might drive the development of new varieties, including innovative hybrids and genetically modified (GM) plants, and the potential expansion into marginal areas with suboptimal environmental conditions. The following gives a brief historical overview and a recent update of breeding achievements in strawberry, focusing mainly on abiotic parameters in the economically most important species *F.* × *ananassa*, *F. chiloensis*, and *F. virginiana*.

In terms of berry production, quality traits play a more pronounced role in *Fragaria* breeding above overall yield, in contrast to many other crop plants. Due to their soft fruit character, strawberries are highly perishable and shelf life quality is a significant breeding trait. But in practice, several traits are ideally combined when it comes to the final selection of new elite cultivars in order to address the demand of suitable plant characters with regard to both plant performance and fruit attributes.

18.2.3.1 Cultivation and Berry Production

Today, major breeding efforts in Europe, the United States, and also in Australia and Asia, have been made to develop varieties that are adapted to regional climatic

and other environmental conditions in order to facilitate or establish profitable strawberry production. Besides yield, fruit shape, color, and harvest-related fruit traits, the innate tolerance and resistance mechanisms toward multiple abiotic and biotic stress factors have particularly been of major concern in strawberry breeding. Typically resistant selections have been made in order to come up with varieties showing strong resistance to one or several types of threatening pathogens such as red stele (*Phytophthora fragariae*) and crown rot (*Phytophthora cactorum*), anthracnose (*Colletotrichum acutatum*), wilt (*Verticillium dahliae*), gray mold (*Botrytis cinerea*), and powdery mildew (*Sphaerotheca macularis*). Another major aspect, the daylight response in *F.* × *ananassa* and the development of suitable short-day and long-day plants and everbearers, has already been discussed. The Californian short-day "Camarosa" might serve as an example due to its international success since this variety was specifically produced for the warmer East coast climates, but simultaneously also grew vigorously in Mediterranean regions and was readily adopted by Spanish and Italian strawberry producers. Furthermore, the aspect of winter hardiness, especially demanded by farmers in temperate and cold-summer regions, eventually led to the development of the "Korona" variety, which has been profitably grown in many Central European and Scandinavian countries for the last two decades.

18.2.3.2 Fresh Market Quality and Consumer Demand

The content of nutritional compounds, phytochemicals with antioxidant activity, and the fruit's pleasant aroma have always been significant traits in *Fragaria* breeding. The balance between sweetness and acidity and the berries' organoleptic properties are important test parameters when it comes to the selection of breeding material. However, one-sided breeding focus toward fruit firmness and shelf life, as discussed in the next section, causes simultaneously a decrease in strawberry aroma and flavor. Demand by consumers and the food industry, however, has reversed this trend with intensified focus on aroma traits [49] and molecular aspects of biosynthesis [50] of strawberry phytochemicals [12].

18.2.3.3 Postharvest and Food Chain

High product prices and thus economic interest in supplying markets outside the production areas led 80 years ago to the development of varieties with increased firmness and skin toughness as well as with acceptable flavor, color, shape, and size. Today, Spain, the United States, Mexico, Belgium, the Netherlands, France, Italy, Turkey, and Morocco [1] are the most important exporters, strongly relying on the use of shipping varieties. Important European types are "Elsanta" (1975), "Sonata" (1998), and "Figaro" (2001), while "Camarosa" (1993), "Aromas" (1996), "Chandler" (1983), and "Selva" (1984) are famous varieties derived from the US breeding programs.

18.2.3.4 Processing and Industry

The launch of the winter-hardy German variety "Senga Sengana" in 1954 started the success story of *Fragaria* breeding efforts. Berries were sold on the fresh

market, and more importantly, the fruit's deep color, strong flavor, and suitable texture made it an ideal raw material for the upcoming freezing and canning industry, and the variety has been grown all over Central and Northern Europe for decades. Nowadays, strawberries are used in many kinds of processed foods from juice, coulis, to dairy products and breakfast cereals. Requirements by specialized branches, for example, by ice cream producers in the United States, might even lead to the development of customized varieties to meet the industry's demand for particular berry qualities.

18.2.3.5 **Classical Breeding of Varieties and Hybrids**

As already intensively discussed, one major drawback of traditional breeding in *Fragaria* has been the one-sided focus on fruit-related traits (yield and shelf life). Other issues complicating breeding efficiency include the complexity and interconnection of traits in view of environmental variables, and finally, the compulsory introduction of plant characteristics from the same species (cross-breeding) or close relatives of the same plant family (hybridization). An important aspect is the efficient and suitable selection of plant material in order to advance breeding processes and variety development for abiotic stress tolerance as in the case of salt stress [51], drought [32], heat [52], and freezing tolerance [2, 53].

Recent genomic approaches for the improvement of agricultural crops are based on technologies and methods in order to understand the genetics behind traits and to generate and utilize functional, structural, and comparative genomic information for breeding purposes, namely, marker-assisted breeding (MAB) and genetic modification (GM).

18.2.3.6 **Marker-Assisted Breeding (MAB)**

While marker-assisted selection (MAS) refers only to the utilization of markers for the purposeful selection of parentals or seedling, MAB implies the use of markers in several steps of the breeding process. DNA fingerprinting and detection of molecular markers, for example, microsatellites and SNPs, allow plant breeders to screen plant populations toward the presence or absence of gene(s) and thus desirable trait(s), based on molecular biological methods rather than on visual observation of trait expression in the plant, as reviewed in Ref. [54]. Important genomic accomplishments in recent years include the publication of the *F. vesca* draft genome [5] and the necessary characterization of germplasm (phenotyping, isozymes, and DNA PCR markers), linkage mapping and quantitative trait locus (QTL) studies, DNA barcoding, and next-generation sequencing technologies as described in Refs [10, 11]. Despite the acceleration of plant breeding using MAB methods, the possible interaction of and overlap between biotic and abiotic responses at the molecular level might pose obstacles, as in the case of the protein osmotin, which is suggested as being involved in salt stress [55], and commonly known from biotic responses upon microbial infection [56]. On the other hand, vast sequence data from model plants within the Rosaceae family, for example, apple and peach, are already available (Table 18.3).

Table 18.3 Some genome projects of higher plants with a total of 18 crops or otherwise important species for feed, fuel, and fiber production, including 2 fruit crop species and 1 plant model within the *Rosaceae* family (given in bold).

Plant name	Organism	Genome (Mb)	Chromosome No.	Status	Release date	Center/consortium
Thale cress	*A. thaliana* Columbia-O	93.7	5	•	2000	Arabidopsis Genome Initiative
Rice (Japanese rice)	*Oryza sativa* Japonica Group Nipponbare	334.8	12	•	2002	International Rice Genome Sequencing Project
Maize	*Zea mays* B73	2970.0	10	•	2009	MaizeSequence.org
Amaranth	*Amaranthus tuberculatus* ACR biotype	4.3		×	2009	University of Illinois at Urbana-Champaign
Rockcress	*Arabidopsis lyrata* ssp. *lyrata* MN47	180.0		×	2009	DOE Joint Genome Institute
Purple false brome	*Brachypodium distachyon*	541.0	5	×	2010	The International Brachypodium Initiative
Papaya	*Carica papaya* SunUp	271.7		×	2008	The Papaya Genome Sequencing Consortium
Cucumber	*Cucumis sativus* 9930	225.9	7	×	2009	The Cucumber Genome Initiative
Eutrema	*Eutrema parvulum* (Schrenk) Al-Shehbaz and Warwick	140.0	7	×	2011	University of Illinois at Urbana-Champaign
Woodland strawberry	***Fragaria vesca* ssp. *vesca* Hawaii 4**	**197.0**	**7**	×	**2010**	**Virginia Bioinformatics Institute**
Soybean	*Glycine max*	1906.1	20	×	2010	DOE Joint Genome Institute
Jatropha	*Jatropha curcas* Palawan	400.0		×	2011	Kazusa
Lotus	*Lotus japonicus* MG-20	147.8	6	×	2008	Kazusa
Apple	***Malus* × *domestica* Golden Delicious**	**526.0**	**17**	×	**2010**	**IASMA Research Center**
Barth's rice	*Oryza barthii* IRRI Acc:105608	~450	12	×	2009	Oryza Chr3 Short Arm Comparat. Sequencing Project
African rice	*Oryza glaberrima* IRGC: 96717	290.0	12	×	2010	Oryza Map Alignment Project (OMAP)

Indian rice	*O. sativa* Indica Group	784.6	12	×	2002	Chinese Academy of Sciences
Japanese rice	*O. sativa* Japonica Group Koshihikari	306.2		×	2010	QTL Genome Research Center, National Institute of Agrobiological Sciences
Japanese rice	*O. sativa* Japonica Group Nipponbare	727.1	12	×	2004	Beijing Genomics Institute
Date plam	*Phoenix dactylifera* cv. Khalas	284.7		×	2009	Cornell University
Physcomitrella moss	*Physcomitrella patens* ssp. *patens*	453.9	27	×	2007	Moss Genome Consortium
Black cottonwood	*P. trichocarpa*	736.6	19	×	2006	DOE Joint Genome Institute
Castor oil plant	*R. communis*	352.3	10	×	2006	J. Craig Venter Institute
Spikemoss	*Selaginella moellendorffii*	210.0		×	2010	Selaginella Consortium
Tomato	*Solanum lycopersicum*	759.0	12	×	2010	Solanaceae Genome Project
Tomato	*Solanum lycopersicum* Heinz 1706	540.6		×	2009	Kazusa
Sorghum	*Sorghum bicolor* BT × 623	658.0	10	×	2009	Sorghum Consortium
Cacao tree	*Theobroma cacao* B97-61/B2	290.0	10	×	2010	*ICGS*
Mung bean	*Vigna radiata* Kamphaeng Saen	580.0		×	2009	National Center Genetic Engineering and Biotechnology
Grape Vine	*V. vinifera* PN40024	467.5	19	×	2007	International Grape Genome Program
Peach	***Prunus persica***	**220.0**		**p**		**UC Davis Genome Center Bioinformatics Core**

Data was compiled from NCBI (2011).
• genome project completed; × genome assembly; **p** genome project in progress.

Moreover, genomic and functional information from other models, such as *Arabidopsis thaliana*, is accessible and in many cases transferable. As an example, one highly conserved mechanism in plants, which typically counteract abiotic stress, includes freezing tolerance, as discussed in Chapter 3. The raffinose pathway is generally induced via the CBF-dependent signaling pathway upon oxidative stress [57] and in particular under cold acclimation/cold stress conditions [58], as reported for many crop plants [7], including woodland and cultivated strawberry [2]. Thus, freezing tolerance mechanisms in *Fragaria* can be targeted by MAB methods addressing the identification of the metabolites involved, cryoprotective proteins, and molecular functions [53].

