Parvaiz Ahmad
Mohd Rafiq Wani *Editors*

Physiological Mechanisms and Adaptation Strategies in Plants Under Changing Environment

Volume 2

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Editors
Parvaiz Ahmad
Department of Botany
Sri Pratap College
Srinagar, Jammu and Kashmir
India

Mohd Rafiq Wani
Department of Botany
Govt. Degree College (Boys)
Anantnag, Jammu and Kashmir
India

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Preface

Environmental stresses such as salinity, drought, flooding, extreme temperatures, nutrient deficiency, or toxicity in addition to deteriorating soil conditions pose major intimidation to agriculture and food security worldwide. The productivity loss is elicited by a series of morphological, physiological, biochemical, and molecular stress-induced changes. Such an adverse situation is in contrast with the mounting global food demand and becomes even more testing in developing countries where they cause severe food insecurity and ruthless poverty for large populations predominantly in rural areas. Global population is escalating at a distressing rate and is anticipated to reach beyond nine billion by the end of 2050. While plant productivity is being seriously constrained by a range of abiotic stresses, feeding the world population under such horrid environment is a major disquiet for all nations. Water stress, on one hand, affects in excess of 70 million hectares of rice-growing land globally, whereas salt and nutrient stresses render more than one hundred million hectares of agricultural land uncultivable, thereby resulting in low outputs, poor human nutrition, and abridged educational and employment avenues. Knowledge and technology in biological science is expanding leaps and bounds, thus it becomes imperative to keep ourselves updated with the advances in plant abiotic stresses to meet the current scientific challenges, particularly the growing food demand for world population. In this scenario, it is urged that such strategies should be adopted which may be used to get maximum crop stand and economic returns from stressful environments. By employing contemporary tools and techniques, strenuous attempts are being made worldwide to understand how plants respond to these stresses. In this context, the book "*Physiological Mechanisms and Adaptation Strategies in Plants under Changing Environment*" *Volume 2* will prove an indispensable source for scientists, students, and others seeking advancements in this area of research.

The present volume comprises of 13 chapters and each chapter has different research scope from the other. Chapter 1 throws light on biochemical and molecular approaches for drought tolerance in plants. Here, the authors scrupulously review the effects of drought stress on biochemical parameters especially proline metabolism in plants besides recounting the mechanism of drought resistance on physiological, molecular, and enzymatic basis. Chapter 2 addresses the heavy-metal

attack on freshwater side: physiological defense strategies of macrophytes and ecotoxicological ops, wherein the authors have comprehensively put in their efforts in elaborating the role of Cd and Cu pollution for inducing heavy-metal stress at all organization levels. The authors reveal that physiological responses remain very sensitive to the xenobiotic levels and constitute the first step towards the development of histological protection against the free radicals. Chapter 3 is about the secondary metabolites and environmental stress in plants: biosynthesis, regulation, and function. In this chapter, recent developments on structural and regulatory genes involved in the biosynthesis of secondary metabolites are explicitly discussed.

Chapter 4 is about the major phytohormones under abiotic stress, where the authors uncover the pivotal role of phytohormones in plants for adapting to changing environments by mediating growth, development, nutrient allocation, and source/sink transitions. Furthermore, the chapter summarizes the recent progress concerning the essential role of phytohormones in plant responses to abiotic stress, which has brought change in transcriptomics, metabolomics, and proteomics. Chapter 5 is regarding the nitric oxide and its role in plants under abiotic stress. In this chapter, the author presents the comprehensive synthesis of nitric oxide and its role in many physiological and developmental processes in addition to signaling molecule interactions with plant hormones and defense gene regulations under environmental stresses. Chapter 6 describes brassinosteroids: improving crop productivity and abiotic stress tolerance. The chapter focuses on the exogenous application of effective doses of brassinosteroids (BRs) in stress-affected plants, which play crucial roles in wide spectrum of biochemical, physiological, growth and developmental processes, besides defending them from adversaries of environmental stresses.

Chapter 7 deals with ethylene and its role in plants under environmental stress. In this chapter, it is highlighted that ethylene acts via complex signaling pathway leading to the activation of *Ethylene Response Factor* (*EtRF*) genes which represent one of the largest transcription factor families in the plant kingdom. Chapter 8 describes the scenario of climate changes in the context of agriculture. Here, the authors painstakingly discuss the contributing factors to global warming in addition to global distribution of synthetic organic compounds, alteration in biochemistry of elemental cycle, and impact of climatic changes on the productivity of plants. Chapter 9 is concerned with the role of protective compounds in stress tolerance. This chapter highlights how protective compounds alleviate the effects of environmental stresses, especially drought and salt and function as metabolic signals for broader influence on physiological responses and metabolic adjustments vis-à-vis stressful conditions.

Chapter 10 deals with the growth patterns of tomato plants subjected to two nonconventional abiotic stresses: UV-C irradiations and electric fields. This chapter covers the effects of the exposition of tomato to UV-C radiation and DC-electric field in bringing the significant alterations in plant growth. The protection of tomato plants against UV-C, combined with growth-promoting effects of electro-culture, could allow farmers to grow better crops in less time and at lower cost. Chapter 11 is about rhizobacteria and the restoration of heavy-metal contaminated soils. In this chapter, the authors enumerate the panoply of mechanisms used by microorganisms to cope

up with metal stress and mobilize their plant growth promotion traits in association with their host plants with special emphasis to actinobacteria in metal contaminated lands. Chapter 12 deals with potassium and sodium transport channels under NaCl stress, where the authors have discussed in detail the pathways for Na^+ and K^+ transport across the plasma membrane, tissue distribution of these ions, and their intracellular compartmentalization. Chapter 13 is about *Jatropha curcas*: an overview. The chapter encompasses different aspects of *Jatropha* plantation and its uses including in soil conservation under stressful conditions. In addition, the chapter also includes the information about phytochemical constituents of *Jatropha* and its possible allelopathic effects.

Chapters contributed in this book have been published keeping intact author's justifications; however, suitable editorial changes were made, wherever considered necessary. In spite of our best efforts, there is a chance of some errors still creeping in the book, for which we seek reader's feedback. We wish to express our appreciation to the well-versed contributors, who readily accepted our invitation to write the chapters. Moreover, we would like to thank Springer Science+Business Media, LLC, New York, particularly Eric Stannard (Editor Botany), Flora Kim (Developmental Editor), Andy Kwan (Assistant Editor), and all the other staff members of Springer, who were directly or indirectly associated with us in the current project for their constant support and efforts in bringing out the timely publication of this volume.

Srinagar, Jammu and Kashmir, India Parvaiz Ahmad
Anantnag, Jammu and Kashmir, India Mohd Rafiq Wani

About the Editors

Dr. Parvaiz Ahmad (Editor) Dr. Parvaiz Ahmad is Assistant Professor in Botany at S. P. College, Srinagar, Jammu and Kashmir, India. He has completed his post-graduation in Botany in 2000 from Jamia Hamdard, New Delhi, India. After receiving Doctorate degree from Indian Institute of Technology (IIT) Delhi, India, he joined International Centre for Genetic Engineering and Biotechnology, New Delhi in 2007. His main research area is Stress Physiology and Molecular Biology. He has published more than 30 research papers in peer-reviewed journals and 19 book chapters. He is also an editor of 6 volumes (5 with Springer NY USA and 1 with Studium Press Pvt. India Ltd., New Delhi, India). He is the recipient of Junior Research Fellowship and Senior Research Fellowship by CSIR, New Delhi, India. Dr. Parvaiz has been awarded Young Scientist Award under Fast Track scheme in 2007 by Department of Science and Technology (DST), Govt. of India. Dr. Parvaiz is actively engaged in studying the molecular and physio-biochemical responses of different agricultural and horticultural plants under environmental stress.

Dr. Mohd Rafiq Wani (Co-editor) Dr. Mohd Rafiq Wani is currently Assistant Professor in Botany at Government Degree College (Boys), Anantnag, University of Kashmir, India. Dr. Wani did his Masters in Botany in 2003 with specialization in "Genetics and Plant Breeding" from Aligarh Muslim University (AMU), Aligarh, UP, India. After obtaining the Degree of Doctorate in 2008 for his research work on "Chemical Mutagenesis in Mungbean" from the same University, he joined Department of Higher Education, Government of Jammu and Kashmir in 2009. He teaches a

range of bioscience-related subjects at undergraduate/post-graduate levels. At present, his research interests are mainly focused on the improvement of pulses through induced mutations and exploring the physiological and biochemical responses of crop plants to a range of biotic and abiotic stresses. As a part of his research endeavour, Dr. Wani has extensively researched and written on the issues of induced chemo-mutagenesis among the food crops, with special reference to pulses. He has around twenty eight (28) research publications to his credit, published in various international and national journals of repute. Moreover, he has also submitted several book chapters to various research-oriented volumes. Dr. Wani, while constantly working for his academic and research interests, is currently in the process of editing many volumes of books on the subjects of plant stress physiology and induced plant mutagenesis with reputed international publishers. In addition, he is an editorial member and reviewer of few online journals pertaining to plant sciences, besides being the life member of various scientific societies like Indian Society of Pulses Research and Development (ISPRD) and Indian Society of Genetics and Plant Breeding (ISGPB).

Contents

Contributors

Elsayed Fathi Abd-Allah Faculty of Food and Agriculture Sciences, Plant Production Department, King Saud University, Riyadh, Saudi Arabia

R.M. Agarwal School of Studies in Botany, Jiwaji University, Gwalior, Madhya Pradesh, India

Mohammad Abass Ahanger School of Studies in Botany, Jiwaji University, Gwalior, Madhya Pradesh, India

Parvaiz Ahmad Department of Botany, Sri Pratap College, Srinagar, Jammu and Kashmir, India

Mohammad Babar Ali Rhizosphere Science Laboratory, Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA

Muhammad Ashraf Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Andrzej Bajguz Institute of Biology, Department of Plant Biochemistry and Toxicology, University of Bialystok, Bialystok, Poland

Shagun Bali Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Renu Bhardwaj Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Donato Castronuovo School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

David Delmail Laboratory of Pharmacognosy and Mycology—UMR CNRS 6226 ISCR PNSCM, University of Rennes 1 (European University of Brittany), Rennes, France

Oxana Dobrovinskaya Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, Colima, Mexico

Magda Formela Department of Plant Physiology, Poznań University of Life Sciences, Wołyńska, Poznań, Poland

Vandana Gautam Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Piotr Goliński Department of Chemistry, Poznań University of Life Sciences, Poznań, Poland

Anber Hamdani Crops Science Institute, National Agricultural Research Center, Islamabad, Pakistan

Asiya Hameed Department of Botany, Jamia Hamdard University, New Delhi, India

Sumiya Jamsheed Department of Botany, Jamia Hamdard University, New Delhi, India

Sumira Jan Department of Botany, Faculty of Science, Jamia Hamdard, New Delhi, India

Dhriti Kapoor Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Ravdeep Kaur Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Alvina Gul Kazi Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Kiyoon Kim Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Ashwani Kumar Mycorrhizal Research Laboratory, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa

Pascal Labrousse Faculty of Pharmacy, Laboratory of Botany and Cryptogamy—GRESE EA 4330, University of Limoges, Limoges, France

Stella Lovelli School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

Van Chung Mai Department of Plant Physiology, Poznań University of Life Sciences, Poznań, Poland

Department of Plant Physiology, Vinh University, Vinh, Vietnam

A.J. Matilla Faculty of Pharmacy, Department of Plant Physiology, University of Santiago de Compostela (USC), Santiago de Compostela, Spain

M.A. Matilla-Vázquez Department of Biochemistry, University of Cambridge, Cambridge, UK

Iwona Morkunas Department of Plant Physiology, Poznań University of Life Sciences, Wołyńska, Poznań, Poland

Aisha Naseem Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Poonam Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Igor Pottosin Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, Mexico

School of Agricultural ScienceUniversity of Tasmania, Hobart, TAS, Australia

Saiema Rasool Department of Botany, Jamia Hamdard University, New Delhi, India

Rida Rehman Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Tongmin Sa Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Aykut Sağlam Molecular Biology and Genetics, Karadeniz Technical University, Trabzon, Turkey

Antonio Scopa School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

Anket Sharma Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Indu Sharma Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Subzar Ahmad Sheikh Department of Botany, Government Degree College (Boys), Anantnag, Jammu and Kashmir, India

Adriano Sofo School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

Parthiban Subramanian Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Giuseppe Tataranni School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

Seifeddine Ben Tekaya Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Sherlyn Tipayno Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Nisha Singh Tomar School of Studies in Botany, Jiwaji University, Gwalior, Madhya Pradesh, India

Ana-María Velarde-Buendía Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, Mexico

Mohd Rafiq Wani Department of Botany, Government Degree College (Boys), Anantnag, Jammu and Kashmir, India

Agnieszka Waśkiewicz Department of Chemistry, Poznań University of Life Sciences, Poznań, Poland

Chapter 1
Biochemical and Molecular Approaches for Drought Tolerance in Plants

Parvaiz Ahmad, Asiya Hameed, Elsayed Fathi Abd-Allah, Subzar Ahmad Sheikh, Mohd Rafiq Wani, Saiema Rasool, Sumiya Jamsheed, and Ashwani Kumar

1 Introduction

Plants are subjected to variety of abiotic stresses such as drought, temperature, salinity, air pollution, heavy metals, UV radiations, etc. (Ahmad et al. 2008a; Ahmad and Prasad 2012a, b). Abiotic stress adversely affects crop production worldwide, decreasing average yields for most of the crops to 50 % (Bray et al. 2000). Abiotic stress hampers all the metabolic processes and affect the normal functioning of plant (Ashraf et al. 2006, 2009; Jaleel et al. 2007a, b, c, 2008a, b, c; Azooz et al. 2009; Koyro et al. 2012; Katare et al. 2012; Ahmad and Prasad 2012a, b). Drought is one of the major abiotic stresses occurring in many parts of the world and is the main limiting factor in crop production (Ashraf et al. 2006, 2009; Jaleel et al. 2007a, b, c). Due to an increasing world population, drought stress will lead to a serious food shortage by 2050 as the population is expected to reach ten billion. Water stress may result either from an insufficient water availability because of

P. Ahmad (✉)
Department of Botany, Sri Pratap College, Srinagar 190 001, Jammu and Kashmir, India
e-mail: parvaizbot@yahoo.com

A. Hameed • S. Rasool • S. Jamsheed
Department of Botany, Jamia Hamdard University, New Delhi, India

E.F. Abd-Allah
Faculty of Food and Agriculture Sciences, Plant Production Department, King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia

S.A. Sheikh • M.R. Wani
Department of Botany, Govt. Degree College (Boys), Anantnag 192 102, Jammu and Kashmir, India
e-mail: botanyrafiq@gmail.com

A. Kumar
Mycorrhizal Research Laboratory, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, PO Box 94, Grahamstown 6140, South Africa

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drought or from an excessive presence of water activity in the plant's environment (Jaleel et al. 2007a, b). Water deficit means the absence of sufficient moisture content necessary for normal plant growth and its life cycle (Zhu 2002; Sankar et al. 2008). Plant experiences drought stress either when the roots face water deficit or because of the enhanced transpiration rate and both these conditions often coincide under arid and semiarid climates (Sankar et al. 2007a; Sakcali et al. 2008).