18.3 Genetically Modified (GM) Plants

The exploitation of genetically modified (GM) crop plants either through introduction of new genes and/or through alteration of existing genetic functions and traits started almost 20 years ago with the introduction and commercialization of the so-called *Flavr Savr* tomato with delayed softening and prolonged shelf life. Today, major GM traits are related to herbicide tolerance and resistance toward insect pests and viral diseases in important crops. The potential to modify plant nutritional traits has attracted more and more interest over the past decade with regard to amino acids (e.g., in maize), fatty acid profiles (e.g., in rapeseed), starch composition (e.g., in potato), and vitamin A-fortified "Golden rice" [59].

The strawberry industries in Europe and the United States have also shown interest in the purposeful modification of strawberry plants regarding nutritional and aroma-related properties of berry fruit, and several patents have been granted [60–62]. Safety issues and perception of GM food plants were emphasized as part of a European study on GM strawberry (genetical background: "Calypso") with enhanced resistance to gray mold (Project: TSP-EEES/QLK5-CT-1999-01479) [63]. Consumer concerns toward GM food were further addressed through the development of cisgenic or intragenic plants [64], that is, the introduction of genes from the same species, and marker-free GM strawberry plants [65]. Only a few attempts have been made toward abiotic parameters and stress amelioration so far. Heterologous expression of cold-inducible transcription factor CBF1 [66, 67], cryoprotective dehydrin WCOR410 [68], and osmotic stress-induced protein LEA3 [69] has resulted in improved freezing tolerance of *F.* × *ananassa* plants. Moreover, efforts have also been made to improve salt tolerance in osmotin-overexpressing GM strawberry [70]. In the long run, commercial use of GM strawberries might be expected, depending on the purpose of GM modification regarding consumer benefits.

18.4
Omics Approaches toward Abiotic Stress in *Fragaria*

Omics technologies have been frequently applied in the study of crop species throughout the past decade. Technological innovations and methodologies were based on the *Arabidopsis* model in the first place, but plant science has moved onward to new plant models, including significant food plants. Though rather few genome sequencing projects of crop species have been completed so far, the assembly of further plant genomes is in progress, and vast genomic information is already available (Table 18.3). Ongoing projects also include three whole-genome sequencing approaches in the Rosaceae family, namely, in apple (*Malus* × *domestica* Borkh.), peach (*Prunus persica* (L.) Batsch), and the Woodland strawberry *F. vesca*, whose draft genome was recently published [5]. *F. vesca* serves primarily as a model species within the *Fragaria* and for other Rosaceae species due to its diploid character, comparatively small genome, and ease of cultivation and propagation.

The major advantage of accessible genome information and gene annotation is the purposeful investigation of crop plants with regard to genetic regulation toward morphological development, phenotype expression, physiological functioning, and stress response, and the study of underlying molecular and cellular processes. In the following sections, the achievements and application of omics technologies in *Fragaria* will be presented with regard to genomic, proteomic, and metabolomic approaches.

18.4.1
Genomic Approaches toward *Fragaria*

The first transcriptional profiling studies were carried out using microarrays of combined *Fragaria* and *Petunia* cDNA in order to address attributes such as aroma biosynthesis [71], development of berries [72], and maturation [73]. In addition to studies of cultivated *F.* × *ananassa*, similar studies were done using the wild-type "model" *F. vesca* [74]. Although the first genomic investigations emphasized berry quality and yield-related properties, more recent studies strongly focused on functional aspects regarding single gene expression, polygenic effects, and QTLs and the evolution of phenotypes. Research in diploid *Fragaria* was intensified toward *Fragaria* genomic structure and the generation of genetic linkage maps [75–77] for mapping purposes with the cultivated strawberry and other Rosaceae species. Moreover, more recent investigations have tried to reconstruct a Rosaceae ancestral genome [78] and studied targeted gene neighborhoods in *F. vesca* [79], and thus established valuable information on the assembly of the *F. vesca* draft genome [5] and molecular breeding. Regarding abiotic stress responses and functional genomics in *Fragaria*, necessary tools and knowledge based on T-DNA insertions [80], microRNA profiling [81], and RNAi silencing [82] have been developed. Utilizing new-generation sequencing technology in combination with multiple abiotic factor treatments (light, day length, water, and temperature) in *F.* × *ananassa*, the

functional annotation of genes (contigs) could be accomplished, and, furthermore, new strawberry-specific transcripts were able to be identified [83].

18.4.1.1 Case I: Genomic Approaches toward Cold Acclimation/Freezing Tolerance in *Fragaria*

In order to support molecular breeding in *Fragaria* through functional genomics research, the authors used genotypes of the diploid model *F. vesca* and octoploid varieties of the cultivated strawberry in order to approach cold acclimation and freezing tolerance traits in strawberry through the application of omics technologies [2]. Biological processes in higher plants, which are triggered by abiotic stimuli, are highly conserved throughout the evolution, indicated by a moderate to strong homology between species regarding stress-responding genes. Moreover, recent results from the publication of the *F. vesca* draft genome [5] underscored the similarity of the *Arabidopsis* and the *Fragaria* genomes (number of protein-coding genes, shared gene families) and, thus, the potential of a heterologous genomics approach. Microarray analyses started with a full-genome *Arabidopsis* oligo array consisting of 26 604 genes. Profiling of cold-acclimated (4 °C) leaves from three Norwegian *F. vesca* clones and one *F.* × *ananassa* variety ("Korona") clearly showed the strong modulation of biological processes such as metabolism, transport, photosynthesis, signaling, and development after 24 and 72 h of cold exposure [2]. A total of 1957 and 1843 differentially regulated *Arabidopsis* gene homologues, respectively, could be detected.

In order to improve data from global transcriptional profiling, a customized *Fragaria* microarray chip was therefore developed as a joint collaboration between the Norwegian breeding company Graminor Breeding Ltd. and the authors' research group at NTNU in Trondheim. About 59 000 publicly available *Fragaria* EST sequences from NCBI were included, together with *F. vesca* cDNA sequences provided by the Center for Genomics and Bioinformatics, Indiana University (>3 million reads from GS-FLX Titanium – Roche/454 Life Sciences sequencing). In addition, about 190 Mb of preliminary draft genome sequences from *F. vesca* [5] were provided by the Strawberry Genome Sequencing Consortium. cDNAs, which served as templates for probe design, were validated by BlastN against the *F. vesca* draft genome, whereas cDNAs of microbial origin were excluded. Screening the draft genome against proteins of *A. thaliana*, *Vitis vinifera*, *Ricinus communis*, and *Populus trichocarpa* carried out the identification of genes not represented in the cDNA collection, and exon sequences from genes not found in the cDNAs were included. In total, 43 723 unique 60-mer probes were printed on microarray in 4 × 44k format Agilent chip design. Cold acclimation experiments using the *Fragaria* chip were focused on short- and long-term effects (48 h and 6 weeks after cold acclimation) in meristematic crown tissue in two varieties with contrasting freezing tolerance. The upregulation of ~100 cold-responsive genes (transcription factors, dehydrins, and enzymes) and transcripts involved in starch breakdown and raffinose biosynthesis was revealed. Besides central metabolism, secondary metabolism was also strongly modulated as seen by changes in the expression of flavonoid biosynthesis-related genes. In total, more than 1400 upregulated and 1100

downregulated genes (at least a 1.5-fold change (log-transformed) in either direction) were detected, establishing the basis for further studies of functional responses and molecular networks based on combined omics approaches (see also Sections 18.4.2.1 and 18.4.3.1).

18.4.2 Proteomic Approaches toward *Fragaria*

Studies toward the proteome in *Fragaria* are based on only a few publications focusing on cold acclimation [2, 53], vegetative propagation and development [84], fruit maturation and quality [85, 86], and allergens [87]. The latter research group reported a total of 97 proteins found in 7 varieties (red fruit) and three numbered genotypes (white) showing a similar protein variation within and between varieties.

18.4.2.1 Case II: Proteomic Approaches toward Cold Acclimation/Freezing Tolerance in *Fragaria*

As already pointed out in *Case I*, integrated approaches in the model *F. vesca* and the crop *F.* × *ananassa* [2] were initiated by our research group, standing out as the only proteomic attempts toward abiotic factors in *Fragaria* to date. Leaf samples of the octoploid variety "Korona," covering the time points 0 h (control), 24 h, and 240 h after the onset of cold acclimation (4 °C), were subjected to 2DGE and liquid chromatography coupled with mass spectrometry/mass spectrometry (LC-MS/MS) analysis. More than 800 proteins were matched in all gels, comprising 7% differentially regulated proteins and indicating a ratio of 3 : 1 between down- and upregulated protein spots (Figure 18.1). Generally, not only chloroplast-associated but also metabolic proteins were affected upon cold acclimation. The upregulation of cryoprotective dehydrin proteins [88] was validated by Western blotting using antibodies for the detection of *Arabidopsis* dehydrin COR47. Detected *Fragaria* dehydrin, designated as *Fa*COR47, was strongly upregulated after long-term cold acclimation (10 days).

These preliminary studies were followed up by ongoing investigations of different octoploid *Fragaria* varieties [53] based on integrated approaches with different omics technologies. Proteins possibly involved in freezing tolerance in strawberry were examined in octoploid strawberry plants (crown meristem) differing in cold tolerance, using either 2-DGE and LC-MS/MS or shotgun MS/MS. A total of 30 potential biomarkers were detected that showed significant changes in response to cold. On comparing the two MS/MS techniques, the shotgun approach was found better suited to reflect protein abundance since posttranslational modifications are less likely to affect the identification of proteins. Moreover, observed differences in protein levels between the varieties after 48 h of cold acclimation (2 °C) were related to decreases in transcripts indicating translational and/or posttranslational regulation. Many metabolic-related proteins were identified, including several enzymes linked to secondary metabolism (flavonoid pathway).