Drought accelerates the effect of other stresses to which plants are subjected to and several different abiotic stresses (like salt and cold stress) result in water stress (Ahmad and Prasad 2012a, b). The general effects of drought on plant growth are well known, but the effects of water deficit at biochemical and molecular levels are not well understood (Shao et al. 2008a). Plant species possess distinctive indicators of stress tolerance at whole plant, tissue, or cellular level (Munns 2002). Sufficient evidences favour the view that adaptive osmotic adjustment is mediated by proline and glycine betaine (GB), that also helps in protecting the subcellular structures in stressed plants. Proline accumulation has been advocated as a selection criterion for stress tolerance (Azooz et al. 2004; Jaleel et al. 2007d; Ahmad et al. 2008b). Proline accumulation has been reported in many plants on exposure to the stresses like temperature, drought, salt, heavy metal, etc. (Sairam et al. 2002; Ahmad et al. 2006, 2011a, 2012a, b; John et al. 2009; Katare et al. 2012). Enhanced proline level enables the plant to maintain low water potentials (Jaleel et al. 2007e, 2008d, e). As the water potential decreases, the compatible osmolytes involved in osmoregulation accumulate, resulting in additional water absorption thus overcoming the immediate effect of water shortages (Azooz 2004; Ahmad and Sharma 2008; Jaleel et al. 2009). The main role of these osmolytes is probably to insulate plant cells against the destructive effects of stress by preserving the osmotic balance by stabilizing the structure of key proteins such as Rubisco, by protecting the macromolecular structure and function and helps to adapt stress injury (Bohnert and Jensen 1996).

Water stress tolerance is a natural phenomenon in all plant species, but it varies from species to species. Improving the efficiency of water use in agriculture is associated with increasing the fraction of the available water resources that is transpired, because of the unavoidable association between yield and water use (Lawlor 2002). During last few decades, lots of physiological works have been conducted under drought stress in crop plants (Shao et al. 2008a, b; Zhao et al. 2008). Although the drought tolerance mechanism is still unclear, but it can be to some extent explained on the basis of ion homeostasis mediated by stress adaptation effectors, toxic radical scavenging, osmolyte biosynthesis, water transport, and long distance response coordination (Reddy et al. 2005). Due to the complexity of the interactions between stress factor and various molecular, biochemical, and physiological phenomena affecting plant growth and development, the abiotic stress tolerance is complex phenomenon (Ashraf and Harris 2004; Ahmad and Sharma 2008; Ahmad et al. 2010a; Hakeem et al. 2012). Some small and electrically neutral molecules act as osmoprotectants and stabilize proteins and membranes against the denaturizing effect of some abiotic stresses and are nontoxic at molar concentrations (Munns 2002).

Natural osmoprotectant concentrations in cytoplasmic compartments are osmotically significant and have pivotal roles in maintaining cell turgidity and the driving

force for water uptake under stress (Rontein et al. 2002). One of the four most common responses against stress in plants is overproduction of different types of compatible solutes. Accumulation of osmotically active biomolecules plays an imperative role to develop the stress tolerance. These are low molecular weight organic metabolites called compatible solutes which do not inhibit other cellular functions. It is an adaptive mechanism that enables protection of cell turgor and restoration of water status of cells without disturbing the normal cellular function. The compatible solutes include proline, sucrose, polyols, trehalose, and quaternary ammonium compounds (QACs) such as gylcine betaine (GB), alanine betaine, proline betaine, choline-*O*-sulphate, hydroxyproline betaine, etc. (Azooz et al. 2004; Ashraf and Foolad 2007; Ahmad and Sharma 2008; Koyro et al. 2012; Rasool et al. 2013). Compatible solutes protect plants from stress through different means including contribution to cellular osmotic adjustment, detoxification of reactive oxygen species (ROS), protection of membrane integrity, and stabilization of enzymes/proteins (Ahmad and Sharma 2008; Koyro et al. 2012; Grant 2012; Sofo et al. 2012; Rasool et al. 2013). Proline and glycine betaine accumulation help to adapt the stress injury as they provide an environment attuned with macromolecular structure and function (Sankar et al. 2007b). Foliar application of various organic solutes enhances tolerance to abiotic stress and this approach significantly contributes in increasing the crop production under stressed environment.

2 Effects of Drought on Biochemical Parameters

2.1 Soluble Proteins

Changes in protein expression, accumulation, and synthesis have been observed in plants on exposure to drought stress (Cheng et al. 1993). Drought stress brings quantitative as well as qualitative changes in proteins (Riccardi et al. 1998). Stress-induced protein accumulation may provide a storage form of nitrogen and is used by the plant later and have been proved to play a role in osmotic adjustment. Nayer and Reza (2008) demonstrated that drought stress induced expression of 50 proteins in two varieties of maize. Riccardi et al. (1998) has reported a significant quantitative variation in 78 out of 413 leaf proteins, with 38 exhibiting differential expression in two genotypes of maize during water deficit. A relationship has been reported to exist between the accumulation of drought-induced proteins and physiological adaptations during water stress (Bray 1993). Dehydrins, the proteins synthesized in response to drought stress, belong to group II late embryogenesis-abundant proteins (Close 1996). These group II proteins defend protein structure and act as molecular chaperones during stress. Four names have been designated for this protein family—RAB, LEA D-11, LEA (II), and DHNs (dehydrins) (Dure et al. 1989).

Dehydrin (dehydration-induced) genes expresses in the embryos during the late stages of embryogenesis. These are also induced in vegetative tissues during normal

growth conditions and in response to stresses like drought, low temperature, and salinity leading to cellular dehydration. They are distributed in a wide range of organisms including algae, yeast, cyanobacteria, and higher plants. Dehydrins are mainly found in cytosol, nucleus mitochondria, vacuole, and the vicinity of plasma membrane (Rorat 2006). Dehydrin gene expression has been observed to be drought-regulated in both drought-tolerant and drought-susceptible cultivars (Wood and Goldsbrough 1997). Dehydrins have been most extensively studied in relation to drought stress. They are believed to play an important role in the stability of membrane proteins and in osmotic adjustment (Dure et al. 1989) like that of compatible solutes. The dehydrins may also be playing the role by binding with ions accumulated (ion sequestering) under drought stress and in controlling the solute concentration in the cytoplasm. Dehydrins may also have a cryo-protective role in macromolecular stabilization by binding water molecules to their hydrophilic surfaces, which reverses or prevents cellular protein denaturation (Jiang and Huang 2002).

In many plants, like some maize cultivars, sorghum, wheat, and cocksfoot, the drought-induced expressions of dehydrin genes have been identified (Nayer and Reza 2008; Shao et al. 2009). Dehydrin-like proteins can be detected in the roots and leaves of drought-stressed plants and probably protect them from further dehydration damage (Tuğçe and Yasemin 2005). Drought either induces earlier expression of dehydrin-like proteins by accelerating the development, or changes the water potential which results in the expression of dehydrin-like proteins (Nayer and Reza 2008). de Rodríguez et al. (2002) observed in sunflower that leaf soluble proteins decreased during water stress. A contrasting result was observed by Ashraf and Mehmood (1990), who reported association between degree of drought resistance and protein contents. According to Irigoyen et al. (1992) and Tahkokorpi et al. (2007) under water stress, the nature of plant species and the type of tissue modulate the concentration of soluble proteins. Under stress, the reduction in protein content may be due to an increase in proline content (Chen et al. 1999). The decreased protein content may be due to the hydrolysis of protein or the inhibition of protein synthesis by oxidative stress leading to the accumulation of proline (Feng et al. 2003). Protein metabolism of the plants has been associated with the adaptation to environmental changes.

2.2 *Free Amino Acids*

Amino acids (protein, non-protein, and amides) have been reported to accumulate in plants subjected to stress (Mansour 2000). The accumulation of free amino acids accounts for most of the osmotic potential changes in sorghum (Yadav et al. 2005). Accumulation of free amino acids in higher contents has been reported under stress conditions in soybean (Fututoku and Yamada 1981), wheat (Munns and Weir 1981; Hamada 2000), durum wheat (Morgan et al. 1986), olive (Anjuthakur et al. 1998), coconut (Kasturi and Rajagopal 2000), groundnut (Asha and Rao 2002), *Vicia faba* (Ismail and Azooz 2002), *Oryza sativa* (Hsu and Kao 2003) and bell pepper

(Nath et al. 2005). Amino acid accumulation plays a crucial role in drought tolerance through osmotic adjustment in different plants such as *Catharanthus roseus* (Jaleel et al. 2007a) and *Abelmoschus esculentus* (Sankar et al. 2007b).

Chartzoulakis et al. (2002) has observed contrasting results by indicating that no significant increase occurs in total free amino acid content under water stress. According to Greenway and Munns (1980), accumulation of amino acids helps plants to overcome water deficit conditions through osmotic adjustment. Amino acids and other soluble nitrogenous compounds play an essential role in plant metabolism by being the primary product of inorganic nitrogen assimilation and precursors of protein and nucleic acids. Because of the importance of soluble nitrogenous compounds, there has been much interest in the influence of environmental stress on their metabolism. One of the main responses of plants to environmental stress is amino acid accumulation (Aspinall and Paleg 1981). The total soluble sugar and free amino acid content increases under stress at all the growth stages which indicate their possible involvement in osmotic adjustment (Yadav et al. 2005). Osmotic adjustment is one of main mechanisms that alleviates some of the detrimental effects of water stress (Morgan 1984) and has been identified as a chief criterion of yield stability and drought tolerance in several crops including sorghum (Chimenti et al. 2002).

2.3 Proline

Proline is an important osmolyte which plays a pivotal role in membrane stabilization and protein structure besides regulate the accumulation of usable nitrogen. Proline is induced in response to various environmental stresses (Ahmad and Jhon 2005; Ahmad et al. 2006, 2007, 2010b, 2011a, 2012a, b) and occurs in cytosol where it helps in osmotic adjustment. Proline production, during salt or water stresses, probably plays a role in tolerance to these stresses in wheat (Azooz 2002), rice (Hsu and Kao 2003), soybean (Porcel et al. 2004), pea (Ahmad and Jhon 2005; Ahmad et al. 2008b), *Vicia faba* (Ismail and Azooz 2002), mulberry (Ahmad et al. 2006) and mustard (Ahmad 2010). Singh et al. (1972) were probably the first who tried to establish a correlation between proline accumulation and drought resistance in barley cultivars. They showed that drought-resistant cultivars of barley accumulated higher quantities of free proline than the susceptible ones. Depending on the species and the extent of stress, the proline accumulation under abiotic stresses accounts for concentrations of few millimolars (Delauney and Verma 1993; Bohnert and Jensen 1996). Two enzymes pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductases (P5CR) play an important part in proline biosynthetic pathway (Delauney and Verma 1993; Koyro et al. 2012).

According to Nanjo (1999), in higher plants, the osmotic stress stimulated free proline accumulation and is regulated by a rate-limiting enzyme P5CS. Further, the antisense transgenics in *Arabidopsis* with P5CS cDNA show morphological alterations in leaves that were hypersensitive to osmotic stress. In *Arabidopsis*, the proline

deficiency has been found to affect specifically the structural proteins of cell walls, suggesting that proline is an osmoregulator in osmotolerance and morphogenesis in plant (Reddy et al. 2004). Many workers have reported that water deficit induced proline accumulation at vegetative stages in sorghum (Yadav et al. 2005), bell pepper (Nath et al. 2005), *Gossypium hirsutum* (Ronde et al. 1999), wheat (Demir 2000; Hamada 2000) and *Cyamopsis tetragonoloba* (Shubhra and Ooswami 2003). In salt-tolerant alfalfa, proline concentration in the root rapidly doubles, while in the salt-sensitive plants, the response is slow (Petrusa and Winicov 1997). Ahmad et al. (1981) reported that salt-tolerant ecotypes of *Agrostis stolonifera* accumulated more proline in response to salinity than salt-sensitive ecotypes. Some other stresses have also been found to be inducing proline accumulation, e.g. chilling in cucumber plant (Feng et al. 2003), chilling and drought in soybean (Heerden and Krüger 2002).

Significant variations in proline content among ten wheat genotypes, under water deficit stress, were reported by Hong-Bo et al. (2006). Sawhney and Singh (2002) reported accumulation of proline under chemical applied desiccation stress in wheat. According to Reddy et al. (2005), proline is known to be involved in reducing photo damage in the thylakoid membranes by scavenging and/or reducing the production of O_2^-. Also, it can be inferred that proline acts as a free radical scavenger and may be more effective in overcoming stress than acting as simple osmolyte (Reddy et al. 2004) as reported in *Catharanthus roseus* (Jaleel et al. 2007a). Proline accumulation in plants is caused not only by the activation of proline biosynthesis, but also by the inactivation of proline degradation, thereby resulting in a decrease in the level of accumulated proline in dehydrated plants like groundnut (Girija et al. 2002). According to Morot-Guadry et al. (2001), reduced leaf water potentials results in manifold increase in proline concentrations and at this stage photosynthesis is known to be quite reduced. Gupta et al. (2000) reported increased proline accumulation, when water stress was followed by simultaneous increase in leaf water potential in chickpea. In water-stressed plants, proline accumulation is maximum at flowering stage and minimum at vegetative stage, with a rapid accumulation in the stem (including sheaths) and roots as compared to the leaves, with the roots being net proline importers (Singh et al. 1973). Overexpression of P5CS in transgenic tobacco plants showed elevated levels of proline and tolerance to salt and drought stress (Kavikishore et al. 1995). Proline accumulation could be used as selection criterion for stress-resistant genotypes. Such studies open a new avenue of research for metabolic engineering in several agriculturally important crop plants for drought resistance (Kavikishore et al. 1995).