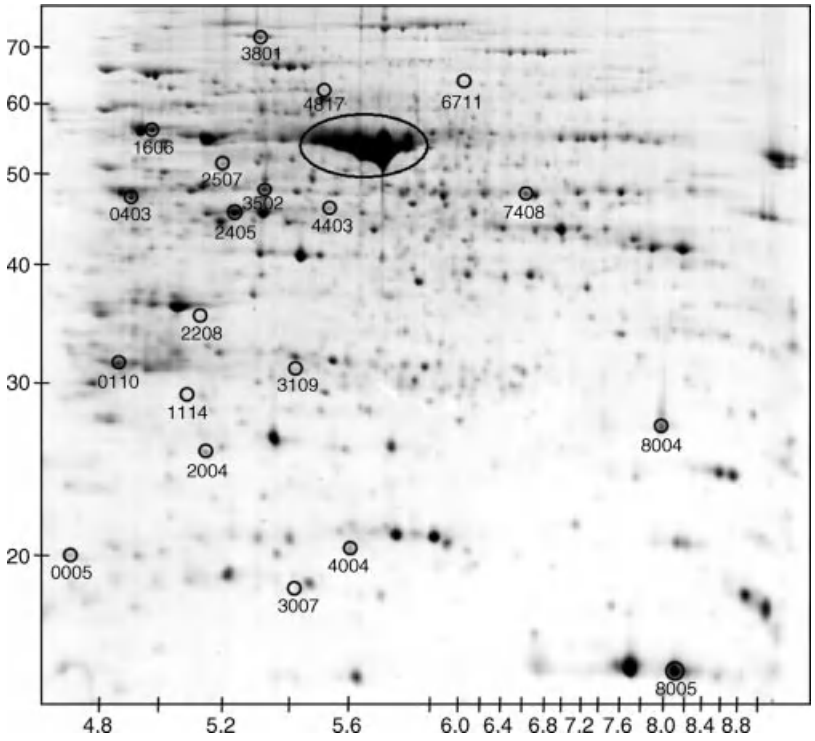

Figure 18.1 Example of 2D gel of leaf tissue extracts from *F. ananassa* "Korona" after 24 h of cold acclimation treatment, showing a high number of spots of which 20 distinct single spots are indicated. Protein sampling for further LC-MS/MS analysis was not performed in the region containing RuBisCO LS (circled region) (*Courtesy*: Stephen K. Randall, Department of Biology, IUPUI, IN).

18.4.3 Metabolomic Approaches toward *Fragaria*

Gene expression profiling based on microarrays has over 15 years of tradition and has had the strongest impact on performance and outcome of molecular–biological studies in modern times. However, the history of extensive metabolite profiling approaches dates back to the 1970s, mainly based on the introduction of gas chromatography (GC) equipment for the high-resolution separation of complex metabolite mixtures using capillary columns. GC in combination with mass spectrometry (MS) further allowed the identification of metabolites based on MS fragmentation patterns and database searches. The earliest applications focused on profiling of the complex strawberry aroma leading to the identification of ~360 volatile compounds. More than 50 volatile structures can easily be detected in a single sample of *F.* × *ananassa* [21, 89] and *F. vesca* [90, 91]. The range of GC-detectable compounds was extended

through so-called derivatization techniques, enabling the detection of highly polar compounds in strawberries such as sugars and acids, using either conventional GC techniques [92] or more advanced, multiparallel GC-MS coupled with time-of-flight technology (GC/TOF-MS) [2, 93]. The first extensive nontargeted metabolic analysis of strawberry reported 5844 unique mass peaks in fruits at four developmental stages based on Fourier transform–mass spectrometry (FT-MS) technology [94]. Developments in liquid chromatography in the past two decades have further facilitated high-resolution separation of plant metabolites with regard to the group of polar (nonvolatile) structures. Combination with MS technology (LC-MS) allows the identification of single compounds from complex mixtures and was recently applied to study different plant tissues in *Fragaria* sp., for example, leaves and roots [2], flower metabolites [95], and strawberry fruit [14, 96]. Furthermore, NMR techniques have been applied in order to approach the chemical composition of *F.* × *ananassa* leaves [97]. *In situ* analysis of metabolites (sugars and acids) using infrared AP MALDI mass spectrometric imaging represents a relatively new method for *in vivo* investigation of small biomolecules and biological processes for functional studies [98].

Despite the applicability and versatility of modern metabolite profiling techniques, few reports describe their use with regard to the effect of the abiotic environment on strawberry plants. The influence of light quality on berry volatiles when cultivating strawberries under plastic rain covers showed that taste and aroma quality slightly changed without negative effects on marketable fruit quality [99]. However, earlier studies with colored plastic mulch (red versus black) clearly pointed out the strong impact of spectral composition of light in the plant's near environment on metabolite profiles of sugars, acids, and aroma volatiles [29]. On the other hand, the distinct effect of abiotic stress factors in strawberry production might be masked by natural year-to-year variation [21] and latitudinal effects (Rohloff *et al.*, unpublished).

18.4.3.1 Case III: Metabolomic Approaches toward Cold Acclimation/Freezing Tolerance in *Fragaria*

As already presented under *Case I* and *Case II*, a recent cold acclimation study of both the cultivated strawberry and the diploid model was carried out using GC/TOF-MS-based profiling. Analyses of short- and long-term effects (time points 0, 3, 24, 72, and 240 h) revealed increased levels of not only carbohydrates (glucose and fructose), amino acids (glutamine, glutamic acid, asparagines, and aspartic acid), and TCA intermediates (malic acid and fumaric acid) but also distinct secondary metabolites and redox functioning (ascorbic acid) [2]. In general, more metabolites were found to be differentially regulated at later time points of the cold acclimation period (72/240 h) compared to short-term regulation after 3/24 h (Figure 18.2). As a common feature in both *Fragaria* species, the raffinose pathway (galactinol and raffinose) was similarly affected in leaves and roots after 72 and 240 h of cold acclimation. The application of networks is discussed in Sec-tion 18.4.4.

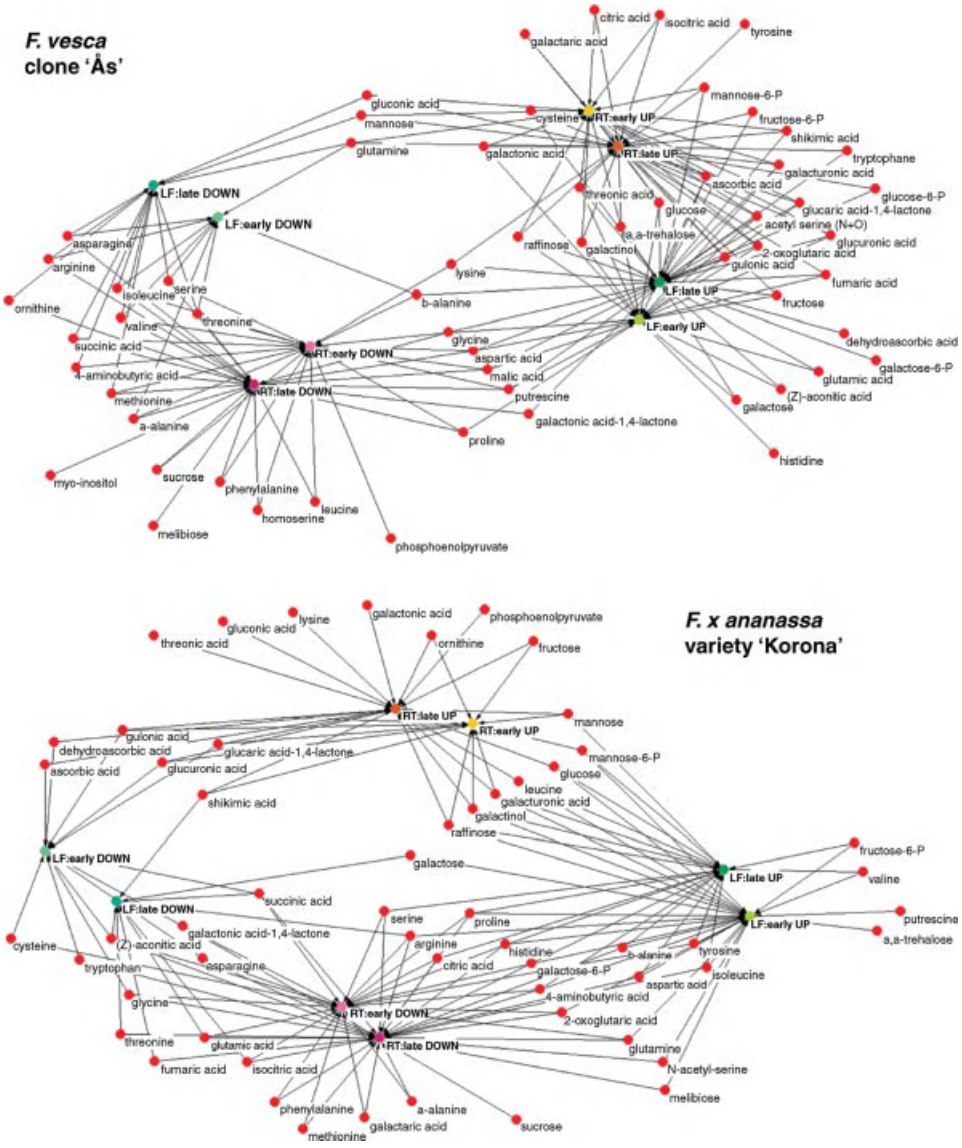

Figure 18.2 Unweighted metabolic networks of *early* (3 and 24 h) and *late* (72 and 240 h) cold response in leaf and root samples of *F. vesca* clone "Ås" and *F.* × *ananassa* variety "Korona." Diagrams were drawn using the Pajek software v. 1.24 (http://pajek.imfm.si/doku.php). Metabolites comprise 61 compounds of the central metabolism. The threshold for considering differentially regulated metabolites was $\geq$1.25-fold increase or $\leq$0.75-fold decrease (log-transformed data) compared to control samples (0 h).

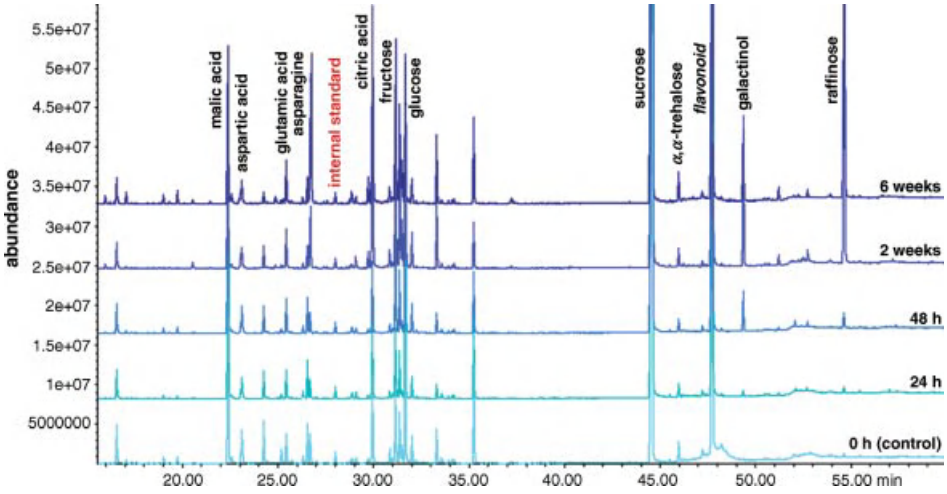

Figure 18.3 Example of GC-MS chromatograms of *F.* × *ananassa* "Jonsok" plants (crown tissue) after 0, 24, and 48 h as well as after 2 and 6 weeks of cold acclimation at 2 °C.

In more recent studies, *F.* × *ananassa* varieties were solely assessed, here also showing the same patterns of metabolic regulation upon plant exposure to cold through upregulation of carbohydrates, polyols, amino acids, TCA intermediates, and distinct secondary metabolites. Variation in metabolic patterns in cultivated strawberry indicated varietal differences and potentially acclimation strategies due to contrasting frost tolerance. The raffinose pathway was particularly affected showing highly raised levels of the central metabolites galactinol (a precursor of raffinose) and raffinose after 2 and 6 weeks of onset of cold, respectively (Figure 18.3).

18.5 Systems Biology as Suitable Tool for Crop Improvement

A system is a group of independent but interconnected components that function together as a unified whole [100]. For the stability and functional robustness of any system, the components have to work together in a coherent manner. In a biological context, systems can be identified at different hierarchical levels such as ecosystems, organisms, organs, tissues, cells, molecules (genes, proteins, and metabolites), and interatomic levels. The interactions within a single hierarchy itself can be tremendously complex. When we consider a system comprising multiple hierarchical levels, functional complexity becomes even more complicated. Over the last decade, systems biology has emerged as a promising field that integrates vast amounts of data from genome-scale technologies and builds computational models to help understand the overall organization and dynamical function of the molecular systems that build and sustain an organism [101, 102]. Systems biology is an approach that breaks down boundaries between biological studies and

merges it with mathematics, physics, chemistry, and computer science. It is difficult to give a universally accepted definition of systems biology; some emphasize the role of dynamic modeling, whereas others emphasize multidimensional data integration, visualization, and analysis. Such a diverse range of opinions is to be expected due to the infant stage of the field and the necessity of interdisciplinary expertise [103–106].

18.5.1
Omics Data Integration for Improving Plant Productivity/Translational Research

As a result of vast technological developments in recent years, different omics approaches are producing huge amounts of biological data. A combinatorial approach using multiple omics platforms and integration of their outcomes is now an effective strategy for understanding molecular systems integral to improving plant productivity. Starting with genomics (sequencing the whole genome of few model organisms) in the early 1990s, the area of omics has now evolved to many more established disciplines such as proteomics ("all" proteins), transcriptomics ("all" transcripts), interactomics ("all" interactions between biomolecules), and metabolomics ("all" metabolites). The first sequence of the model plant *A. thaliana* was completed and published in 2000 by the Arabidopsis Genome Initiatives (AGI) [107]. If we compare the situation over a decade later, there are 405 instances of genome projects in species of *Viridiplantae* that include both green algae and land plants, including many agronomically important crops, as of March 2011 (Table 18.3) (http://www.ncbi.nlm.nih.gov/sites/entrez). Whole-genome sequence data have become the primary set of resources for designing microarrays, tilling arrays, or molecular markers and are also an important reference for integrating other omics-derived information with genome sequences. Several comparative genomics projects among green plants such as *Phytozome* (http://www.phytozome.net/) and *Gramene* [108] (http://www.gramene.org/) have proven to be successful to help grasp the biological properties of each species and to accelerate gene discovery and the functional analyses of genes. Current version (v6.0) of *Phytozome* has 25 plant species and *Gramene* (release #32) has genomic information on 20 grass species. As a part of *International Structural Genomics Organization Initiatives* (ISGO) (http://www.isgo.org), the phylogenetic relationships between the different *Fragaria* species have been studied [109]. Several large-scale plant proteomics (http://www.masc-proteomics.org/) and plant metabolomics (www.plantmetabolomics.org) projects are currently being carried out. Large-scale determination of protein 3D structures is crucial for understanding mechanisms of protein functions in structural complexes or reaction cascades. Apart from X-ray crystallography and NMR spectroscopy-based structural proteomics methods, several dedicated research groups are involved in determining large-scale structures of plant proteins. Thus, the number of solved protein structures from plants appearing in the Protein Data Bank (PDB) (http://www.pdb.org/), which is the most popular resource for biomolecule structure data sets, has dramatically increased over the past decade [110].

Nowadays few more new branches are evolving in *omics* such as phenomics ("all" the phenotypes related to gene mutants) [111] or bibliomics (the study of complete literature on a biological topic) [112]. The ultimate aim of all these omics approaches is to generate a holistic view of the interplay between biomolecules. Rapid development of technologies such as *in vivo* NMR, next-generation sequencing, and high-throughput molecular imaging will generate copious amounts of data in the future years. The enormous complexity of the data sets generated through these techniques requires an extension of our current bioinformatics/computational analytical abilities for data integration [113]. Hence, the most challenging research at this moment is the design of man-made intelligent systems (such as computers, software, and robots). These systems will require advanced features necessary for adaptable, robust performance and to be capable of independent operation over long periods of time. As high-throughput profiling delivers data from genome to transcriptome, proteome, and metabolome, bioinformatics will provide us with the integrated information upon which models for the inner workings of the cell can be developed. Robust bioinformatics tools, genome databases, and integration of information from different fields enable the identification of genes and gene products and elucidate the functional relationships between genotype and observed phenotype [114]. In a recent review by Gehlenborg *et al*. [115], some of the best available integrated visualization tools for omics-scale data are discussed. Conventional MAB or MAS will gradually evolve into "omics-assisted breeding" for crop improvement. The most important necessity at this time is to develop user-friendly visualization tools that will help the biologist to interpret more clearly and easily the complex multidimensional biological networks of genes and their relationships with phenotypes. The construction of "graphical genotypes" of each plant or progeny row would allow the breeder to determine which chromosome sections are inherited from each parent to facilitate the selection process and to design superior genotypes *in silico*. This will perhaps dramatically reduce the time needed for extensive field tests [116].

The *F. vesca* genome is recently sequenced [2]. The genome information is available at http://www.strawberrygenome.org. The Genome Database for Rosaceae (GDR) [4, 117], which is a curated and integrated web-based relational database containing comprehensive data of the genetically anchored peach physical map, annotated EST databases of apple, peach, almond, cherry, rose, raspberry and strawberry, Rosaceae maps and markers, and all publicly available Rosaceae sequences. In a recent comparative study, considerable microsynteny has been discovered between *Fragaria* and other plant model genomes such as *P. trichocarpa*, *Medicago truncatula*, *V. vinifera*, and *A. thaliana* reflecting a close evolutionary distance [118]. This information might be extremely important for translational research, allowing the information on marker, gene, or QTL position from one of these species to be used in the other [119, 120]. In an another study, a high-resolution depiction of targeted gene neighborhoods in strawberry genome was performed, which will aid whole-genome sequence assembly,

provide valuable tools for plant breeders, and advance the understanding of strawberry genome evolution [79]. Here, a specific set of genomic sequences and gene neighborhoods were targeted for study because they contain genes of likely relevance to horticultural and fruit quality traits with emphasis on metabolic pathways, flowering-related genes, and disease resistance-related genes. From the previous success stories in other crop species, it can be predicted that high-throughput omics data integration will be extremely useful for improving crop productivity in *Fragaria*.

18.5.2 Plant/Crops Systems Biology

Plants are unable to escape from adverse environmental conditions. As a consequence, they have developed an incredible level of flexibility in their responses to environmental challenges caused by abiotic and biotic factors such as sunlight/UV, drought, temperature, nutritional stress, and pathogen and pest attacks. This flexibility of plants is known as a genotype by environment response. Genotype reflects a genetic organization optimized for survival in a range of environmental conditions. Studying the systems level responses of whole plants to environmental conditions is crucial for agricultural productivity. Crop scientists have been using a systems approach to investigate whole-crop physiology and crop ecology, morphology, and so on for decades [100]. Thus, plant systems biology is not to be considered an entirely new field. Systems biology has made this practice genome-scale, offering solutions to a more complete analysis by allowing virtual experimentation and hypothesis testing [121].

Recently, Yin and Struik [8] proposed a practicable concept, *crop systems biology*, which aims at modeling complex crop-level traits relevant to global food production and energy supply, by integrating omics-level information, underlying biochemical understanding, and physiological component processes. This crop systems biology follows the combined approach of modern functional genomics and traditional sciences (such as crop physiology and biochemistry) in understanding and manipulating crop phenotypes relevant to agriculture. The integrated systems approach to crop improvement incorporates advanced technologies in molecular markers, statistics, bioinformatics, crop physiology, and modeling. Hammer and coworkers have proposed such an integrated crop improvement program that can enhance the efficiency of crop improvement relative to conventional phenotypic selection by changing the focus from the paradigm of identifying superior varieties to a focus on identifying superior combinations of genetic regions and management systems [122]. This proposed integration program is considered to work across different levels of biological organization from genetic to plant growth, development, and yield without compromising with main goals of functional breeding and crop improvement program.

18.5.3
Pathway Modeling and the Concept of "Virtual Plant"

Plant tissues consist of heterogeneous cells with multiple cellular compartments. Consequently, diverse gene and pathway types exist in terms of complex molecular concentration gradients in different tissues. These properties of highly compartmentalized intercellular networks and the resulting complexity arising from the environmental interactions create great challenges for plant biologists [123]. Plant systems biology helps in predicting functions of many genes and find missing links and crosstalk between various pathways and their effect on the plant [102]. With the advent of high-throughput omics data and informatics tools, one can now build mathematical models of different biochemical or physiological processes [124]. A model should preferably include the possibility of exploiting the dynamic processes in plant cells and tissues. With the help of information available from metabolic pathway databases such as *KEGG* [125], *MetaCyc* [126], and *AraCyc* [127], the building of topologically accurate models is possible for various organisms. Based on such information, stoichiometric models (e.g., flux balance analysis) [128, 129] are built that assume physicochemical constraints that have to be satisfied when an organism reaches a steady state (e.g., mass and energy balance, system boundaries, and flux limitations). The limiting factor is that such constraints are not known for every organism and every environmental condition. So the possible steady-state solutions have to be further restricted by optimization with regard to an assumed objective (e.g., optimal growth, minimization of damage by excess light, or maximization of nutrient uptake). The effect of various combinations of stress in a particular crop variety and the interplay with other signaling systems that are activated or deactivated in response to stress remain difficult to predict [130, 131]. All these concerns have inspired efforts to build *Virtual Plant* (www.virtualplant.org) [121, 132] or plant part models [133, 134]. These plant and plant part models will be of utmost value for research in plants and will provide unique modeling opportunities for developing better crop varieties. Model-based predictions and hypothesis generation will help to rationalize the efforts and resources spent on wet lab research.