2.4 Glycine Betaine

Glycine betaine (GB) is one of the most abundant QACs produced in higher plants like onion (Mansour 1998), rice (Mohanty et al. 2002), sorghum (Yang et al. 2003), mustard (Ahmad 2010) and mulberry (Ahmad et al. 2010b) under stressful environmental conditions. Overexpression of betaine aldehyde dehydrogenase (BADH)

induced by stress, leads to GB synthesis, e.g. in barley (Nakamura et al. 2001) and sunflower (Manivannan et al. 2007). According to Rhodes and Hanson (1993), in higher plants, GB is synthesized in chloroplast from serine via ethanolamine, choline, and betaine aldehyde. Choline monooxygenase (CMO) converts choline to betaine aldehyde, which is then converted to GB by BADH. This pathway has been identified in all GB-accumulating plant species (Weretilnyk et al. 1989; Luo et al. 2012). During dehydration stress, GB localized in chloroplasts showed increase in concentration and plays an important role in chloroplast adjustment and protection of thylakoid membranes which leads to maintenance of photosynthetic efficiency and membrane integrity (Yokoi et al. 2002). Different plants experience GB accumulation in response to different stresses, e.g. sugar beet, spinach, barley, wheat, sorghum, and maize (Hunag et al. 2000; Yang et al. 2003; Zhang et al. 2012). Exogenous application of GB to naturally low-accumulating or non-accumulating plants, may help to ameliorate the adverse effects of environmental stresses (Yang and Lu 2005; Reddy et al. 2013).

Exogenous application of GB ameliorates the adverse effects and improved the growth of temperature-stressed plants, e.g. *Lycopersicon esculentum* (Makela et al. 1998a, b; Park et al. 2006), salt-stressed *Oryza sativa* (Lutts 2000), *Lolium perenne* (Hu et al. 2012) and drought-stressed *Lycopersicon esculentum* (Rezaei et al. 2012), *Carica papaya* (Mahouachi et al. 2012). Foliar application of GB improved the growth and yield of water-stressed plants viz. *Nicotiania tobaccum* (Agboma et al. 1997b), *Gossypium hirsutum* (Gorham et al. 2000), *Glycine max* (Agboma et al. 1997c), *Zea mays* (Agboma et al. 1997a) and *Triticum aestivum* (Aldesuquy et al. 2012). According to Storey and Wyn-Jones (1975), the accumulation of glycine betaine might be serving as an extracellular osmoticum and could be closely correlated with the elevation of osmotic pressure as in *Spartina x townsendii*. According to Kavikishore et al. (1995), glycine betaine can maintain the osmoticum of plant, provided its basal metabolism sustains a high rate of GB synthesis to facilitate osmotic adjustment for water stress tolerance. Under stress, glycine betaine protects membranes, metabolic enzymes, and also stabilizes PSII protein pigment complexes (Papageorgiou and Morata 1995). Yang et al. (2003), while working on sorghum revealed that the level of glycine betaine biosynthesis is dependent on the nature and severity of environmental stresses. However, there are few reports which demonstrated that certain plants do not show such positive correlation with exogenous application of GB (Meek et al. 2003).

3 Proline Metabolizing Enzymes

Several workers have discussed proline metabolism from time to time (Ahmad et al. 2010b, 2012a, b). A number of plants respond to osmotic stress by accumulating high concentration of proline mainly because of stimulated proline biosynthesis (Rudulier et al. 1984). In plants like soybean and moth bean, proline biosynthetic pathway has been well characterized (Delauney and Verma 1993; Hu et al. 1992).

Two proline biosynthetic pathways are present in plants: the glutamate pathway and orinithine pathway; the former appears to play a predominant role under osmotic stress (Rhodes 1987; Ahmad and Sharma 2008; Koyro et al. 2012). In glutamate pathway, enzyme complex pyrroline-5-carboxylate synthetase (P5CS) converts glutamic acid into γ-semialdehyde (GSA). The glutamic acid γ-semialdehyde is converted into pyrroline-5 carboxylic acid (P5C) by non-enzymatic cyclization. The enzyme γ-1-pyrroline-5-carboxylate reductase (P5CR) converts P5C into proline (Treichel 1986; Fujita et al. 2003). The P5C synthetase probably regulates proline synthesis (Boggess et al. 1976). The enzymes γ-glutamyl kinase and γ-glutamyl phosphate reductase form an enzyme complex called P5C synthetase as the resulting product. Glutamic GSA is non-enzymatically converted to γ-1-pyrroline-5-carboxylate (P5C). The conversion of ornithine to proline in plants with P2C or P5C as intermediate has been debated by many workers for long (Adams and Frank 1980; Stewart 1981).

3.1 γ-Glutamyl Kinase

The γ-glutamyl kinase is an important enzyme regulating proline synthesis. The induction of proline accumulation may be due to a stimulated proline synthesis through glutamate pathway involving activity of many enzymes like γ-glutamyl kinase, glutamyl phosphate reductase, and Δ-pyroline-5-carboxylate reductase (Girija et al. 2002). The enzyme γ-glutamyl kinase belongs to an amino acid kinase family and its predicted three-dimensional model has been constructed on the basis of crystal structures of three related kinases (Fujita et al. 2003). In the glutamate pathway, enzyme γ-glutamyl phosphate reductase converts glutamate to GSA. This product spontaneously cyclizes to (P5C) γ-1-pyrroline-5-carboxylate which is then reduced by NADPH to proline by the enzyme γ-1-pyrroline-5-carboxylate reductase (Fujita et al. 2003). Muthukumarasamy et al. (2000) reported higher γ-glutamyl kinase activity in NaCl-stressed radish. Variation in γ-glutamyl kinase activities has been reported in tomato in different regions (Fujita et al. 2003) and mulberry (Ahmad et al. 2012b).

3.2 Proline Oxidase

Under water stress, a drastic reduction in proline oxidation was observed by Flowers and Hanson (1969) in beans, by Sells and Koeppe (1981) in *Zea mays* and by Ahmad et al. (2010b) in *Morus alba*. Proline is converted to glutamate by proline oxidase. Thus proline oxidase also influences the free proline level. In plant proline biosynthesis, enzyme γ-1-pyrroline-5-carboxylate synthetase is the rate-limiting enzyme and is subjected to feedback inhibition by proline. Under stress conditions, the feedback regulation of P5CS is lost in plants (Hong et al. 2000).

4 Polyamines in Response to Drought Stress

Environmental stress factors like salinity, temperature, drought affects the growth and development of plants globally. Abiotic stress causes an accumulation of excess concentrations of active oxygen species (AOS) resulting in oxidative damage at cellular level. AOS being highly toxic, damages many biomolecules such as DNA, RNA, lipids, and protein (Smirnoff 1993; Pourtaghi et al. 2011; Masoumi et al. 2011; Ahmad et al. 2010a, 2011b; Ahmad and Umar 2011). Osmotic imbalance and membrane stability damage are the most frequent symptoms during dehydration stress. Drought is one of the important stresses responsible for crop loss worldwide. Water scarcity threatens the agricultural systems and limits the crop production. To enhance the high tolerance of crops for better productivity, polyamines (putriscine, spermidine, and spermine) play a key role to overcome this problem as these are known to accumulate to a higher concentration under unfavourable conditions (Ahmad et al. 2012c). Polyamines have protective role as scavengers of AOS, consequently, results in an improved adaptation ability and growth of plants under drought stress (Türkan and Demiral 2009). Polyamines are low molecular weight natural compounds present in all living cells that are nontoxic at higher concentration, protecting them from dehydration injury, thereby acts as osmoprotectants and compatible solutes (Reddy et al. 2004; Shao et al. 2005; Ahmad et al. 2012c; Todorova et al. 2013; Lutts et al. 2013).

Putriscine, spermidine, and spermine are polymines that occur in free form as cations but are often conjugated to micromolecules and also to various macromolecules. Polyamines are implicated in a variety of fundamental and developmental processes of plants, including transcription, RNA modification, protein synthesis, and modulation of enzyme activities (Tonon et al. 2004). It has been observed that spermine, spermidine contents in shoots of *Phaseolus vulgaris* have been found to increase substantially under drought stress alone or in combination with pretreatment of H_2O_2 (Abass and Mohamed 2011). Loka et al. (2013) also reported that spermidine, putriscine, and total polyamines increased significantly in cotton flower and its sun tending leaf under water deficit stress. However, it has been observed that exogenous spermidine and spermine stimulated the growth and reduced the membrane damage in jack pine seedlings (Rajasekaran and Blake 1999). Hence, spermidine may serve as signalling regulator in stress signalling pathway, thus developing stress tolerance mechanism in plants. Furthermore, polyamines have been shown to prevent senescence due to their acid-neutralizing and antioxidant properties and also for their membrane and cell wall stabilizing abilities (Zhao and Yang 2008). Besides, it also plays an important role in regulating the plants defense response to drought stress (Yamaguchi et al. 2007).

The mechanism of drought resistance, through which roots are most likely associated, is drought avoidance. Genotypes comprising deep, coarse roots with capability of branching and penetration, higher root to shoot ratio, elasticity in leaf rolling, early stomatal closure, and high cuticular resistance are reported as main constituents of drought avoidance (Samson et al. 2002; Wang and Yamauchi 2006).

To meet the growing water shortage, it becomes necessary to accomplish drought resistance in plants and needs a deeper understanding of drought resistance mechanisms (Serraj et al. 2011). Significant knowledge in the physiology of drought response can also contribute to plant breeding efforts towards drought resistance cultivars (Serraj et al. 2009). Root traits are known to be critical for increasing yield under soil-related stresses (Serraj et al. 2004; Lynch 2007). Polyamines have been reported in different plant cultivars in response to drought stress (Galston et al. 1997; Bouchereau et al. 1999; Guerrier et al. 2000). Their accumulation in plants causes the removal of ROS resulting in better survival in subsequent stresses, maintaining turgor (Islam et al. 2003) and photosynthetic activity (Galston et al. 1997). It has been well documented that genetic transformation with several polyamine biosynthetic genes (ADC, ODC, SAMDC, SPDC) have been shown to improve significantly the environmental stress tolerances in different plant species (Liu et al. 2007).

Transgenic plants overexpressing ADC, SPD have been reported to endure multiple stresses including drought. Transgenic approaches demonstrate that polyamines play essential roles in stress tolerance and open up the possibility to design new strategies to increase the plant survival in adverse environments. Variation in polyamine contents has been examined significantly in plants exposed to single as well as combined stresses (Capell et al. 2004; Kasukabe et al. 2004; Liu et al. 2007). As compared with stress-intolerant plants, Kasukabe et al. (2004) revealed that stress-tolerant plants showed twofold enhancement in polyamine biosynthesis. Polyamines linked to phospholipids function as membrane surface stabilizers (Wang et al. 2006), protect against stress via stabilizing protein structure to prevent proteins from degradation by conjugating to proteins (Waie and Rajam 2003; Verma and Mishra 2005). Water deficit inhibited the seed germination and seedling growth and indicating that the root zone extensively affects the growth and development, thereby leading to poor productivity or death of the plant (Grzesiak et al. 1996; Dhanda et al. 2004). Similar reports have been found in *Vicia faba* by El-Tayeb (2006). Okçu et al. (2005) investigated the reduction of shoot growth of pea as compared to root growth under water stress. Photosynthesis under drought stress has been observed to inhibit by causing changes in chlorophyll contents, damage photosynthetic apparatus, decreases the activities of Calvin cycle enzymes and ultimately the crop yield (Monakhova and Chernyadev 2002).

In recent years, several reports established that thylakoid-bound polyamines help in the regulation of structure and function of the photosynthetic apparatus (Imai et al. 2004). Zlatev and Yordanov (2004) have observed a considerable inhibition of photosynthesis owing to stomatal closure under drought stress. To deal with this stress, plants instigate reprogramming of transcriptional, post-transcriptional, and metabolite processes that restrict water loss. Application of exogenous polyamines has been reported to improve drought tolerance against the perturbation of biochemical processes (Yang et al. 2007; Alcázar et al. 2010), but mechanisms of their action in modulating physiological phenomena especially in photosynthesis are not fully understood (Bae et al. 2008). Both photosynthetic rate and water-use efficiency in leaves of rice subjected to dehydration stress for 7 days were extensively enhanced by spraying plants with 10 μM Put, Spd, and Spm solutions, among which

Spm was found to be the most effective (Farooq et al. 2009). Moreover, use of 0.1 mM exogenous Spd has been found to increase the yield of tomato seedling by preventing stomatal closure and stimulating CO_2 uptake during the later period under drought stress (Zhang et al. 2010).

Absorption of polyamines by the seedlings seems to be effective in improving root and shoot growth thereby, balancing the cellular membrane and showed vigorous growth. Therefore, they have been implicated in a wide array of biological processes, growth, development, and abiotic stress responses including drought (Galston et al. 1997; Liu et al. 2007; Do et al. 2013) in plants. Liu et al. (2007) also ascertained that stress-tolerant plants increase their endogenous polyamine levels to a finer extent than sensitive ones. The polyamine overproducing transgenics have greater stress tolerance (Galston et al. 1997) and uses of exogenous polyamines confer protection from many abiotic stresses (Nayyar and Chander 2004). By means of genetic manipulation, modifications in PA biosynthesis resulted in enhanced abiotic stress tolerance. Kasukabe et al. (2004) reported that the transgenic *Arabidopsis*, overexpressed SPDS of *Cucurbita ficifolia* under 35S promoter, showed enhanced drought tolerance.

Capell et al. (2004) found that transgenic rice expressing ADC of *Datura* (stress tolerant) accumulated a much higher level of polyamines than its wild type, hence achieving higher water stress tolerance. Use of biosynthetic inhibitors like DFMA, DFMO, and CHA affects the growth of several crop species by increasing the stress injury and reducing the water content of roots. Liu et al. (2004), Amooaghaie and Moghym (2011) and Amooaghaie (2011) found that the addition of exogenous PAs to water-stressed soybean seedlings retarded growth reduction and inhibited the loss of membrane stabilization. These results clearly indicate that polyamines are involved in stress-adaptive responses and, thereby act as tolerance enhancers in various crops. Under drought stress, osmotic stress induced a greater increase in putriscine and spermidine contents in tolerant species of *Lycopersicon pennellic* than the sensitive *Lycopersicon esculentum* (Santa-Cruz et al. 1997). Likewise, tolerant sugarcane varieties due to greater activity of arginine decarboxylase and ornithine decarboxylase induced a greater increase of putriscine synthesis (Zhang et al. 1996). Further evidence is supported by the fact that the adaptive role of polyamines was higher in terrestrial reeds than in swamp reed ecotypes (Wang et al. 1995).

The transformation of tobacco with *S*-adenosylmethionine decarboxylase led to increased polyamine biosynthesis and improved drought tolerance (Waie and Rajam 2003). Due to increase of spermine content and the level of other polyamines, yield of soybean plant has been found to grow tremendously; suggesting polyamines an efficient protection against drought-induced stress (Simon-Sarkadi et al. 2006). Putriscine content from transformed soybean also brought an enhancement in poplar (Guerrier et al. 2000) and rape plant (Aziz and Larher 1995). As a result, exogenous polyamines in transgenic plants, during stress periods, might contribute to their greater ability to alleviate stress damage. Spermine content known to prevent senescence has been well studied in different plants (Kaur-Sawhney and Galston 1991; Tiburcio et al. 1994). This induces increase in protein, RNA, and DNA synthesis, reduces RNAase activity and chlorophyll loss (Kaur-Sawhney and Galston 1979) and inhibits specific protease activity of senescing oat leaves (Kaur-Sawhney et al. 1982).