18.5.4
Network-Based Approaches

Networks have been proven to be a vibrant tool to describe, visualize, and simulate complex systems [135]. Each component of a complex system can be described as a node and connections or relationships can be drawn between these nodes based on certain criteria (Figure 18.2). In biology, this relationship is referred to as *emergent behavior*: a function that derives from the interaction of network components [100]. Because of the complex multiple levels of hierarchical organization present in biological systems, biological networks have been classified into two categories – microscopic (molecular level, process level, tissue level, and organ level) and

macroscopic (organism level and ecosystem level). Under natural conditions, plants/crops encounter combinations of stress and, therefore, have to mount an integrated response toward this stress via synergistic and antagonistic actions. Various stress signaling pathways are likely able to crosstalk and adjust their contribution to the total response. Plants have evolved intricate mechanisms to perceive external signals, allowing optimal response to environmental conditions. As described earlier, crop systems biology employs a global approach to model complex crop traits relevant to global food production and energy supply, via establishing the links between omics-level information, underlying biochemical understanding, and physiological component processes in related environmental conditions. Network-based approaches offer extreme flexibility in the study of such complex multiscale crosstalking events.

Building meaningful networks from biological data such as sequences, transcripts, proteins, or metabolites is tricky. Much more information such as interactions or process of regulation is necessary to build up a network between the components of the system. Such a network brings heterogeneous data into a single model. Some user-friendly software tools, for example, *Cytoscape* [136] and *CellDesigner*™ [137], have been developed to construct and visualize such networks. Many different methods and algorithms have been developed to explore biologically relevant information from these networks [138–140]. Several such network-based approaches are dealt with in the following sections.

18.5.4.1 Correlation Studies Using Multivariate Data

Genes and metabolites showing similar behavior or clustering pattern suggest that they participate in the same biological process (this is a basic network concept). Clustering algorithms such as PCA (principal component analysis), SOM (self-organizing maps), and ICA (independent component analysis) are benchmarked methods to construct such gene to metabolite networks.

18.5.4.2 Protein–Protein Interaction (PPI) Networks

Interactions between various proteins at a physical level are determined by techniques such as Y2H (yeast two hybrid) systems and *in vivo* methods such as FRET (fluorescence resonance energy transfer), BiFC (bimolecular fluorescence complementation), and TAP-tagging (tandem affinity purification protein-tagging). Based on these experimental and predicted information, protein–protein interaction (PPI) networks are built and stored in various databases, for example, *IntAct* [140, 141] and *MIPS* [142].

18.5.4.3 Gene Regulatory Networks

A gene regulatory network describes how genes interact with other regulatory genes during the biological process to perform a function [143]. The relationships between the genes in the network can be defined in terms of activation, repression, and other types of functional interactions. Taking the concept from control engineering, such networks can be represented as circuit diagrams. The transcription regulatory network describes the interaction between transcriptional regulatory

genes and downstream genes [144]. The data are often based on interactions between regulatory genes and downstream genes, as defined by mutant studies, global gene expression profiling, computational prediction of *cis*-elements, and protein–DNA interaction studies using gel-shift assays.

18.5.4.4 **Coexpression Networks**

Coexpressed or coregulated genes indicate their involvement in similar biological processes, meaning that individual modules can be attributed to specific biological processes. Using this basic concept, group of genes with similar expression pattern in different space, time, genotype, or experimental conditions can be put together to build modules. Such modular network topology-based analysis has been proven to be useful in identifying functional modules [145]. In a coexpression study, Weston *et al*. [146] have shown how a mechanistic understanding of adaptive physiological responses to abiotic stress can provide plant researchers with a tool of great predictive value in understanding species and population level adaptation to climate change. In another study by Lee *et al*. [147], abiotic stress phenotypes were linked with gene coexpression network analyses to find out the genes responsible for a particular phenotype. With the availability of more high-quality microarray or next-generation sequencing data, such methods will be more and more efficient in coming days.

18.6
Conclusions and Future Prospects

One major obstacle in traditional crop breeding has been the complexity of traits on the one hand and the limitation to address only distinct traits based on gene identification by forward and reverse genetics on the other. The feasibility of QTL mapping has opened up the successful identification of multiple traits, not least due to the availability of sequence and necessary marker information. Yet, the genetic architecture of quantitative traits still needs to be elucidated and trait effects to be evaluated by population mapping, which is a costly and time-consuming method. Detailed SNP information in particular allowed association mapping (*linkage disequilibrium*) through correlation of genetic markers with phenotype within a collection of genotypes. Nowadays, the combination of comprehensive SNP sequencing [148] with information from *F. vesca* T-DNA mutant lines for the elucidation of gene function [80, 149] have moved trait-oriented mapping toward the so-called *genome mapping* and *genomic selection*. This implies that sequence contigs can be positioned on a SNP-based linkage map, and targeted gene loci are monitored genetically with regard to solely SNP diversity, and not, as usual, phenotypic (trait) variation [148]. Moreover, individuals might be selected much earlier in the breeding cycle, for example, before the reproductive stage, thus reducing work load and speeding up the breeding process [150]. In the following, recent innovations and developments are summarized, which may further aid and guide the breeding of cultivated strawberry and *Fragaria* sp. in the future.

18.6.1
Technology-Driven Innovations for Fragaria Breeding and Development

- New-generation sequencing technologies [151] for potential identification of genes, markers, and biological functions.
- Quantitative proteomic profiling using label-based and label-free approaches [152] for the potential identification of biomarkers.
- Metabolomics in integrated approaches [153] for coexpression network analysis, functional annotation, identification of phytochemicals, and metabolome QTL (mQTL) analysis for breeding purposes.
- Curation of publicly available databases and information resources [4, 154], and further extending accessible information of genome and proteome data by also including metabolome database information.

18.6.2
Biology-Related Issues for Improvements in the *Fragaria* Genus

- Marker-assisted selection and marker-assisted breeding in cultivated strawberry based on linkage mapping with diploid *F. vesca* [155] for multitrait transfer into elite selections and variety development.
- Reconstruction of today's strawberry through interspecific hybridization [156, 157] for *Fragaria* crop improvement.
- Innovative hybrids due to heterosis and ploidy not only toward fruit and yield traits [158] but also with regard to abiotic stress tolerance [52].

Acknowledgments

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19
Rose: Improvement for Crop Productivity

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Abstract

Roses are economically important and much in demand in domestic and international markets and in perfumery industry. The genus *Rosa* contains 200 species and more than 18 000 cultivars. Light, temperature, humidity, and mineral nutrition are essential factors for rose production. Abiotic (salt and drought) stresses adversely affect growth and productivity of rose. Drought is the most common stress responsible for elevated release of ethylene resulting in senescence and abscission. Much work has been done on rootstocks and emphasis has been laid on their potential to be used for increased productivity. Senescence as a result of drought in rose petals and other plant parts is associated with overproduction of reactive oxygen species (ROS) that damage protein, lipids, carbohydrates, and DNA. Substantial progress has been made in the identification of genes involved in abiotic stress tolerance and many stress-related genes are derived from petals in *Rosa* spp. Osmotin, a stress-responsive protein adapted to salinity and desiccation, accumulates in salt-adapted cells. Overexpression of osmotin gene induces proline accumulation and tolerance to osmotic stress. Stable integration of osmotin gene in *Rosa bourboniana*, a scented rose species, was successfully attempted. Most of the prevailing rootstocks in our country do not respond to cold stress of winter. CSIR-IHBT has developed winter-active rootstock selections that are successfully tested over a decade and deployed in the northern region. In roses, effect of different types of stresses has been studied on cut flowers, potted plants, and on plants under field conditions.

19.1
Introduction

The genus *Rosa* (family Rosaceae) is endemic to temperate regions of Northern Hemisphere. Many broad rose classes are known that have been grouped into

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European roses, China roses, Hybrid Perpetuals, Tea roses, and Hybrid Tea roses [1]. The poetess Sappho has addressed roses as "Queen of Flowers" [2].

Roses are generally used for beautifying the gardens and walkways as cut flowers and for essential oils. Internationally, the cut floral industry, of which roses account for two-thirds of all selections, exceeds $40 billion per year. Commercial flower growers cultivate Hybrid Tea and Floribunda-type roses for the cut flower market and for domestic and industrial landscaping. In addition to *Rosa hybrida*, a number of species are cultivated as ornamentals and for the food and medicinal value of their hips. Many ornamental roses are also grown for attractive or scented foliage (*Rosa glauca* and *Rosa rubiginosa*), ornamental thorns (*Rosa sericea*), or for their showy fruit (*Rosa moyesii*). Ornamental roses have been cultivated for millennia, the earliest known cultivation dates back to 500 BC in Mediterranean countries, Persia, and China [3].

At present, three species of the scented roses, namely, *Rosa damascena*, *Rosa centifolia*, and *Rosa gallica* are used for the production of quality perfumery products. However, rose oil distilled from the Bulgarian *R. damascena* is traditionally sought after [4, 5]. Rose water is very soothing to irritated skin and is very valuable as an antiseptic in eye infections. The rose syrup, most commonly made from an extract of rose petals, is much in use in France. In the United States, the French rose syrup is used to make rose scones and marshmallows. In India, "gulkand" made by the mixture of rose petals and sugar in equal proportion is used as a tonic and also as a laxative [6]. The Bourbon roses (*Rosa bourboniana*) also belong to scented class of roses having a wide range of growth habit in form and height. *R. bourboniana* is a natural crossing between the China rose (repeat blooming) and the Autumn Damask rose. With their sublime fragrance, tolerance for cold temperatures, and recurrent blooming, bourbons are among the most distinctive of all roses.

Rose seed is an achene with thick hard pericarp and germination is always lower than 50%. The amount of ABA in rose achene is 10–1000 times more than that in other plants. The gibberrellic acid needed to interrupt dormancy is higher in immature seeds and decreases as the seeds mature. Different treatments have been used to enhance seed germination, for example, gibberrellic acids and cytokinins, stratification, and scarification to remove physical obstacles from the pericarp. In nature, microorganisms on dead leaves overcome the physical problems of achene pericarp. In *Rosa corymbifera* "Laxa," seed germination rate was enhanced considerably upon addition of commercial compost activator to the sowing compost. However, in surface sterilized seeds, similar effect was not seen, thus indicating the role of microorganisms already present on achene pericarp [7].

Modern roses are a product of many years of selection and careful hybridization. This makes it impractical to propagate true to types from seeds. True to type cultivars can be achieved using vegetative methods, that is, budding, cutting, stenting, layering, and also through *in vitro* culture for mass multiplication of disease-free plants. Plants from cuttings take longer to reach a saleable size (mostly for garden roses) and not all cvs. or varieties can be grown vigorously without a rootstock. Slow growing roses often produce smaller and fewer blooms than their grafts. Hence, in much of the work reported, particularly in relation to stress, rootstocks have been used in experiments.

Rosa indica var. *odorata* and *R. multiflora* are the two rootstocks most widely utilized in India for obtaining plants with quality and quantity flowers. Different rootstocks have been used with great success across the world, for example, *R. multiflora* (the United States, Zimbabwe, Japan, and Zambia), *Rosa canina, R. laxa, R. manetti, R. multiflora, and R. rugosa* (Europe). In Kenya, however, *R. multiflora* has been found to be prone to nutrition deficiency problems [8]. Most of prevailing rootstocks in our country do not respond to cold stress of winter. In this regard, CSIR-IHBT has recently developed winter-active rootstock selections that are successfully tested for over a decade and deployed in the northern region. Details of this are mentioned in Section 19.2.4.

19.2
Abiotic Stress and Rose Yield

As in other plants, abiotic stresses adversely affect growth and productivity of roses. These include drought, floods, high soil salinity, soils poor in nutrients, extreme temperatures (too high or too low), reduced light level, or excess of UV radiation. Cultivated plants are more sensitive to abiotic stress conditions than their wild ancestors. Nearly all species of roses are able to tolerate hot summers and low temperatures even up to −15 °C [9]. Some of the species found in China do not withstand cold temperatures (−10 °C), including *R. gigantea*. This species was used in the development of hybrid tea roses and as a result hybrid teas are also susceptible to cold climates [9]. Different abiotic stresses experienced by the genus *Rosa* are summarized in Table 19.1. Herein plant responses to various types of abiotic stresses that are manifested at morphological, physiological, biochemical, and molecular levels are summarized.

19.2.1
Drought Stress

Water deficit is a key factor limiting plant growth and development during both the vegetative and reproductive stages, leading to decreased productivity [29, 30]. The degree of drought tolerance exhibited by a plant is related to its ability to respond to adverse conditions. Under water deficit conditions, the availability of water in supporting materials such as soil, vermiculite, perlite, or peat moss is restricted, thereby causing low water use efficiency in plant cells [30, 31]. Williams *et al.* [10] studied the response of potted miniature roses (*R. hybrida*) to reduced water availability during production. All plants produced under water deficit conditions were more compact than the control plants and the cyclic grown plants (cyclic water-deficit treatments) most closely resembled commercially produced plants. It was observed that all drought treatments significantly reduced the number of flower buds, but the time to flowering was not influenced. Importantly, plants produced with reduced water availability were more efficient in dry matter production per volume of water consumed compared to that with controls. Niu *et al.* [12] compared the growth and physiological responses to drought stress in four rose rootstocks,

Table 19.1 Response of genus *Rosa* to various abiotic stresses.

Type of stress	Species/cultivar	Response	References
Drought stress	Potted miniature roses (*R. hybrida*)	More compact plants, reduced number of flower buds	[10]
		In vitro flowering and the number of flowers per plantlet declined significantly, pigment degradation, chlorophyll a fluorescence, and net photosynthetic rate (Pn) greatest in plantlets exposed to a water deficit stress Increased proline levels Development of reproductive organs suppressed	[11]
	R. odorata, *R. multiflora*, and cv. "Dr. Huey"	Plant growth reduction	[12]
	Roses cv. "Madelon"	Flower quality affected Malformed buds with no carpels and stamens tightly packed on the center of the receptacle	[13]
	Pot cultured rose, soilless cultures, and *in vitro* cultures	Development of reproductive organs suppressed	[14–16]
	R. chinensis	No flowering, induction of dormancy, leaf injury, leaf necrosis and chlorosis	[17, 18]
Salt stress	Pot cultured rose, soilless cultures, and *in vitro* cultures	Development of reproductive organs suppressed	[14–16]
	R. chinensis	No flowering, induction of dormancy, leaf injury, leaf necrosis and chlorosis	[17, 18]
	R. hybrida	Aberrant color changes in flowers	[19]
	Miniature roses	*In vitro* flowering sensitive to salt stress, low numbers of flowers per plant and low flower initiation, maximum pigment degradation, low photosynthetic abilities and maximum growth reduction	[16, 20, 21]
	R. hybrida cv. "Hot Lady"	Chl a and Chl b pigments damaged significantly	[19]
		Sodium enrichment at cellular level leading to induced program cell death	[22]
		Inhibition in fresh weight, dry weight, and leaf area	[16, 23]
	R. fortuniana, *R. multiflora*, and *R. odorata*	Significant growth reduction in rootstocks, chlorophyll concentration reduced	[24]

(*continued*)

Table 19.1 (*Continued*)

Type of stress	Species/cultivar	Response	References
	R. chinensis	No flowering, induction of dormancy, leaf injury – leaf necrosis and chlorosis	[17, 18]
Deicing salts	*R. rugosa*	Decrease of chlorophyll content, potential photochemical efficiency, performance index, and biomass accumulation	[25]
Light stress (quantity and quality)	Roses	High light interception increases the photosynthesis rate, dry mass gain, ultimately flowering shoot quality improved in terms of larger stem and bud diameter and increased fresh weight	[26]
		In Mercedes roses, the bent shoot technique reduced the number of leaves by 25%	[26]
		The bent stems form a floor of green matter like a dense crop and transmit and reflect more strongly on FR than on R wave band resulting in smaller R:FR ratio that advances flowering	[27]
Low-temperature stress	Roses cv. "Madelon"	Flower quality affected Malformed buds with no carpels and stamens tightly packed on the center of the receptacle	[13]
High-temperature stress	*Rosa* sp.	Increased gamete ploidy level, leaves turn yellow	[28]
	R. chinensis	No flowering, induction of dormancy, leaf injury – leaf necrosis and chlorosis	[17, 18]

namely, *R. hybrida* cv. "Dr. Huey," *Rosa fortuniana*, *R. multiflora*, and *R. odorata*. Well-irrigated plants were subjected to mild drought stress for five to six cycles. Maximum growth reduction occurred in *R. odorata*, followed by *R. multiflora* and *R. hybrida* cv. "Dr. Huey." *R. fortuniana* was found to be the most tolerant and *R. odorata* to be the least tolerant to drought stress. Two cultivars of potted miniature roses, Apollo Parade and Charming Parade (R), were exposed to repeated drought stress and recovery cycles from second cut to flowering [32]. Maximum responses were recorded following the first exposure to drought. This first exposure to drought had a conditioning effect on the plants, which also improved their tolerance to subsequent exposure to drought. The two cultivars used different

mechanisms to respond to drought in that cv. "Apollo" utilized osmotic adjustment, while cv. "Charming" modified its stomatal closure.

Reproductive development has been reported as being susceptible to water deficit stress in pot-cultured rose [13, 14] soilless cultures [15], and *in vitro* cultures [33]. Cha-Um and Kirdmanee [11] studied tolerance to water deficit in miniature roses. *In vitro* flowering and the number of flowers per plantlet declined significantly when miniature rose plantlets were exposed to water deficit stress. Such knowledge would be useful for selection of water deficit-tolerant genotypes in miniature rose breeding programs.

Under water deficit conditions, varying degree of biochemical changes occur. These include decreased ribulose-1,5-bisphosphatase carboxylase/oxygenase (Rubisco) activity, reduced photochemical efficiency and net photosynthetic rate (Pn), accumulation of stress metabolites, increased activities of antioxidant enzymes, and reductions in the levels of reactive oxygen species (ROS). Proline is a vital compatible solute. Its accumulation in water-deficit stressed plants has roles in cellular osmotic adjustment and in the scavenging of toxic oxidants [34–36].

Jin *et al.* [37] investigated the role of ascorbate peroxidase (APX) in protecting flowers of cut rose cv. Samantha from the damage caused by water deficit. The water deficit inhibited flower opening process and caused a decrease in APX activity. In addition, both pretreatments affected superoxide dismutase (SOD) activity. APX activity was induced by water deficit stress and reached the peak in 9–12 h, but SOD activity remained low until 9 h after water deficit stress and then increased. This shows that antioxidant systems, at least APX and SOD, cooperatively act in a defense system under water deficit conditions. The antioxidants improved flower tolerance to water deficit stress, and increased SOD and POD (peroxidase) activities [38]. The flowers were pretreated with ascorbic acid (AsA) and β-aminophenol before water deficit stress. AsA has been widely used as a basic component in many preservatives of cut flowers for several decades. Jin *et al.* [37] suggested that not only AsA but also its relevant artificial substrates with powerful functions of enhancing APX activity could be used to further improve the tolerance to water deficit stress in cut rose flowers. AsA tolerance to water deficit stress is partially through the regulation of Rh-APX1 at transcript level. Several studies have shown that overexpression of APX gene in transgenic plants can enhance their tolerance to oxidative stress [39–41] and water deficit stress [42]. It seems feasible to improve the tolerance to water deficit stress in cut roses through overexpressing of APX genes.

19.2.1.1 **Ethylene Biosynthesis**

Water deficit stress elevates the release of ethylene, resulting in senescence and abscission. Morgan *et al.* [43] stated that most reports of increase of ethylene production by water deficit stress have involved detached plant parts and that several studies with intact plants found no increase of ethylene production. Based upon their findings, use of intact plant for experimentation was emphasized. Such an approach seems essential if normal plant behavior is in view.

Ethylene is also an important signaling response hormone for many abiotic stresses and pathogen interactions [44, 45]. The two steps of ethylene biosynthesis, that is, conversion of *S*-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and its subsequent oxidation to ethylene, are regulated by ACC synthase (ACS) and ACC oxidase (ACO), respectively [46, 47]. These enzymes are encoded by multigene families present in all plant species and consist of at least nine and four members, respectively, in *Arabidopsis* and tomato [48, 49]. Sucrose treatment also decreased the ethylene responsiveness of carnation petals and has been correlated with decreased activities of ACC oxidase and ACC synthase [50]. Kumar *et al.* [51] also reported that ethanol plus sucrose treatment suppressed ethylene production, thereby delaying aging in cut rose (*R. hybrida*).

19.2.2
Salt Stress

Salinity toxicity is a worldwide agricultural and an ecoenvironmental problem. Approximately one-third of the world land surface is arid and semiarid, of which one half is affected by salinity [52]. In cut flower species, the number of flowers per plant and total number of flowering plants are reduced when exposed to salt stress [53, 54]. Reproductive developmental processes in salt-stressed roses in pot culture have been reported as being salt susceptible [19, 55]. The reproductive stage of *in vitro* miniature roses was also found to be sensitive to salt stress [16] due to abnormal flower development, maximum pigment degradation, low photosynthetic abilities, and maximum growth reduction. In "Hot Lady" hybrid rose, the Chl "a" and Chl "b" pigments were damaged significantly under salt stress (25 mM NaCl) and were lower than the control by 46.32% [19]. Under salt stress conditions, Na^+ is generally enriched at the cellular level, leading to induced program cell death [22] and inhibiting growth characters, especially fresh weight, dry weight, and leaf area [23].

In climatic conditions of Northern Hemisphere, use of salt for road deicing causes increased salinity of road side soils. Wrochna *et al.* [25] studied the effect of deicing substances on *R. rugosa* and observed decrease of chlorophyll content, potential photochemical efficiency, performance index, and biomass accumulation.