During water stress, the activation of ethylene hormone causes membrane leakage, eventually leads to senescence in plants (Hipkins and Hillman 1985). Ethylene evolution increases the rate of degradation of chlorophyll in *Cucumin sativus* cotyledons (Abeles and Dunn 1989). Here, polyamines impede senescence by inhibiting ethylene production (Apelbaum et al. 1985) or by stabilizing cell membrane against enzymatic degradation and solute leakage (Kaur-Sawhney et al. 1978). To determine the specificity of ROS-driven transcript expression, Gadjev et al. (2006) showed how the accumulation ROS in a variety of subcellular compartments altered gene expression. Their experiments included transgenic plants with certain disorder of ions and enhanced activities of antioxidant enzymes (catalase, cytosolic ascorbate peroxidase, or copper/zinc superoxide dismutase) by exogenous application of oxidative stress-causing agents (methyl viologen, *Alternaria alternata* toxin, 3-aminotriazol, and ozone) to plants. The disruptions in antioxidant enzymes included experiments in which the enzyme activity was reduced or completely abolished. This analysis showed that a majority of the transcripts responding to the stress were altered only in one experiment, i.e. by one species of ROS. The authors considered these transcripts to be "hallmarks for a specific oxidative stress characterized by the chemical identity of the produced ROS and/or the subcellular site of its production" (Gadjev et al. 2006). A bulk of genes that had a change in expression level responded only in one experiment, highlighting that the type of ROS and/or the subcellular location of its generation determines the gene response. The genes that had the largest change in expression were three ethylene-responsive element-binding proteins, supporting a connection between ethylene and singlet oxygen as was previously observed by Danon et al. (2005), who observed that by blocking ethylene production, the cell death that normally occurs in flu mutants upon moving from dark to light was partially blocked. These genome-wide expression inventories have shed light on early response and downstream transcripts, especially altered in their expression, by a particular type of ROS and hinted at transcripts or pathways that serve as integrative points of ROS-mediated plant responses. The identification of ROS sensors and signalling components which are responsible for this remarkable selectivity and specificity of ROS signalling within the cell remains a major challenge.

5 Genetic Engineering and Drought Stress

Tolerance to drought stress is controlled by many genes to improve the yield of crops. In this regard, scientists are under a challenge to develop the drought-tolerant plants that mitigate the water limitation and thereby, accelerate the production to meet the global needs (Ozturk et al. 2002; Yang et al. 2004; Montalvo-Hernández et al. 2008; Macková et al. 2013). During scarcity of water, changes in gene expression patterns have been observed from early response gene (signal transduction, transcription and translation factors) to late response genes, i.e. water transport, osmotic balance, oxidative stress, and damage repair (Ahmad and Prasad 2012a, b;

Yu et al. 2013). Bray (2002) observed an adaptive response in plants as a consequence of such changes. Gene knowledge initially obtained from *Arabidopsis* have been transformed to important food plants to certain extent and have shown to develop stress tolerance against drought condition (Zhang et al. 2004; Rai et al. 2013; Yu et al. 2013; Li et al. 2013). Such transgenics, with several stress inducible genes, resulted in their increased tolerance to drought and other abiotic stresses (Umezawa et al. 2006a; Kumar et al. 2013). Seki et al. (2003) observed the expression pattern of about 1,300 genes in *Arabidopsis* also demonstrated that many stress inducible genes like osmoprotectant, chaperons, and detoxification enzymes directly protect against environmental stress. The ability of responsive genes to survive under these stress conditions has become exceptionally important (Chinnusamy et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007).

Various metabolites viz., polyamines, carbohydrates, proline, glycine betaine, and trehalose have been shown to be associated with drought resistance and utilizes the related genes to transfer them to sensitive plants that have been evolved through different biochemical pathways (Ahmad et al. 2013). The gene TPS1 encoding trehalose-6-phosphate, crucial for the biosynthesis of trehalose was engineered into tobacco (Romero et al. 1997). It was shown that transgenic plants exhibited drought tolerance by determining the water loss from detached leaves. Simultaneously, transgenic potato plants encoding gene TPS1 also showed higher drought resistance (Yeo et al. 2000). These studies have been correlated with the accumulation of polyols in many plants and animals (Bohnert and Jensen 1996). Polyols act as osmolytes as well as scavengers against water deficit (Bohnert et al. 1995). An enzyme pyrroline-5-carboxylate synthetase for proline synthesis encodes gene P5CS. Overexpression of this gene in transgenic tobacco plants resulted in accumulation of proline, conferring drought resistance and showing better growth over control plants (Kavikishore et al. 1995). This overproduction of proline showed enhancement in root biomass and flower development under dehydration stress (Kavikishore et al. 1995). Same gene has been found to be incorporated into rice, petunia as well as in soybean by Su and Wu (2004), Yamada et al. (2005), de Ronde et al. (2004) respectively. Similarly, the bacterial gene SacB in *Bacillus subtilis*, encoding for levan-sucrase, was used for transformation of *Nicotiana* plants, the resultant transgenics produced, showed accumulation of bacterial fructans and hence better performance under PEG-mediated drought stress over control (Pilon-Smits et al. 1995). Both the genes betA encoding for choline dehydrogenase and betB encoding for betaine dehydrogenase are involved in the biosynthesis of glycine betaine. Holmstrom et al. (1994) showed that transformation of betB gene to tobacco plant caused the accumulation of glycine betaine conferring drought resistance. This build-up of glycine betaine in transgenic plant provides an adaptive response to water stress and can be attributed to protein stabilization, scavenging oxygen radicals as well as regulation of osmotic effects.

Bacterial mannitol phosphate dehydrogenase engineered (mE1D) gene in tobacco plants showed increased biomass due to the accumulation of mannitol in the cytoplasm (Tarczynski et al. 1992, 1993). Similar gene in the egg plant was observed to endure drought stress (Prabhavathi and Rajam 2007). In polyamine

biosynthetic pathways, three genes TcODC, TcADC, and TcSAMDC have been observed to be responsible to multiple environmental stresses including drought (Bae et al. 2008). These genes show some differential expression due to developmental stage and tissue specificity and are constitutively expressed in the entire cacao tissues studied (Yoo et al. 2004; Hao et al. 2005). Transgenic tobacco plants with ODC genes from yeast (Hamill et al. 1990), ADC genes from oat (Masgrau et al. 1997) and SAMDC gene from humans (Noh and Minocha 1994) have also been reported. Furthermore, overexpression of these three genes have been observed in rice and egg plant conferring increased putrescine levels in ODC and ADC and enhancement of both spermidine as well as putrescine contents in SAMDC transgenics (Prabhavathi and Rajam 2007). HVA1 gene encoding late embryogenesis, abundant with barley, produced transgenic rice. These LEA proteins are accumulated in vegetative organs to develop resistance against drought stress (Dure 1993). Same gene was transformed into wheat, and the transgenic wheat produced showed greater biomass productivity and water use efficiency over the untransformed plants (Sivamani et al. 2000). Tobacco transgenics with *imt1* encoding myo-inositol-O-methyl transferase resulted in an accumulation of ononitol and confers better tolerance against dehydration stress than control plants (Shevelena et al. 1997).

Various transgenic plants have been produced in different crop species by different scientists viz., DQ663481 encodes for lea gene in tobacco (Wang et al. 2006), OsLEA3-1 encodes for the synthesis of lea gene in rice (Xiao et al. 2007), ME-leaNY coding for LEA protein in *Arabidopsis* (Figueras et al. 2004) and ZmDREB2A encodes HSP and LEA protein in *Arabidopsis* (Qin et al. 2007). Expressions of the transgenic rice and mulberry have been documented to improve tolerance with overexpression of *Arabidopsis* intracellular Na^+/H^+ antiporter AtNHX5 gene (Bassil et al. 2011; Li et al. 2011). Enhancement of grain has also been observed due to overexpression of OSNAC10 under root-specific promoter (RCc3) (Jeong et al. 2010). Similarly, transgenic maize expressing ZMNF-YB2 showed better tolerance to severe drought stress (Nelson et al. 2007). To combat the stress, dehydration responsive element (DRE) helps to regulate gene expression (Yamaguchi-Shinozaki and Shinozaki 1994). It has been documented that transcription factor DREB1A specifically interact with DRE and induces an expression of stress tolerance genes. Overexpression of DREB1A cDNA in transgenic plants has shown to activate the expression of many stress-tolerant genes under normal growth conditions, hence improved tolerance against stress conditions (Kasuga et al. 1999). Overexpression of CBF1/DREB1B gene has been observed in many crop plants like rice, wheat, and canola (Dubouzet et al. 2003; Jaglo et al. 2001) leading to development of tolerance in response to drought. Lesser water loss due to decrease in stomatal opening has been demonstrated in transgenic tomatoes expressing Sly-miR169c and thereby endure drought stress (Zhang et al. 2011). Overexpression of aldehyde dehydrogenase AtALDH3 gene in *Arabidopsis* confers tolerance to drought (Sunkar et al. 2003) and this enzyme is known to maintain membrane integrity under osmotic stress.

One of the major and predominant metabolite produced in plants against drought stress is the hormone ABA (abscisic acid) (Bartels and Sunkar 2005). Associated with ABA biosynthetic gene (NCED3) (Luchi et al. 2001), a cytochrome

P450CYP707A family identified as ABA 8/-hydroxylase has been shown to regulate ABA levels during seed imbibition and dehydration stress (Saito et al. 2004; Kushiro et al. 2004). This control of ABA level leads to an improvement in engineering of drought tolerance in plants. Also, it has been documented that among few CYP707A members, insertional mutant of CYP707A3 exhibited higher degree of drought tolerance with a concomitant reduction of transpiration rate (Umezawa et al. 2006b). Transgenic plants expressing the phosphorylated active form of AREB1 resulted in the induction of many ABA-responsive genes and have potential to contribute drought tolerance through gene transfer (Furihata et al. 2006). Recently, Kuromori et al. (2011) identified gene called AtABCG22 from *Arabidopsis*, expressed predominantly in guard cells, implies that this gene plays a key role in stomatal regulation as well as protecting plants against drought stress. With increasing cytoplasmic Ca^{2+} levels in plant cells during drought stress, signals are likely to be stimulated by protein phosphorylation/dephosphorylation cascades, the majority of which is Ca^{2+}-stimulated protein phosphorylation carried out mainly by members of Ca^{2+}-dependent protein kinase (CDPK) family in plants (Ahmad et al. 2008a, 2012d; Sarwat et al. 2013). Selected members of CDPK family have been shown to be responsible for the activation of stress/ABA responsive promoter (Sheen 1996; Ahmad et al. 2012d; Sarwat et al. 2013). It is prudent to find out the outcome of new alternatives to generate rice transgenics with altered levels of this protein. Protein OsCDPK7, overexpressing in transgenic rice, enhances the tolerance level to drought and signifying that the manipulation of CDPK activity has been the great endeavour with regard to stress tolerance.

6 Conclusions and Future Perspective

In the light of the overview presented, it could be concluded that drought stress in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes. Plants subjected to drought stress undergo some detrimental effects on their growth and metabolic processes. The responses of plants to drought stress depend on the plant species, levels of drought, soil characteristics, and the stages of growth. Many plants possess different constitutive processes to minimize the detrimental effects of drought stress. These processes include accumulation or regulation of biosynthesis of osmotic solutes (osmoprotectants) like soluble proteins, free amino acids, proline, and glycine betaine as well as proline metabolizing enzymes (γ-glutamyl kinase and proline oxidase), which improve the osmoregulation and increase the osmotic potential of the cells. High levels of these osmotic solutes enable plants to maintain low water potentials. By lowering water potentials, the accumulation of osmotic solutes involved in osmoregulation allows additional water to be taken up from the environment, thus modulating the immediate effect of water shortages within the stressed plants. In many plants, drought tolerance is improved through conventional selection and breeding techniques. Variability in drought tolerance among plant species

has been reported. The physiological responses to drought stress are varied quantitatively or qualitatively between drought-tolerant and drought-sensitive plants. Drought stress increases or decreases the content of soluble proteins in plants. Free amino acids were significantly accumulated. Many plant species naturally accumulate glycine betaine and proline as major organic osmolytes, when subjected to drought stresses. These compounds are thought to play adaptive roles in mediating osmotic adjustment and protecting subcellular structures in stressed plants. Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants. Accumulation of glycine betaine also occurs in many plants during drought stress, indicating its role in protecting plant cell mechanism under drought conditions. However, not all the plants accumulate glycine betaine or proline in sufficient amounts to help averting adverse effects of drought stress. Thus, different approaches have been contemplated to increase the concentrations of these compounds in plants grown under drought stress conditions to increase their stress tolerance.

The above conclusions open a new avenue of researches for metabolic engineering in several agriculturally important crop plants for drought resistance. The future studies must focus on the role of antioxidant systems and gene expression for a better understanding of the alteration and osmoregulation in plants subjected to drought stress. Moreover, future priorities should be aimed to see a much clearer picture of drought stress signal transduction pathways and genetic improvement of drought stress tolerance through tuning plant sensing and signalling systems. Finally, attempts should be made to identify the molecules connecting different pathways in this system and key components in each pathway determined to enlighten the problem more clearly. The tolerance of plant to drought stress can be greatly refined by characterization of individual genes and assessing their contribution to drought stress tolerance.

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Chapter 2
Heavy-Metal Attack on Freshwater Side: Physiological Defense Strategies of Macrophytes and Ecotoxicological Ops

David Delmail and Pascal Labrousse

1 Introduction

Elements considered as heavy metals are natural metal with a density over than 4–5 g cm^{-3}. These elements are present in all environmental compartments but in low concentrations and are designated as trace elements. However, high concentrations of heavy metals can be measured locally, e.g., near granitic stations. Human activities may also lead to their release in the environment, thus increasing their levels. Due to (1) their ubiquitous presence all over the Earth, (2) the essentiality of certain metals to organisms, and (3) their involvement in acclimation, selection, and adaptation of living forms, heavy metals represent a specific class of chemical substances.