The effects of salt on rose depend on cultivar [56] and on the type of culture used, for example, soil or hydroponics [57]. Improvement of nitrate uptake and assimilation by rose plants have better implications in terms of increased flower production. Thus, the relationship between production and any element that could interfere with nitrate assimilation is very important. Under high sodium concentrations, the uptake of nitrate, potassium, and phosphate is adversely affected and nitrate reductase (NR) activity decreased by up to 50%. An increase of NR activity and leaf glutathione synthase (GS) takes place at low sodium concentrations [58].

In rootstock roses, namely, *R. chinensis* cv. "Major," Na^+ in the leaf tissues caused injury to the leaves (90.8%) when exposed to 30 mM NaCl for 13 months [17]. In hydroponic culture of roses (*R. hybrida* L.), Na^+ in the salt-stressed leaves reached

150 mM/kg after cultivation for 100 d in 40 mM NaCl salt treatment [59]. In *in vitro* cultivated rootstocks of roses, Na^+ has been reported as a toxic ion that accumulates in the leaf tissues leading to leaf necrosis and chlorosis symptoms [17]. Vegetative growth and quality of cut flowers were affected by NaCl treatment in *R. chinensis* cv. "Major," *R. rubiginosa*, and *R. hybrida* cv. "Kardinal" [60]. Leaf injury was more pronounced in *R. chinensis* cv. "Major" compared to the other two genotypes. The rootstock *R. rubiginosa* showed a higher tolerance to the NaCl stress than *R. chinensis* cv. "Major." The tolerance in the cultivar *R. hybrida* cv. "Kardinal" and rootstock *R. rubiginosa* was approximately the same. The survival of the plants under increased duration of NaCl stress as well as extent of leaf injury could be used in the determination of tolerance of the rose genotypes. Increasing of NaCl concentration leads to a significant reduction in the length of the cut flowers in *R. hybrida* cv. "Kardinal". Niu *et al*. [24] studied the effect of saline water irrigation on growth and physiological responses of three rose rootstocks, namely, *R. fortuniana*, *R. multiflora*, and *R. odorata*. The elevated salinity of the irrigation water reduced the relative chlorophyll concentration and maximal photochemical efficiency of photosystem II (PSII) and minimal fluorescence (F-o)/maximum fluorescence (F-v/F-m), with largest reduction in F-v/F-m at 2.4%. Based on growth and visual quality, *R. fortuniana* was relatively more salt tolerant followed by *R. odorata* and *R. multiflora*. Cabrera *et al*. [61] evaluated the yield and quality and the ion accumulation responses of roses grafted on various rootstocks to increasing salinity stress. Salt concentration increases significantly and affected the biomass, cut flower production, and foliage and quality of the roses, but the responses were modulated by rootstock selection. Cultivar "Manetti" plants in general sustained better absolute and relative biomass and flower yields, accumulated less Na^+ and Cl^- in its tissues, and showed less toxicity symptoms with increasing salinity compared to *R. odorata* (Andr.) cv. "Natal Briar" and cv. "Dr. Huey" rootstocks.

Salt tolerance of many crop plants is reported to be enhanced by addition of silicon. The nutritional properties of silicon in plant growth are not well established. The stimulation of growth by silicon may be either indirect, owing to the protective effects of Si against pathogens [62, 63], or direct originating from implications of silicon to both morphological changes and physiological processes in plants.

The quality determinants of cut flowers depend upon their visual appearance. Various factors are responsible for aberrant color changes in flowers. Reezi *et al*. [19] reported that silicon controlled salt stress, decreased malondialdehyde content, reduced chlorophyll content, and affected petal color of cut rose (*R. hybrida* L.) cv. "Hot Lady." Addition of 50 ppm Si increased the flower number in plants grown under both salinity and unstressed conditions. It may act to alleviate salt stress in rose by decreasing permeability of plasma membranes and membrane lipid peroxidation and by maintaining the membrane integrity and function. Liang *et al*. [52, 64] reported that silicon decreased the permeability of plasma membrane of leaf cells and significantly improved the ultrastructure of chloroplasts that were badly damaged by the added NaCl [65].

Proline accumulation in the salt-stressed leaves is a defense response of plants to salt stress [66]. Alternatively, proline, well known as an antioxidant or

alleviator of oxidative stress, is identified by antioxidant enzyme regulation [67] and malondialdehyde content [68]. In addition, endogenous and exogenous proline in the petals of roses may play a key role in delaying senescence in cut flowers [20, 21].

19.2.3 Light Stress

A perusal of literature reveals a correlation between radiation [69–71] plantation density and radiation interception [72] and Leaf Area Index (LAI) [73] on rose production. Decrease in light intensity and the duration of light period owing to the seasonal changes or shading reduce the yield of roses, total plant weight, and growth of plant. Flower bud abortion in rose is affected by low light intensity at early stages of shoot development. High light intensity is necessary for anthocyanin formation and hence color in rose cultivars. The plants regulate their metabolism and development depending upon the quantity and quality of light [74]. The interception by the plant photosynthetically active radiation (PAR) and LAI is very important because it is correlated with canopy photosynthesis and the dry mass production in plants that grow free of biotic or abiotic stress [75].

Mascarini *et al*. [27] assessed the changes in light quality, Leaf Area Index, plant water content and its impact on rose flowering, shoot quality, and yield using spectral reflectance in different rose canopy management. The bent stems form a floor of green matter like a dense crop and transmit and reflect more strongly on FR than on R wave band resulting in smaller R: FR ratio that advances flowering. High light interception increases the photosynthesis rate, thereby enhancing dry mass gain [76]. Ultimately, flowering shoot quality improved in terms of larger stem and bud diameter and increased fresh weight. In Mercedes roses, the bent shoot technique reduced the number of leaves by 25%, but light interception was 89% greater in comparison to traditional management. Whole plant net photosynthetic rate was 28% higher in plants grown with shoot bending than in those grown with traditional upright growing technique. The bent shoots also provide good hiding places for beneficial predators and parasites between the flushes, which is an advantage in the biological pest control of cut roses. Bending the blind shoots in the traditional cultivation technique is recommended for cultivars like “Mercedes” that are not as strongly growing cultivars as “Frisco.” However, blind shoot bending results in long flowering stems in both cultivars [73].

Under greenhouse conditions in hot climates, high summer temperatures limit productivity of roses due to heat stress. Common approaches to reduce the damage are using shadow screens or white washing having 50% reflectivity across the whole solar spectrum including PAR, thereby reducing photosynthesis and crop production. Baille *et al*. [77] found that glass whitening had positive effect on crop yield of *R. hybrida* cv. First Red. However, Stanghellini *et al*. [78] suggested filtering of near-infrared (NIR) range (400–700 nm) to reduce warming of greenhouse and hence transpiration without affecting crop photosynthesis and yield. They discussed the application of NIR selective filters

in *R. hybrida* cv. Passion under greenhouse conditions for increasing photosynthesis and crop productivity.

19.2.4
Low-Temperature Stress

Chlorophyll fluorescence (CF) is an indicator to assess chilling injury in leaves of roses [79]. The technique provides a rapid method to prescreen genotypes for chilling tolerance in *Rosa* sp., as required in plant breeding. As the temperature decreases, variable fluorescence (Fv) decreases dramatically in the more susceptible genotypes, whereas it is more stable and decreases more slowly in the less susceptible genotypes.

Wild roses growing in and around Palampur area of Kangra district of Himachal Pradesh were collected and domesticated at CSIR-IHBT for their identification, conservation, and possible future utilization in the development of new variants of roses through breeding, selection of suitable types for rose rootstocks, and maintenance of potential indigenous rose germplasm having other desirable characters. Based on taxonomic and morphological studies, the accessions were identified and kept in to their respective grouped species. Furthermore, experiments were carried out for the evaluation of better compatibility, easy to multiply, and winter-active strains of wild rose from the domesticated population for selection and utilization as suitable germplasm.

A well-known rose rootstock (*R. indica* var. *odorata*) and scion bud of Scarlet Queen Elizabeth were used as a control for all the experiments. Based on information generated by these experiments, out of 21 wild strains 4 strains belonging to the 4 different species were selected as potential wild germplasm of roses. These selected germplasm are better or *at par* in terms of compatibility, propagation, plant vigor, production of higher number and better height of flowering shoots, and active growth during severe winter (November 15–February 15) months under Palampur conditions than the control. The selected germplasm can be utilized as rose rootstocks and in the improvement of roses and rootstocks through breeding. These four domesticated and potential germplasm accessions of IHBT were awarded registration numbers and national identity number also by the National Germplasm Registration Committee, New Delhi. Details of these winter-active rootstocks are given in Tables 19.2 and 19.3.

19.2.5
High-Temperature Stress

Heat stress during summer months causes leaf yellowing in roses. Recently planted roses with less developed strong root system are most susceptible to heat stress due to increased rate of transpiration. Heat stress causes scorched leaf margins, wilting, and dark-edged rose petals. Under heat stress, rose buds that start to develop turn brown and drop off. When plants are under heat stress, they stop

Table 19.2 Winter-active wild rootstocks cultivated at CSIR-IHBT Palampur.

Rose accession/germplasm registration number/National Identity Number	Floral characteristics
IHBT- WR-24/INGR 08066/IC549905 (wild strain of *R. multiflora*)	 Winter-active, easy to multiply, compatible to scion, and produced better number and height of flowering shoots than the standard rootstock of *R. indica var. odorata* under Palampur conditions. Potential indigenous rootstock. Flower white double, diameter 5.64 cm, flowers/panicle 54.2 and number of petals/flower 29.2, sepals and thalamus hairy. Number of hips/panicle 18.8, size of hips 0.73 cm. No. of seeds/hip 10.3
IHBT-WR-16/INGR 08067/IC549906 (wild strain of *Rosa brunonii*)	 Vigorous, winter-active, easy to multiply through stem cuttings, compatible to scion, and produced number and height of flowering shoots at par to the standard rootstock of *R. indica* under Palampur conditions. The strain has potential to be utilized as rootstock and further improvement of roses through breeding. Flower white, single, diameter 5.31 cm. Number of flowers/panicle 44.7 and number of petals/flower 5–9. Sepals and thalamus glandular. Number of hips/panicle 34.80, size of hips 0.73 cm, and number of seeds/hip 11.2.
IHBT-WR-23/INGR 08068/IC549907 (wild strain of *Rosa cathayensis*)	 Winter-active, easy to multiply, better compatible to scion, and produced better number and height of flowering shoots than the standard rootstock of *R. indica var. odorata* under Palampur conditions; Potential suitable rootstock. Flowers double, pink color, diameter 4.16 cm, number of petals/flower 28, number of flowers/panicle 32. Sepals hairy and glandular, thalamus hairy, number of hips/panicle 1, size of hips 0.6 cm, and number of seeds/hip 7.0

(*continued*)

Table 19.2 (*Continued*)

Rose accession/germplasm registration number/National Identity Number	Floral characteristics
IHBT-WR-21/INGR 08069/IC549908 (wild strain of *Rosa alba*)	 Winter-active, easy to multiply, compatible to scion, and produced better number and height of flowering shoots than the standard rootstock of *R. indica* under Palampur conditions. Potential suitable rootstock for improvement of roses. Flower white with pinkish tinge in early stage, double, diameter 4.16 cm, number of flowers/panicle 16.7, and number of petals/flower 75. Sepals and thalamus hairy. Number of hips/panicle 4.1, size of hips 0.6 cm, and number of seeds/hip 7.0.
IHBT-0 (Control) *R. indica var. odorata*	 Grows up to 8–10 feet and suckers easily. Nonrecurrent flowering behavior. The flower color whitish pink. Commonly used rootstock, for easy multiplication of roses under north Indian conditions, performs well in soil with pH above 7.5–8.

their less essential functions – such as producing flowers – in favor of simple survival. Expression and accumulation of sHSPs in plants can be triggered by various abiotic stress conditions, such as extreme temperatures [80–83], salinity [84], drought [85], and osmotic [86] and oxidative stresses [87–89]. RcHSP17.8 expression in *R. chinensis* was induced by heat, cold, salt, drought, and osmotic and oxidative stresses.