When stored, some heavy metals are known to be toxic for organisms when present in high amounts. Their bioaccumulation leads to the continuous increase of their concentrations along the food web, i.e., biomagnification (Angerville 2009). Metal pollution may induce deep disturbances in ecosystem functioning, water cycle, plant growth, and animal development. Urbanization and industrial development-linked anthropogenic releases of metals in natural localities constitute one of the main threats for environment and for public health (due to high toxicity, persistence, and biomagnification) (Gentès et al. 2013). The main problem related to metal occurrence in nature is the inefficiency of biological degradation processes. Furthermore, in aquatic environments heavy metals are present under many forms, i.e., chemical, free and complex, with high mobility, and they could be detected downstream far from their release point (Miquel 2003).

D. Delmail (✉)
Laboratory of Pharmacognosy and Mycology—UMR CNRS 6226 ISCR PNSCM, University of Rennes 1 (European University of Brittany), Rennes 35043, France
e-mail: david.delmail@univ-rennes1.fr

P. Labrousse
Faculty of Pharmacy, Laboratory of Botany and Cryptogamy—GRESE EA 4330, University of Limoges, Limoges 87025, France

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Since several years, industrialized countries focus on the consequences of these toxic compounds in waters. To increase stream quality, the Clean Water Act of the United States of America have established in 1997 a black list of 14 heavy metals mostly detrimental for the environments: Ag^{+}, As^{3+}, Be^{2+}, Cd^{2+}, Cr^{3+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, Se^{4+}, Tl^{+}, and Zn^{2+} (CWA 1977). Despite that, some metals like Hg or Pb are non-essential and toxic for macrophytes, while some others are essential to their metabolism: Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Mo^{2+}, Ni^{2+}, V^{5+}, and Zn^{2+}. However, an optimal concentration exists for each metal to allow cell homeostasis and to prevent from any metabolic disorders due to excess or lack of the concerned element (Nagajyoti et al. 2010).

In this study on macrophyte adaptations, we will mainly focus on two heavy metals highly represented in stream environments: one essential, Cu, and one non-essential, Cd.

2 Heavy-Metal Origins and Dispersals

2.1 Cadmium

Releases of Cd in the atmosphere may have many origins such as anthropogenic and natural sources. This element occurs in Earths' crust under chlorides ($CdCl_2$), oxides (CdO), sulfates ($CdSO_4$), and carbonates in Zn, Pb, and Cu minerals. The Cd is naturally dispersed through eolian erosion and volcanism. Earths' crust degradation and atmospheric fallout enrich telluric and aquatic ecosystems with Cd. However, industry (e.g., metal refining, coal combustion, metalworking industry, garbage incineration) is the main anthropogenic source of Cd in all natural compartments, and especially water. Indeed, in aquatic environments, this heavy metal is provided by eolian erosion, water leach, industrial garbage and effluents (Pichard et al. 2005a).

2.2 Copper

Copper is naturally present in the environment under many mineral forms. This element is ubiquitous and is often measured in superficial and underground waters. The main exposed compartment is the lithosphere: 97 % of total Cu versus 3 % in hydrosphere and 0.04 % in atmosphere. The soil contamination is mainly due to mining by-products in which Cu is under sulfide (CuS and Cu_2S) and insoluble silicate ($CuSiO_3$) forms. Secondary sources of contamination are sludge from water treatment and electrotyping plants, metalworking industry, plumbing and electrical installation. In waters, Cu comes from soil erosion by streams (68 %), phytosanitary $CuSO_4$ releases (13 %), and sewages (Pichard et al. 2005b).

2.3 Speciation

In waters, physicochemical properties of heavy metals and environmental features (dissolved organic matter, carbonates, ions, pH, and salinity) influence the qualitative occurrence of these elements. This phenomenon is called metallic speciation which influences heavy metal effects on living forms. Heavy metals could be found under several forms in waters: (1) free ionic forms, (2) complex forms with inorganic matter, and (3) adsorbed forms on colloids/organisms (Lead and Wilkinson 2006). To estimate the disturbances of environmental contaminations, it is important to measure each metal-species rates. However, ecotoxicological models to evaluate effects on biocoenosis are mainly focused on the free ionic form. This metal species, as the most bioavailable one, is considered as the best representative feature of the disturbance level in biocoenosis (Lead and Wilkinson 2006).

In hydrology, free ionic forms are related to environmental chemistry, physicochemical parameters, and abiotic parameters. Indeed, heavy metals are complexed to a wide variety of organic and inorganic ligands (e.g., colloids, macromolecules). Heavy-metal speciation is influenced by physicochemical features like ligand nature, their concentration, pH and biotic factors (e.g., bacteria and protists influence speciation through chelator synthesis and release of complexant exopolysaccharides) (Diallo et al. 2005). It is important to underline that all these organic compounds are considered as dissolved organic matter and that they play an indirect important role in linkages with heavy metals involving inorganic compounds (Lead and Wilkinson 2006).

2.4 Bioavailability for Aquatic Photosynthetic Organisms

Heavy-metal bioavailability is defined as the physicochemical capability of a metal to pass through the biological protective layers (plasmalemma and cell wall) and to interact with an organism (Lyubenova et al. 2013). This implies the chemical dissociation/association of different complexes, their adsorption on macrophyte-specific receptors and their transfer through these layers (Fig. 2.1). The adsorption is known as fast and reversible, despite absorption is slow and limited. In these conditions, equilibrium is running between the plant cell surface and the heavy metal in solution. The whole absorption process may be influenced by (1) cell-membrane characteristics, (2) heavy-metal interactions with this membrane, (3) presence of other heavy metals or cations (e.g., Ca^{2+}, Mg^{2+}) that reduce/stimulate the metal absorption, and (4) existence of a ligand which influences the heavy-metal activity (Slaveykova and Wilkinson 2002; Worms et al. 2006). Then to allow the metal biouptake, some specific transporters are involved in the transport of Cd and/or Cu (Memon and Schröder 2009): the P-Type ATPase (Cd/Cu) from the expression of genes *AhHMA3-4*, *AtHMA1-8*, *GmHMA8*, *OsHMA9*, and *TcHMA4*; the Nramp (Natural resistance-associated macrophage protein) (Cd) coded by *AhNRAMP3*, *AtNRAMP1-6*, and *LeNRAMP1-3*; the IRT (Iron-Regulated Transporter) from the expression of *AtIRT1*, *LeIRT1-2*, *NtIRT1*, *OsIRT1-2*, and *TcIRT1-2*.

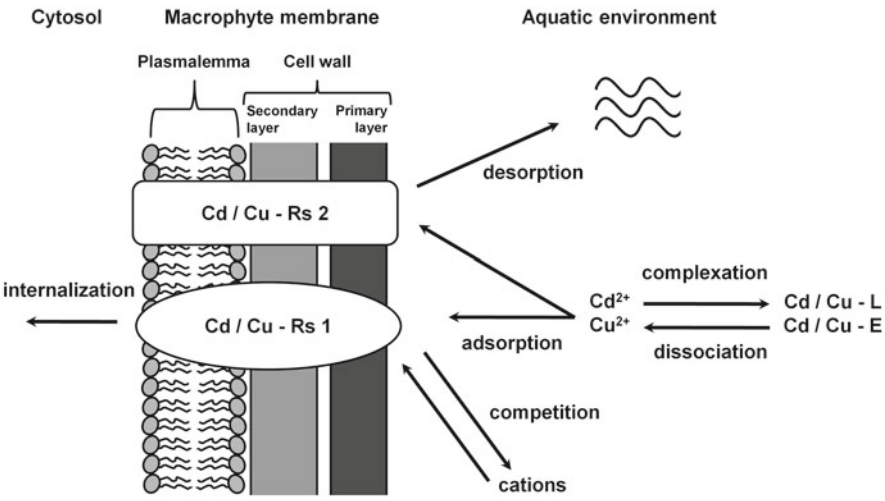

Fig. 2.1 General model of physicochemical processes involved in the Cd and Cu uptake by macrophytes in aquatic environments (modified and adapted from Delmail 2011). In the followings, replace M by Cd or Cu: M^{2+}, free ionic form; M-E or -L, heavy metal complexed to an exopolysaccharide or a ligand; M/Rs, heavy metal bounded to membrane receptors allowing uptake (Rs1) or not (Rs2)

Occurrence of humic substances may reduce the metal absorption by aquatic organisms as it was observed in the freshwater angiosperm *Vallisneria spiralis* (Hydrocharitaceae) with a reduction of Cd^{2+} and Cu^{2+} absorption in presence of humic acids (Wang et al. 2009). However, humic substances are able to adsorb on biological surfaces and to modify the membrane permeability and its ionic charge. They may affect the bioavailability of heavy metals and promote the absorption of free ionic forms (Lamelas et al. 2005). Like humic substances, exopolysaccharides influence significantly the environmental speciation. The consequence is the reduction of metal bioavailability through the decrease of free-ionic-form concentrations. This is observed in *Chlamydomonas reinhardtii* (Chlamydomonadaceae) where Cu^{2+} is complexed with exopolysaccharides leading to a bioavailability loss (Sunda and Huntsman 1998). In the same way, *Pseudokirchneriella subcapitata* (Ankistrodesmaceae) synthesizes exudates complexating Cd, Cu, Pb, and Zn, and reduces the rates of free-ionic forms (Koukal et al. 2007).

3 Toxicity in Macrophytes and Biomonitoring

The environmental health is often measured by the presence/absence, abundance, and physiological state of a species, called indicator species, specific to certain environmental conditions. This indicator species is a taxon with steno-affinities (sometimes eury—if studies are conducted at a wider scale) with distribution/state

providing information on environmental parameters. However, it does not allow estimating if ecosystem health is optimal. It provides only information on ecological features from the studied ecosystem. Normalized indicators developed for freshwater environments from macrophytes are mainly focused on trophic pollutions.

3.1 Using Macrophytes in Ecotoxicology

Aquatic plants considered as "macrophytes" are photosynthetic organisms with a size (or with a colony) visible to the naked eye. It includes immersed, floating, or emergent phanerogams, bryophytes, microalgae with filamentous/thallus/globular colonies, macroalgae (Characeae), pteridophytes, and in lesser part lichens and fungal/bacterial colonies (Chauvin et al. 2008).

Aquatic macrophytes remain important for the functioning of ecosystems and, like diatoms, aquatic macrophytes are fixed on a substrate (Kleeberg 2013). In consequence, they assimilate modifications and disturbances of their environment (Souza et al. 2013). Plant communities respond to natural and anthropogenic environmental conditions through variations in diversity and abundance. Since many years, phytosociological studies highlight the deep relationships between nutrient levels and distribution of phytocoenosis in streams and lakes (Hinojosa-Garro et al. 2008; Trémolières et al. 2008). Bioindication scales are based on macrophyte communities and their responses to eutrophication; they constitute a reference in water-quality categories defined by their lithological and chemical characteristics, in specific physical contexts (Trémolières et al. 2008). On the other hand, pollution by xenobiotics, like heavy metals, eliminates sensitive plants and/or induces a metal accumulation in cell wall and vacuole leading to physiological disruption, especially among bryophytes and lichens. Indeed, certain macrophytes present some accumulator phenotypes for one or several heavy metals (Kamal et al. 2004; Pio et al. 2013; Saygideger et al. 2013; Xie et al. 2013). These plants could store metal at concentrations near 100,000-fold higher than those from the surrounded environment and some of them were used to exclude these toxic compounds from natural ecosystems (e.g., *Eichhornia crassipes* [Pontederiaceae], *Pistia stratiotes* [Araceae], *Spirodela polyrhiza* [Araceae]) (Mishra and Tripathi 2008). However, these plants are restricted to ponds as floating forms and no consideration of streams is possible. Only a few studies have focused on the heavy-metal disposal in waters (Mechora et al. 2013) compared to those on soil phytoremediation (Marchand et al. 2010) considering plant models modified genetically (*Thlaspi caerulescens* TcHMA4 [Brassicaceae] (Papoyan and Kochian 2004)) or not (*Arabidopsis halleri ssp. gemmifera* [Brassicaceae] (Kashem et al. 2010)). Until now, only one study highlights in immersed macrophytes "hyperaccumulator" capabilities (in *Myriophyllum alterniflorum* [Haloragaceae]) analogous to those from continental species (Delmail et al. 2013). It allows encouraging ecotoxicological perspectives in freshwater environments, especially as this species prefers streams. In bioaccumulation, the bioconcentration factor (BCF) is widely used to estimate the accumulation capacity of a plant

and several authors indicated that a plant with a BCF over 1,000 could be considered as a hyperaccumulator (Zhu et al. 1999; Bunluesin et al. 2004; Lu et al. 2011). In *M. alterniflorum*, Delmail et al. (2013) measure a Cu BCF up to 226,024 after 28 days of contamination and a Cd BCF up to 10,377 after a 21-day exposure. These *in situ* BCF indicate that this species hyperaccumulated these heavy metals in ranges similar to those observed by Lu et al. (2011) for *P. stratiotes*. In that sense, *M. alterniflorum* appears as a promising species for phytoremediation of running freshwaters.

Only a few studies underlined the potential of biodetection of heavy-metal contaminations by aquatic macrophytes, e.g., Chatenet and Botineau (2001) on the lichen *Dermatocarpon luridum* (Verrucariaceae), Harguinteguy et al. (2013) on *Myriophyllum aquaticum* (Haloragaceae). In the same way, only one standardized biological index (Relative Treatment Efficiency Index [RTEI]) was recently developed by Marchand et al. (2010) to quantify the impact of phytoremediation on the elimination of a dozen heavy metals by macrophytes, e.g., *Ceratophyllum demersum* (Ceratophyllaceae), *Cyperus alternifolius* (Cyperaceae), *Eichhornia crassipes*, *Lemna gibba* (Araceae), *Pistia stratiotes*, *Salvinia herzogii* (Salviniaceae). Currently, macrophytes are mainly used for their bioaccumulation capabilities to phytoremediate heavy-metal disturbed environments rather than their bioindicator sensitivity to an environmental disturbance.

However, from the beginning of the bioindication, managers of aquatic ecosystems would use and develop methodologies using plants into the conception of multiparametric tools for stream-quality evaluation. In consequence, the trophic pollution was studied more intensively. A first range of six macrophytic indexes was developed by Haury et al. (1996) to assess the water trophic quality from 240 taxa. This method could consider the spreading, supraaquatic, and eurytopic species. Then, after a fastidious synthesis work led by the Groupement d'Intérêt Scientifique Macrophytes des Eaux Continentales (GIS MEC), a new index was created: the Indice Biologique Macrophytique en Rivière (IBMR; normalized NF T 90-395) (Haury et al. 2006). This new protocol takes into account the spreading and the ecological valence (adaptation degree of organism to environmental changes) in parallel with the environmental trophic status.

3.2 Specific Physiological Responses and Biomarkers

Since 20 years, the "biomarker" notion has evolved and presents now many definitions. According to Depledge (1993), a biomarker is "a biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples or at the level of the whole organism (either individuals or populations), that provides evidence of exposure and/or effects of one or more chemical pollutants (and/or radiation)." These characteristics provide information about the specimen exposed to toxic compounds and they evaluate its response to xenobiotics. Then Van Gestel and Van Brummelen (1996) have attempted to define biomarkers and bioindicators. According to these authors, the term "biomarker" must be restricted to

"any biological response to an environmental chemical at the individual level, measured inside an organism or in its products, indicating a deviation from the normal status that cannot be detected in the intact organism." However, some specialists would consider some parameter responses (e.g., morphology) that may be used in an attempt of risk evaluation and which could influence not only the specimen but also its population (through presence, absence, and behavior). In consequence, when biological responses are measured using whole organisms, Van Gestel and Van Brummelen (1996) refer to bioindicators instead of biomarkers. Since 4 decades, progresses in biochemistry and molecular toxicology increased our knowledge on the toxicity mechanisms of xenobiotics, especially among mammalians. Sensitive and specific biochemical effects were highlighted in species with an ecological interest when exposed to certain pollutants. Most of the ecotoxicological studies focused on aquatic environments, but mainly on marine ecosystems as they remain the last receptacle of all pollutions (Roméo and Giambérini 2008).

4 Heavy-Metal Effects and Macrophyte Physiological Responses

When internalized in macrophytes, heavy metals induce a chain reaction of physiological mechanisms beginning with the synthesis of reactive oxygen species (ROS) and a "chemicalfall" of physiological responses. All physiological adaptations may be considered as potential biomarkers in ecotoxicological studies due to their high sensitivity as it will be shown further.

4.1 Reactive Oxygen Species and Detoxification Care

Photosynthetic organisms are aerobic and so use the dioxygen as a source of energy for their growth. As a consequence, this process leads to the synthesis of ROS which are diversified chemically reactive molecules made from oxygen. These ROS are a natural metabolism byproduct and play important roles in cell homeostasis and cell signaling. However, under environmental stress like heavy metals, their intracellular levels increase leading to destruction of organites and cell wall (Delmail and Labrousse 2012). As example, Yu et al. (2007) have observed a 50 % increase of ROS concentration in *Microcystis aeruginosa* (Chroococcaceae) after a 48 h exposure at 6 μg L^{-1} Cu. Then occurrence of these elements in macrophytes leads to the disruption of electronic transports and the disturbance of metabolic pathways.

A partial reduction of O_2 through the respiratory-chain cytochromes implies the ROS production as singlet oxygen ($^1O_2$) and superoxide radical ($O_2^{\bullet -}$) which leads to the synthesis of hydroxyl radical ($^{\bullet}OH$), hydroperoxyl radical ($^{\bullet}O_2H$), and hydrogen peroxide (H_2O_2) (Fig. 2.2). The radicals alkoxyl ($RO^{\bullet}$) and peroxyl ($RO_2^{\bullet}$) result from membrane-phospholipid peroxidation (or lipoperoxidation) by previous ROS

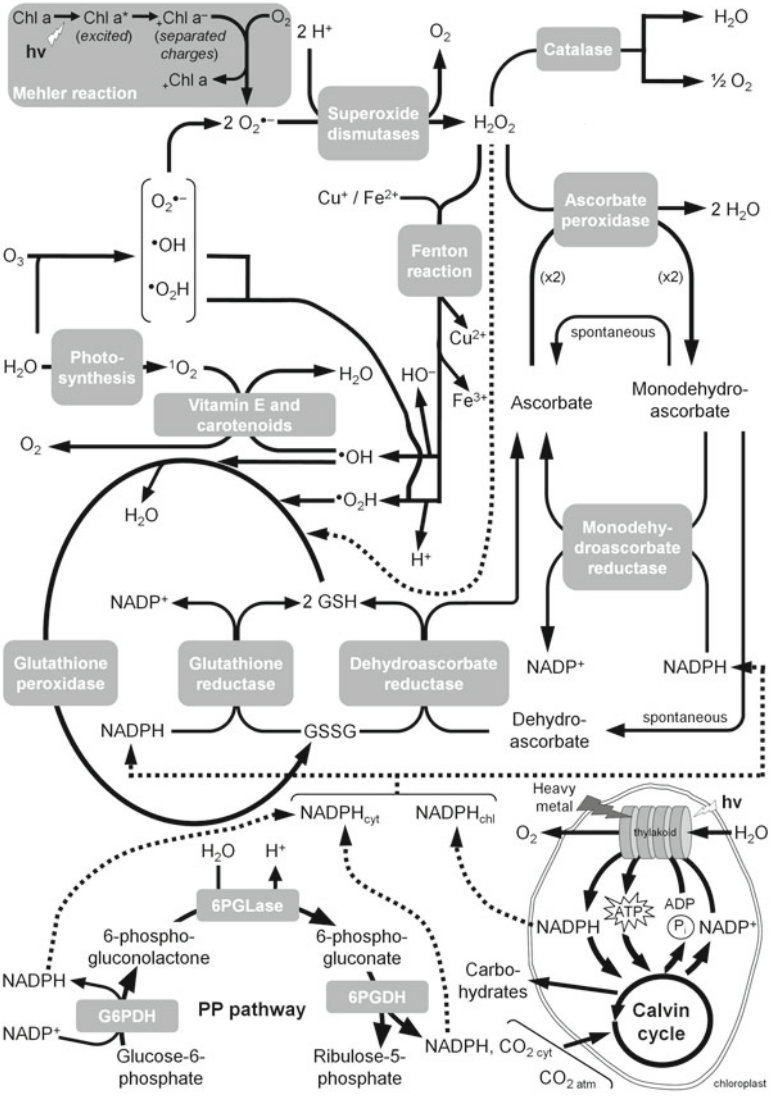

Fig. 2.2 Main antioxidant pathways in macrophytes including enzymes and scavengers (based on Delmail and Labrousse 2012). For easier comprehension, certain reactions are not equilibrated. *Chl a* chlorophyll a, $CO_{2atm/cyt}$ atmospheric/cytosolic CO_2, *G6PDH* glucose-6-phosphate dehydrogenase, *GSH* glutathione, *GSSG* glutathione disulfide, $NADPH_{chl/cyt}$ chloroplastic/cytosolic NADPH, *6PGLase* 6-phosphogluconolactonase, *6PGDH* 6-phosphogluconate dehydrogenase, P_i inorganic phosphate, *PP pathway* pentose-phosphate pathway

(Fig. 2.3) (Thompson et al. 1987; Li et al. 1994; Lagadic et al. 1997; Edreva 2005; Delmail and Labrousse 2012). At the same time, high levels of ROS are produced by the photosynthetic electron transport chains. Indeed, the electrons tetravalently reduce the intracellular oxygen to water. However, some of them may leak from

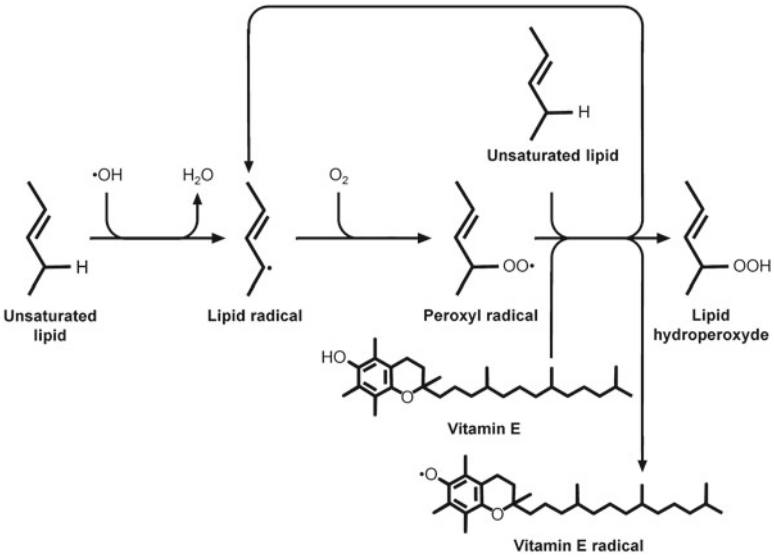

Fig. 2.3 Mechanisms of lipid peroxidation in biological membranes of macrophytes (based on Delmail and Labrousse 2012). The produced peroxyl radicals could react either with another lipid to supply the lipoperoxidative chain reaction mechanism or with a scavenger like vitamin E which disrupts and stops the oxidative process

many sites along the transport chain, resulting in a univalent reduction of O_2 to give $O_2^{\bullet -}$ which could be thereafter dismutated to form H_2O_2 (Alscher et al. 2002; Delmail and Labrousse 2012). This dismutation remains spontaneous or can be catalyzed by one of the three superoxide dismutases (SODs) (Fig. 2.2) depending on the cell compartment where the reaction occurs: manganese-superoxide dismutase (mitochondria, peroxisome), iron-superoxide dismutase (chloroplast), or copper/zinc-superoxide dismutase (chloroplast, cytosol) (Fornazier et al. 2002; Pereira et al. 2002; Gill and Tuteja 2010; Delmail and Labrousse 2012).

Considering all its matched electrons, H_2O_2 is not a free radical. However, it presents a strong toxicity potential due to its long lifespan and high diffusibility far from its synthesis site. It can pass through biological membranes via aquaporins as it presents a chemical structure close to water (Bienert et al. 2006, 2007; Parent et al. 2008; Delmail and Labrousse 2012). The level of H_2O_2 is regulated by antioxidant enzymes like ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GSH-PX) (Fig. 2.2). These proteins use the chloroplastic nicotinamide adenine dinucleotide phosphate (NADPH) produced during the photosynthesis for their functioning (Fig. 2.2). However, the ROS could disrupt the photosynthetic electron transport chains in thylakoid membranes and some electrons may be deflected leading to the chloroplastic-NADPH-supply disruption and the use of NADPH from catabolic pentose-phosphate pathway (Fig. 2.2) (Kruger and von Schaewen 2003; Delmail 2011; Delmail and Labrousse 2012). The H_2O_2 could be also produced through the bivalent reduction of the dioxygen by oxidases like

peroxisomal glycolate oxidase or amine oxidase (Parent et al. 2008; Delmail and Labrousse 2012). The toxicity of H_2O_2 is also correlated to its involvement in the hydroxyl-and hydroperoxyl-radical synthesis through the Haber-Weiss and Fenton reactions (Fig. 2.2). Like their ROS precursor, these short-lifespan radicals are very diffusive through biological membranes and they could affect all organites and cell compartments. They are also mainly implied in the lipoperoxidation (Fig. 2.3) (Lagadic et al. 1997; Edreva 2005; Delmail and Labrousse 2012). The produced fatty-acid radical reacts with molecular oxygen, thereby creating a peroxyl fatty acid radical. This last one reacts with another phospholipid, producing a new radical and lipid peroxide, or cyclic peroxide if it reacts with itself. This cycle continues as a chain reaction mechanism (Schaich 2005; Delmail and Labrousse 2012). This process ends up when two radicals react and produce a non-radical compound and happens when the concentrations of radicals is high enough. Living organisms have evolved different molecules that speed up termination by catching ROS (Paramesha et al. 2011; Delmail and Labrousse 2012). Among such antioxidants, the most important are the scavengers mainly constituted with α-tocopherol (or vitamin E) and carotenoids (β-carotene, xantophylls) (Figs. 2.2 and 2.3) (Delmail et al. 2011a, b, c; Delmail and Labrousse 2012).

Considering all these elements, the ROS are considered as phytotoxic compounds. However, it is currently admitted that their synthesis, in relation to the respiratory and photosynthetic metabolisms, plays an essential role in plant-cell life and death. Indeed, they might play an alternative role and act as cell signalization molecules to establish some defense mechanisms toward xenobiotic stress (Parent et al. 2008; Delmail and Labrousse 2012).

4.2 Activities of Antioxidant Enzymes

Teisseire and Guy (2000) highlight that Cu induced an increase of peroxidase and CAT activities in *Lemna minor* (Araceae): APX (132 % at 319 µg L^{-1} Cu followed by a reduction of 72 % at 1.6 mg L^{-1} Cu), guaiacol peroxidase (G-PX) (553 % at 798 µg L^{-1}), pyrogallol peroxidase (P-PX) (166 % at 1.6 mg L^{-1} Cu), catalase (347 % at 1.6 mg L^{-1}). Furthermore, Teisseire et al. (1998) underline that a significant growth inhibition for half of the exposed organisms is observed but at higher Cu levels than high enzymatic response (EC_{50} = 160 µg L^{-1} vs. 100 % CAT-activity increase at 100 µg L^{-1}). These results confirm the sensitivity of the antioxidant system toward the ROS synthesis. Babu et al. (2005) also reported this phenomenon in *L. gibba* when exposed to 479 mg L^{-1} Cu, as SODs biosynthesis increases of 200 % despite a lower plant growth (−70 %). Among metabolic enzymes, glutathione *S*-transferases (GSTs) perform detoxification of pollutants by conjugation with a sulfhydryl antioxidant, the glutathione (GSH), able to chelate and neutralize ionic elements like heavy metals (Edwards and Dixon 2000). Many xenobiotics are handled by these enzymes, e.g., Cd and Zn induce an activity increase of 14 % at 1.8 mg L^{-1} in *C. demersum* (Aravind and Prasad 2005), and GST activity rises from 125 % in *L. minor* at 80 µg L^{-1} Cu (Teisseire and Guy 2000).

4.3 Responses of Other Enzymes

Parallel to antioxidant-protein responses, several variations could be measured among other enzymes. Among them, two are involved in physiological processes necessary for the protection against stress and well-functioning of the organisms. The esterases constitute a group of hydrolases catalyzing the cleavage and the formation of carboxyester bonds. They are involved in cell-wall synthesis, xenobiotic degradation, and cell signalization. These enzymes are sometimes used as biomarkers of plant viability (Víteček et al. 2007). Depending on the species resistance toward Cd and Cu, the esterase activity increases in tolerant organisms, e.g., *L. minor* (Mukherjee et al. 2004). Another enzyme is the carbonic anhydrase, a Zn metalloprotein important to bring CO_2 for photosynthesis. This protein catalyzes the reversible interconversion of CO_2 and water to hydrogenocarbonate (HCO_3^-) and protons (H^+). Its activity is positively correlated with Zn level due to its deep constitutive dependence. However, Cd could replace Zn in this protein leading to a low enzyme activity. This substitution is possible as Zn is bonded to two nitrogen atoms from the histidine imidazole aromatic heterocycle, and to the two glutamate carboxylates. This phenomenon is observed in *C. demersum* (Aravind and Prasad 2005).