Table 19.3 Comparative evaluation of winter-active rootstocks.

S. No.	Accession	Species	% Winter-active growth	% Compatibility
1	IHBT-WR-23	*Rosa cathayensis*	44.68	47.19
2	IHBT-WR-24	*R. multiflora*	4.41	42.58
3	IHBT-WR-16	*R. brunonii*	8.39	22.21
4	IHBT-WR-21	*R. alba*	53.46	23.14
5	IHBT-0 (Control)	*R. indica* var. *odorata*	2.45	24.99

19.3
Abiotic Stress and Reactive Oxygen Species

Various abiotic stresses [90–92] lead to the overproduction of reactive oxygen species in plants that are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates, and DNA, which ultimately results in oxidative stress [93]. SOD is the key enzyme in plant defense against the damage caused by $O_2^{\bullet -}$ [94]. Various isoforms of superoxide dismutase were detected in senescing rose petals. Changes in SOD activity are induced by the substrate $O_2^{\bullet -}$, which increases under stress conditions. Higher SOD activity leads to an increased synthesis of H_2O_2. This signal molecule then induces the activities of other antioxidant enzymes. The balance between SOD and the different H_2O_2 scavenging enzymes in cells is crucial in determining the steady-state levels of $O_2^{\bullet -}$ and H_2O_2. Petal senescence, the final stage in petal development, involves an array of physiological and metabolical changes. Senescence in rose petals is associated with increased production of $O_2^{\bullet -}$ radicals up to stage-5 of flower bud development. SODs efficiently remove $O_2^{\bullet -}$ but generate H_2O_2 as a by-product [95]. Higher concentration of H_2O_2 was detected during senescence of rose petals [96, 97], which may have inactivated both Cu–Zn–SOD and Fe–SOD [98]. Being resistant to H_2O_2 concentrations, Mn–SOD remains functional at higher concentrations of H_2O_2 and protects the petals from deleterious effect of more toxic $O_2^{\bullet -}$.

In plants, proline constitutes less than 5% of the total pool of free amino acids under normal conditions, but under stress it is 80% of the total amino acid pool [99], indicating a positive relation between intracellular proline levels and resistance to oxidative stress. Proline metabolism imparts a protective effect during petal senescence in rose. Exogenous proline suppressed the oxidative stress and increased the vase life of cv. "Grand Gala" rose (*R. hybrida* L.) [21] and cv. "First Red" cut rose [100]. Proline dehydrogenase (PDH) activity was also very high in proline-treated flowers. Proline catabolism might provide higher energy to delay the aging of flower petals. Similarly, higher reduced glutathione (GSH) content and GSH/GSSG (oxidized glutathione) ratio showed the existence of reduced redox state in cells of proline-treated flower petals. Total SOD activity was substantially higher in treated flower, which might suppress the rate of $O_2^{\bullet -}$ generation. Use of proline is therefore recommended in commercial formulation for enhancing vase life of roses.

19.4
Stress-Related Genes Associated with Abiotic Stress Tolerance in Rose and Attempts to Transgenic Development

Currently, transgenic technology provides the best hope for improvement in plants where traditional breeding is not feasible due to polyploidy that complicates breeding efforts. Substantial progress has been made in the identification of genes involved in abiotic stress tolerance and in their transfer to crops of economic

Table 19.4 Stress-related genes in genus *Rosa*.

Gene (s)	Species	Response	Reference
Ethylene receptor genes *Rh-ETR1* *Rh-ETR3* CTR-like protein kinase genes *Rh-CTR1* *Rh-CTR2*	*R. hybrida*	Ethylene regulates flower opening through expression of two ethylene receptor genes (Rh-ETR1 and Rh-ETR3) and two CTR (Rh-CTR1 and Rh-CTR2) genes	[102]
CTR genes *Rh-CTR1* *Rh-CTR2*	*R. hybrida*	RhCTR1expression increases during flower senescence RhCTR2 constitutively expressed during flower development Expression of both the genes increased in response to ethylene	[103]
RcHSP17.8	*R. chinensis*	Overexpression of gene simultaneously enhances thermotolerance, oxidative and osmotic stress tolerance intransformed *Escherichia coli*, yeast and *Arabidopsis thaliana*	[104]
Vacuolar Na^+/K^+ antiporter gene *RhNHX1*	*R. hybrida*	Expression increases in presence of NaCl. The gene product functions as Na^+/H^+ antiporter in plants	[105]
Plasma membrane aquaporin gene: *Rh-PIP2;1*	*R. hybrida*	Plays an important role in petal expansion. Ethylene inhibits petal expansion by suppressing Rh-PIP2;1 expression	[106]
Sucrose transporter gene: *Rh-SUC2*	*R. hybrida*	Modulation of sucrose import during bud break	[107]
Rh-TIP1;1	*R. hybrida*	Gene expression during flower opening influenced by ethylene and water deficit	[108]
Rh-DREB1A and *Rh-DREB1B*	*R. hybrida*	Involved in signaling pathway of water deficit stress	[18]
Ascorbate peroxidase gene: *Rh-APX1*	*R. hybrida*	Regulation of APX at the transcript level may be involved in the response to water deficit stress	[18]

importance for increased stress tolerance. Many stress-related genes are active in developing rose petals. The information available on stress-related genes in the genus *Rosa* is summarized in Table 19.4. A large number of stress-related proteins were identified in developing petals, including low and high molecular weight HSPs, peroxidase, superoxide dismutase, and catalase [101]. The accumulation of stress-related proteins in petals may be indicative of the requirement for protection

of the cell during flower development against either intracellular stress (e.g., an oxidative environment) or external stresses.

Osmotin is a stress-responsive protein adapted to salinity, desiccation, and low temperature and accumulates in salt-adapted cells [109]. Overexpression of osmotin was reported to induce proline accumulation and tolerance to osmotic stress in transgenic tobacco [110]. Husaini and Abdin [111] introduced osmotin gene directly into strawberry and transgenic plants tolerant to salt stress were produced. Osmotin is a multifunctional 24 kDa basic protein belonging to PR-5 protein family. It provides osmotolerance to plants probably by facilitating compartmentation of solutes or by being involved in metabolic or structural alterations during osmotic adjustment. Singh *et al.* [112] hypothesized that the synthesis of osmotin protein could induce synthesis and accumulation of certain solutes or could be involved in metabolic or structural changes. At CSIR-IHBT, biolistic-mediated transformation of *R. bourboniana* somatic embryos was carried out employing the osmotin gene of *Nicotiana tabacum*, under the control of constitutive 35S CaMV promoter. The transformed plants grown in the contained facility were tested for the stable integration of the gene using PCR and Southern blot hybridization. The transgenic lines were tested for salt and drought stress using NaCl (0, 400 and 800 mM) and PEG (0%, 5%, and 10%).

Experiments were conducted with leaf disks from transformed and untransformed plants. In case of untransformed plants, the leaf disks turned brown after 4 days, whereas transformed ones remained green. The chlorophyll content was also higher in transgenic plants, indicating an increased salt tolerance of the transgenic plants of *R. bourboniana*. At 400 and 800 mM NaCl concentration, total chlorophyll content of leaf disks derived from transgenic plants (T_1 and T_2) ranged from 2.6 to 2.77 mg/g fr. wt. compared to 2.3–2.4 mg/g fr.wt in leaf disks derived from untransformed ones, indicating increased salt tolerance of the transgenic *R. bourboniana*. Cut shoots from transgenic plants cultured in NaCl solutions (0, 400, and 800 mM) for 4 days showed higher total chlorophyll and relative water content at all levels of treatment compared to untransformed. Cut shoots cultured in solutions of 0%, 5%, and 10% polyethylene glycol (PEG 6000) at 25 °C under 16 h photoperiod for 24 h were analyzed for total chlorophyll and relative water content. At all levels of treatment, chlorophyll content and RWC of cut shoots derived from transgenic plants were found higher than untransformed, thereby suggesting increased tolerance to drought stress.

19.5 Conclusions

High economic value and widespread cultivation of rose makes it one of the commercially important ornamental crops. Rose crop production is strongly affected by stresses like drought, soil salinity, heat, and cold. Susceptibility to different abiotic stresses varies according to the species, the scion, the rootstock

and their interaction, and the developmental stage of the plant. The possibility of growing rose crops on marginal land would depend on the successful selection of varieties tolerant to these abiotic stresses. In view of global scarcity of water resources and the increased salinization of soil and water, abiotic stress is a major limiting factor in plant growth. The need of the hour is to integrate biotechnology with classical physiology and breeding. Understanding of the underlying physiological, biochemical, and molecular events leading to stress in plants is much warranted.

Our limited knowledge of stress-associated metabolism is still a major gap in our understanding of stress tolerance in many plant species including roses. Therefore, comprehensive profiling of stress-associated metabolites, combined with stress metabolomics of major crop plants, will be a key factor in molecular breeding for tolerance. Another area for future studies will be the detailed analysis of physiological and molecular mechanisms underlying abiotic stress tolerance in salt-tolerant model species, which will enable future molecular dissection of salt tolerance mechanisms in important crop plants.

The use of transgenic approaches to improve the tolerance of roses to abiotic stresses remains an attractive option. Options targeting multiple gene regulation appear better than targeting single gene regulation. A well-focused approach combining the molecular, physiological, and metabolic aspects of abiotic stress tolerance is required for bridging the knowledge gaps between the molecular or cellular expression of the genes and the whole plant phenotype under stress.

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