4.4 Photosynthetic Pigments

Chlorophylls and carotenoids are the main pigments able to collect and use light among photosynthetic organisms. They are located in thylakoid membranes in chloroplasts. Their role is to use this energy to run the photosynthesis process. The carotenoids have another function as they also protect the photosynthetic pathways from irradiance excess. Indeed, this phenomenon may lead to a surplus of excited electrons which would saturate the photosynthetic electron transport chain and induce the ROS synthesis. Heavy metals could promote the ROS synthesis through the disruption of the electron transport as they act as inhibitors of photosystem-II (PSII) metallo-sensitive sites (Clijsters and Van Assche 1985). This influences carotenoids to act as scavengers to reduce ROS, which inactivates and prevents them from contributing to photosynthesis (Gill and Tuteja 2010).

Moreover, the inhibition of chlorophyll synthesis is linked to heavy-metal occurrence which reduces drastically their concentrations (Noriega et al. 2007). In presence of 10 mg L^{-1} Cd, John et al. (2008) have noted in the macrophyte *Spirodela polyrrhiza* (Araceae) a strong decrease in chlorophylls a and b (−46 % and −62 %, respectively) associated with a fresh-weight loss (−52 %). In the same way, Teisseire et al. (1998) have observed a significant loss of growth capabilities and a reduction of total-chlorophyll level (EC_{50} = 160 µg L^{-1} Cu) in *L. minor*. The chlorophylls seem to be as highly sensitive as growth parameters. Concerning the carotenoids, Malec et al. (2010) have also reported a negative correlation between these pigments (−42 %) and the Cd concentration (250 µg L^{-1}) in *Lemna trisulca* (Araceae). In *Myriophyllum alterniflorum*, chlorophyll-content decrease results from the Cd and

Cu inhibition of metabolic enzyme like enzymes of the chlorophyll biosynthesis pathways (δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydratase) (Delmail et al. 2011b, c). Carotenoids protect photosystems from metal-induced oxidative stress and the imbalance between carotenoid production and carotenoid oxidation in case of intense stress (i.e., strong production of ROS) leads to a decrease in carotenoid content in *M. alterniflorum* (Delmail et al. 2011b, c).

4.5 Heat Shock Proteins

In response to a stress, many authors have stated that living organisms produce stress proteins to fight environmental disturbances like UV, hypoxia, anoxia, temperature, and osmolarity modifications (Brain and Cedergreen 2009). These proteins were described as heat shock proteins (Hsps) by Ritossa (1962) and their wide family depending on their molecular weight acts as chaperons to maintain cell homeostasis. They are involved in denaturated-protein repair and allow a good protection against ROS (Lewis et al. 2001). Potentially interesting for biomonitoring due to their high sensitivity to heavy metals, Hsp70 strongly helps to protect cell components during a metal contamination (Lewis et al. 2001). Our first results in *M. alterniflorum* highlight a sevenfold increase in Hsp70 after 7 days of a 10-µg L^{-1}-Cd treatment and a 240-fold increase in Hsp70 after 5 days of a 100-µg L^{-1}-Cu treatment. In ponds, Ireland et al. (2004) observe a rise in the production of Hsps70 (+400 %) in *L. minor* during 3.7 g L^{-1} Cd pollution. In the same way, Lewis et al. (2001) noted the same physiological response in the green alga *Enteromorpha intestinalis* (Ulvaceae) in presence of Cu (+55 % Hsps70 at 100 µg L^{-1}).

4.6 Phytochelatins

Among macrophytes, phytochelatins are involved in the detoxification of heavy metals to maintain cell homeostasis. The phytochelatin-synthase activity allows assembly of GSH block (from 2 [PC_2] to 11 [PC_{11}]). Phytochelatins are linear polymers made of γ-glutamylcysteine to form a γ-L-glutamyl-L-cysteinylglycine (Fig. 2.4), they can only be measured in presence of heavy metals (especially Ag, Au, Bi, Cd, Cu, Hg, Pb, and Zn) despite some others have no effect (Al, Ca, Fe, Mg, Mn, Na, and K) (Brain and Cedergreen 2009; Grill et al. 1989). Phytochelatins, as specific to these elements, are important and so have high response sensitivity even during multi-pollutant contamination. This phenomenon is observed by Pawlik-Skowrońska (2001) in the genus alga *Stigeoclonium* (Chaetophoraceae) during mining-effluent releases made of Cd, Cu, Pb, and Zn. The phytochelatin synthesis in this alga is linked to heavy-metal speciation correlated to water pH: the more acidic is the pH, the more free-ionic forms will be available in the environment, and the higher phytochelatin levels will be.

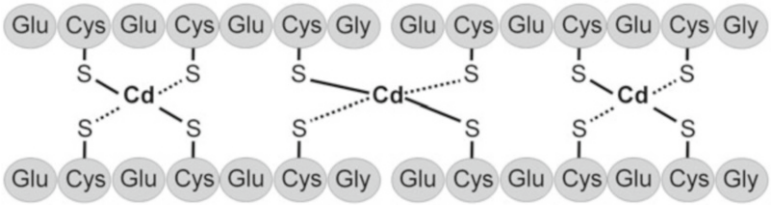

Fig. 2.4 Phytochelatins (PC_2) and Cd chelation. *Cys* L-cysteine, *Glu* L-glutamate, *Gly* glycine. *Continuous line* covalent bond; *broken line* electrostatic interaction

When biomonitoring Cu, levels of PC_2 and PC_3 of *Hydrilla verticillata* (Hydrocharitaceae) (Srivastava et al. 2006) and *Lemna aequinoctialis* (Araceae) (Yin et al. 2002) show a significant increase related to metal concentrations (50 % and 160 % at 1.6 mg L^{-1}, respectively). Considering total phytochelatins, Branco et al. (2010) also noted a positive correlation between Cd level and phytochelatin synthesis (+600 % at 300 µg L^{-1}) in the diatom *Nitzschia palea* (Bacillariaceae). In addition, our preliminary results of phytochelatin production in *M. alterniflorum* during Cd stress indicate that these molecules could probably be used as biomarker. Indeed, a 15-fold increase in PC_2 concentration between days 3 and 7 after exposure to 10 µM Cd is noted. PC_3 becomes detectable after 4 days whereas PC_4 appears only at 7 days.

4.7 Flavonoids

Flavonoids are phenolic compounds derived from phenylpropanoids made of phenylalanine. They can be organized as monomers, dimers, and oligomers: chalcones, aurones, flavonones, isoflavonoids, flavones, flavonols, leucoanthocyanidins, catechins, and anthocyanins. They are involved in several mechanisms like reproduction, signaling, and radiation protection (Croteau et al. 2000; Iwashina 2000; Brain and Cedergreen 2009). The synthesis of these metabolites is related to various environmental stresses but the main one remains the heavy metals (Babu et al. 2003; Kidd et al. 2001). However, the involvement of pollutants on the flavonoid biosynthesis pathway is currently poorly known (Babu et al. 2003; Brain and Cedergreen 2009). No information is available concerning effects on chalcone synthase or intermediary phenylpropanoid byproducts. Only variation could be measured on biosynthesis activity with a 200 % increase of chalcone-synthase activity at 1.28 mg L^{-1} Cu in *L. gibba* (Babu et al. 2003).

4.8 Genotoxicity

From many years, the release of potentially genotoxic xenobiotics in aquatic environments increase tragically leading to obvious or unseen deleterious effects on

aquatic organisms. For example, the total industrial water release in the United States of America for the year 2001 is estimated to 100,153 t. Among them, the release of both inorganic and organic compounds is of great concern as Pb and formaldehyde reach 164.3 and 152.5 t, respectively (Ohe et al. 2004). To assess the genotoxic effects of these compounds, several methods were developed and certain are used in routine testing; they consider several organisms like bacteria, yeast, fungi, insect, mammalian cells in culture or laboratory animals, and specifically in waters fish, bivalves protozoa, microalgae, and higher plants (Majer et al. 2005). The main techniques for evidencing genotoxicity in water samples are the Single Cell Gel Electrophoresis assay (SCGE) best known as the COMET assay, the Polymerase Chain Reaction-Random Amplification of Polymorphic DNA (PCR-RAPD) and the Micro Nucleus test (MN). However, these water-sample tests consider a few aquatic plants species as the MN test is mainly adapted for inland species *Tradescantia sp*. (Commelinaceae), *Allium sp*. (Amaryllidaceae), *Lactuca sativa* (Asteraceae), or *Vicia faba* (Fabaceae) (Majer et al. 2005; Giorgetti et al. 2011). Scarce examples of aquatic plants can be found in the literature, e.g., the wetland macrophyte *Bidens laevis* (Asteraceae) is recently used for testing several organic compounds (Pérez et al. 2008, 2011), the macrophytes *H. verticillata* and *C. demersum* are used for RAPD test during Cd and Cu exposures (Gupta and Sarin 2009).

In terrestrial plants, PCR-RAPD assay is currently used to characterize the effect of various heavy metals since the use of the DNA-fingerprinting technique to detect genotoxic effect is highlighted by Savva (1998). In the same year, this technique is applied on two generations of *Arabidopsis thaliana* (Brassicaceae) exposed to Cd, Pb, and Mn by Conte et al. (1998). More recently, Aydin et al. (2012) focus on population parameters and RAPD band-profiles/genomic template stability (GTS) to assess the genotoxic effects of Cu and Zn in *Cucumis sativus* (Cucurbitaceae). Similar observations are done by Liu et al. (2009) in *Hordeum vulgare* (Poaceae) exposed to Cd. All these authors indicate that the GTS reflecting changes in RAPD fingerprinting is in concordance with the traditional indices such as growth and soluble-protein level. Moreover, in As-treated *Oryza sativa* (Poaceae) specimens, Ahmad et al. (2012) noted that the apparent inhibitions in chlorophyll and protein contents are well correlated with the changes in GTS. In *Solanum melongena* (Solanaceae) during a Cu contamination, the inhibition in root growth is correlated with the changes in root dry weight and total soluble-protein content as population biomarkers, and with RAPD profiles as molecular biomarker (Korpe and Aras 2011). Finally, Cenkci et al. (2009) evaluated the effect of several heavy metals on *Phaseolus vulgaris* (Fabaceae) using RAPD and conclude that DNA alterations detected by RAPD offered a useful biomarker assay for the genotoxic-effect evaluation of B, Cr, Hg, and Zn pollutions in plants.

Genotoxic test may also be done using another assay close to RAPD, the amplified fragment length polymorphism (AFLP) which is rarely used. AFLP allows Labra et al. (2003) to evidence the effect of potassium dichromate and 9,10-dihydrophenanthrene in the genus *Arabidopsis*. One year later, the same team (Labra et al. 2004) evidences in *Brassica napus* (Brassicaceae) the effect of potassium dichromate joining the selective amplification of polymorphic loci (SAMPL)

assay to AFLP. Aina et al. (2006) used the AFLP assay to test the effect of organic pollutants in *Trifolium repens* (Fabaceae). In addition to the commonly used RAPD assay, the animal-cell-widely-applied COMET assay (Dhawan et al. 2009) is developed for plant by some research groups. Indeed, Rodriguez et al. (2011) evaluated the toxicity of Cr on *Pisum sativum* (Fabaceae) by this assay and consider COMET and flow cytometry as reliable endpoints for this metal toxicity in plants. Previously, Gichner et al. (2008a) tested the influence of Cd, organic pollutants, and radiations on *Solanum tuberosum* (Solanaceae) and the effect of Cd, Cu, Pb, and Zn on tobacco and potato plants (Gichner et al. 2006) using the COMET assay. The same team (Gichner et al. 2008b) evaluates the effect of Pb in tobacco and conclude that the COMET assay does probably not represent a suitable method for monitoring genotoxicity of environmental pollutants using plants growing *in situ*. Nowadays, to our knowledge, the COMET assay for plant is only used in laboratory conditions to clarify the relationship between several pathways as was done by Pourrut et al. (2011) between ROS, DNA strand-breaks, and chromosome aberrations induced by Pb. Finally, the micronucleus assay, commonly used on *Tradescantia* or *Allium* root tips, was adapted sometimes to other species. For example, Guo et al. (2010) studied the effect of nitrobenzene on *Glycine max* (Fabaceae) in laboratory experiments. The effect of municipal landfill leachate on *H. vulgare* root tips was done by Sang et al. (2006). These authors noted that leachate caused significant increases of micronucleus frequencies in a concentration- and a time-dependent manner. For the aquatic plant *E. crassipes*, Mishra et al. (2009) indicated that the number of micronuclei is directly proportional to Cr and increases with the metal concentration to which plants are exposed. In contrast, in the hyperaccumulator species *Brassica juncea* (Brassicaceae), Seth et al. (2012) indicated that the induction of micronuclei noted in the root tips treated by Cd, Cr, Cu, and Pb for 24 h show a concentration-wise recovery in cells examined at 24-h post-exposure.

Preliminary result led on the running-freshwater macrophyte *M. alterniflorum* concerning the genotoxicity of Cd and Cu is obtained recently by our team. PCR-RAPD are done with the MWG-Operon random primers *OPB05*, *OPB20*, *OPC19*, and *OPG10*, generating band pattern fluctuating with the heavy-metal concentration as it was shown in Fig. 2.5 for Cd with *OPC19*. The GTS is calculated as described previously by Aydin et al. (2012) using RAPD band-profiles generated by the PCR-RAPD and the formula below:

$$GTS = \left(1 - a / n\right) \times 100$$

where a indicates the RAPD polymorphic bands (appearing + disappearing bands) in each sample and n is the total number of bands in the control.

In *M. alterniflorum*, the GTS strongly decreases during Cd treatment even at low contamination (Fig. 2.6). This observation is in agreement with various previous studies on terrestrial and aquatic species mentioned above. This effect is expected because as a toxic element, Cd has various deleterious effects at different levels of the plant (Nagajyoti et al. 2010; Gallego et al. 2012). Curiously, an increase of GTS is noted at 10 μg L^{-1} Cd probably corresponding to an artifact even if a recovery of

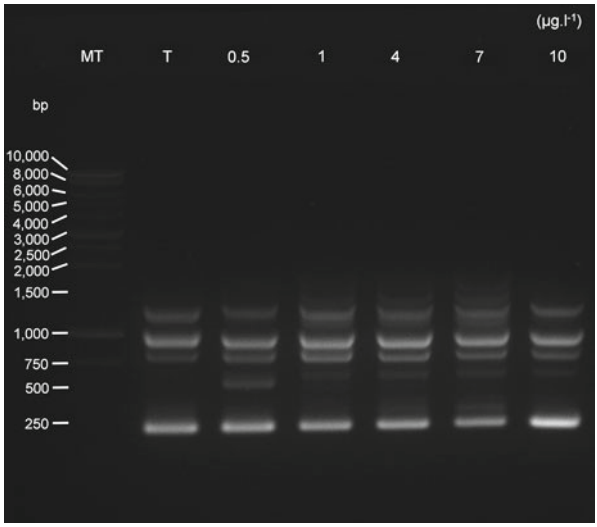

Fig. 2.5 PCR-RAPD of *Myriophyllum alterniflorum* contaminated with Cd during 25 days with random primer *OPC19* (0.5, 1, 4, 7, and 10 µg L^{-1}). MT: 1 kb DNA ladder; T: control

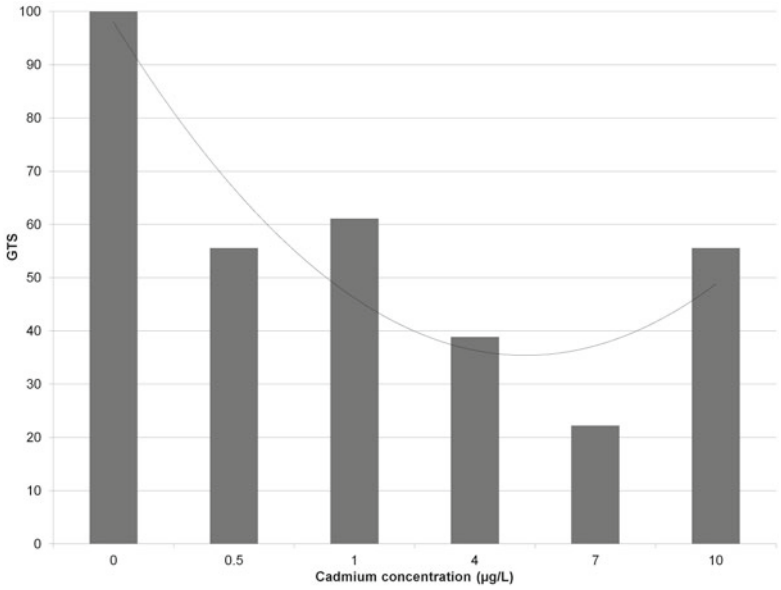

Fig. 2.6 Genomic template stability (GTS) of *Myriophyllum alterniflorum* contaminated with Cd (0, 0.5, 1, 4, 7, and 10 µg L^{-1}) during 25 days, RAPD with random primer *OPB05*, *OPB20*, *OPC19*, and *OPG10*

watermilfoil similar to those described in *B. juncea* by Seth et al. (2012) cannot be dismissed. In contrast to the effect of Cd, Cu has only a slight impact on the GTS of *M. alterniflorum* (Fig. 2.7); even at relatively high concentrations (100 µg L^{-1}), GTS is only reduced by 10 %. This is quiet logical as the Cu is an essential micronutrient

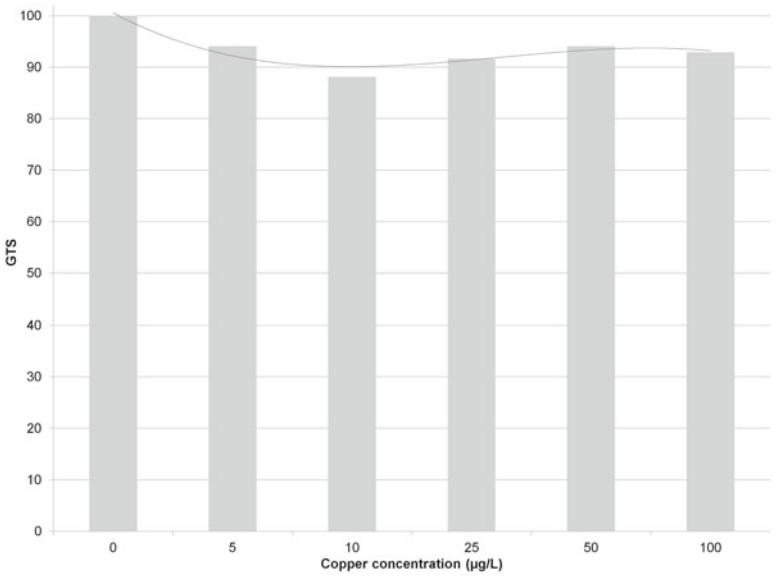

Fig. 2.7 GTS of *Myriophyllum alterniflorum* contaminated with Cu (0, 0.5, 10, 25, 50, and 100 µg L^{-1}) during 25 days, RAPD with random primer *OPB05*, *OPB20*, *OPC19*, and *OPG10*

for plant metabolism (Nagajyoti et al. 2010) and, as a consequence, watermilfoil can regulate his metabolism to maintain Cu homeostasis.

In conclusion, it seems that aquatic macrophytes constitute good model organisms to study genotoxic effects in freshwater environment, even if up to now only a few studies were conducted. This increased interest in aquatic macrophytes is recently highlighted by Arts et al. (2010) but it concerns mainly organic pollutants like pesticides.

4.9 Water Loss and Anatomical Adaptations

Hydric potential is deeply modified in heavy-metal-treated macrophytes and especially *M. alterniflorum* where adaptations are set up. Leaf modifications are observable in hydathodal conductance, osmotic potential, and water content. A heterophyllous response occurs depending on the age of the photosynthetic organs, as heavy-metal distribution in the macrophyte is not homogenous. The related oversized stomatal structures such as hydathodes are incapable of regulating pore aperture and remain always opened to release water under guttation and dissolved solutes from the xylem (Pillitteri et al. 2008). So the decrease of hydathodal conductance in all leaves in presence of Cd or Cu is certainly induced by metals. The physiological reason leading to this transpiration-rate reduction in macrophyte is currently unknown and remains one of the most interesting phenomena to be explained.

To counterbalance this water loss, the macrophyte synthesizes osmocompatible solutes involved in osmotic adjustment to protect its leaf tissues from ionic deleterious effects (Sanità di Toppi and Gabrielli 1999; Delmail et al. 2011b, c). An interesting osmolyte is the free proline as this cytosolic amino acid is involved in two regulation processes: the cellular osmotic adjustment and the scavenging of ROS (Delmail et al. 2011b, c). Gas exchanges are affected by anatomic traits of leaves and Cd and Cu induce the development of xerophytic features in leaves, e.g., a significant increase of the lower epidermis thickness disrupts the stomata functioning by isolating more gaseous-exchange area (Delmail et al. 2011b, c). The consequence is a carbon gain associated with a lower water loss, considered as one of the most xerophytic characteristic of leaves (Shi and Cai 2008).

To face dehydration, a novel anatomic adaptation appears in mature leaves of *M. alterniflorum*, when exposed to Cd and Cu. This double endodermis presents two cell layers with true Casparian strips made of suberin and separated by foliar mesophyll. It improves the leaf water and air tightness in macrophytes, as the cell layer next to the single vascular bundle prevents from sap losses and the cell layer below the epidermis allows a gaseous homeostasis in mesophyll (Delmail et al. 2011b, c). This adaptation is important in macrophytes especially as xylem-vessel diameter is lower in presence of heavy metals due to ontogenic disruptions, and as resorption gaps appear after vessel lignin degradation during oxidative stress (Delmail et al. 2011b, c).

5 Conclusion and Future Perspectives

Our current knowledge relative to Cd and Cu pollution on macrophytes underlines the intensity and the diversity of impacts induced by these heavy metals at all organization levels. From cell to individual level, many strategies are used to fight against metal stress, especially in streams, where pollutant inputs are continuous. After metal internalization, deep physiological disturbances occur in whole macrophyte organs and antioxidants as enzymes and scavengers are largely involved in the oxidative-stress regulation as they represent the main antioxidant barrier. These physiological responses remain very sensitive to the xenobiotic levels and constitute the first step to the development of histological protection against the free radicals. From these observations, recent Cd/Cu ecotoxicological investigations are conducted on running-freshwater macrophyte species. Furthermore, the research of new biomarkers to detect early metal pollutions is under progress to complete classic biomonitoring methods. Finally, it is quite probable that in the near future, freshwater macrophytes constitute the priceless “infantryman” of phytoremediation technologies for running and standing freshwaters. Indeed, current researches conducted on metal-hyperaccumulative macrophyte micropropagation are encouraging and allow high biomass production, and then the successful macrophyte reintroduction in polluted areas allows ecological restoration and biomonitoring.

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Chapter 3
Secondary Metabolites and Environmental Stress in Plants: Biosynthesis, Regulation, and Function

Mohammad Babar Ali

1 Introduction

Secondary metabolites (SMs) are organic molecules synthesized by plants that have crucial roles in plant growth and development (primary metabolites) and are therefore present in all plants. These organic molecules including nucleotides (RNA, DNA), amino acids, fatty acids, proteins, polysaccharides, and lipids play key roles in the survival of the plants. The term "secondary" was used only in the sense that these compounds are different in chemical structure and vary from species to species (Pichersky and Gang 2000). On the basis of their chemical structure more than 10,000 different flavonoids have been identified (Packer 2001; Ferrer et al. 2008; Agati and Tattini 2010; Pollastri and Tattini 2011; Agati et al. 2012; Brunetti et al. 2013) and more than 200,000 different types of SMs are likely to be identified in future (Fiehn et al. 2000; Dixon and Strack 2003; Yonekura-Sakakibara and Saito 2009). The most common or abundant SMs are terpenoids, phenylpropanoids, flavonoids, and alkaloids. Among them, flavonoids are highly studied and are the most biologically active polyphenolic compounds widely distributed in plants. They are responsible for flower color, taste, scent, to attract pollinators, seed dispersal, UV light protectants, signaling molecules, regulators of auxin transport, reactive oxygen species (ROS) scavenging, antimicrobial and antioxidant activities (Dixon and Paiva 1995; Shin et al. 2013; Agati et al. 2013; Benmalek et al. 2013). They are also important for plant–microbe interactions, plant immunity (Hassan and Mathesius 2012) and symbiotic associations (Wang et al. 2011a, b). Flavonoids, phenylalanine-derived secondary metabolites, have protective and regulatory functions in plants. For example, some flavonoids acts by reducing oxidation of lipoprotein and the

M.B. Ali (✉)
Rhizosphere Science Laboratory, Department of Plant and Soil Sciences, University of KentucKY, Lexington, KY 40546, USA
e-mail: alimb@rediffmail.com; mohammad.ali2@uky.edu

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aggregation of platelets resulting in reduced risk of certain diseases (atherosclerosis and cancer) was improved (Zern and Fernandez 2005; Aron and Kennedy 2008; Brown et al. 2009; Paredes-Lopez et al. 2010; Khan et al. 2010; Lu et al. 2013). This chapter summarizes the field of phenylpropanoid metabolism which includes flavonoids, with an emphasis on the types of molecules involved along with the enzymes and pathways associated with their biosynthesis.

2 The Classes of Secondary Metabolites

SMs can be divided into three main groups: (1) Nitrogen containing—a more diverse group, mostly synthesized from amino acids (alkaloids and glucosinolates), (2) non-nitrogen containing—phenolic compounds with an aromatic ring substituted with a hydroxyl group (e.g., phenolic acids, coumarins, stilbenes, flavonoids, tannins, and lignin), and (3) terpenes—compounds consider to be derived from isoprenes which are composed almost entirely of carbon and hydrogen (e.g., plant volatiles, cardiac glycosides, carotenoids, and sterols). Among these compounds, flavonoids are the most highly studied components and are found ubiquitously in plant foods. However, some individual's flavonoids may obtain dietary flavonoids from only a few plant-based foods. On the basis of the molecular structure, flavonoids can be divided into various classes (Rice-Evans et al. 1996) and some of the main groups of flavonoids are listed in Table 3.1.

3 The Current Knowledge of a Flavonoid Biosynthetic Pathway

Flavonoids are large family of phenolic compounds widely distributed in plants. Flavonoid biosynthesis appears to be induced by a variety of biotic and abiotic factors, including light, fungal elicitors, UV radiation, microorganisms, wounding, metal stress, low temperature, drought and deficiency of nutrients such as phosphorus (P) and nitrogen (N) (Dixon and Paiva 1995; Ververidis et al. 2007). The flavonoid pathway begins with phenylalanine conversion to cinnamic acid by four isoforms of phenylalanine ammonia lyase (PAL) and diverges into several branches at *p*-coumaroyl CoA in *Arabidopsis thaliana* (Fig. 3.1). One branch is the flavonoid biosynthesis pathway, in which chalcone synthase (CHS) plays an important role in the formation of flavonoid skeleton, and subsequently leads to biosynthesis of flavonoids, flavonols, and anthocyanins biosynthesis. The other branches beginning from *p*-coumaroyl CoA are crucial for the production of lignin monomers, essential precursors for lignin biosynthesis. Several structural genes such as CHS, chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and leucoanthocyanidin oxidase (LDOX) lead to the formation of the anthocyanidins (Borevitz et al. 2000; Winkel-Shirley 2001; Qi et al. 2011).

Table 3.1 Main groups of flavonoids, the individual compounds, and sources of food

Groups/flavonoids subclass	Dietary flavonoids/compound	Common food sources
Anthocyanin	Cyanidin	Berries
	Delphinidin	Cherries
	Malvidin	Grapes
	Pelargonidin	Raspberries
	Peonidin	Red grapes
	Petunidin	Red wine, tea, strawberries
Flavonols	Quercetin	Apple, lettuce, grape, broccoli, tea (black and green), bean, tomato
	Kaempferol	Broccoli, tea (black and green)
	Myricetin	Grape, lettuce, parsley, tea (black and green), coffee
	Isorhamnetin	Onion
	Rutin	Cranberries, rhizomes of *Selliguea feei*
Flavones	Apigenin	Apple skins, celery, lettuce
	Luteolin	Celery, Brussels sprout, beetroot
	Isovitexin (apigenin 6-*C*-glucoside)	
	Isoorientin	Pears, onion
Flavanones	Hesperetin	Oranges, beetroot, celery, cauliflower, spinach
	Naringenin	Oranges, grapefruit
	Eriodictyol	Lemon, celery, parsley, green tea
	Festin	Citrus fruit
	Taxifolin	
Flavan-3-ols	Catechin	Apple, red wine, tea (black and green), plum, grape, peach, nectarine
	Epicatechin	Apple, tea, pears, grape, tea (black an green), plum
	Epigallocatechin	Tea (black and green)
	Epicatechin-3-gallate	Tea (black and green)
	Epigallocatechin-3-gallate	Tea (black and green)

3.1 Phenylalanine Ammonia Lyase (EC 4.3.1.5)

PAL is the first key enzyme in the phenylpropanoid pathway and it catalyzes formation of transcinnamic acid by using L-phenylalanine (L-Phe), a common substrate for different phenylpropanoid derivatives. PAL exists at the branch point of phenylpropanoid derivative metabolism, and therefore PAL is considered to be one of the key enzymes for the formation of monolignols/lignin, sinapate esters, condensed tannins, anthocyanins, coumarins, benzoic acids, flavonoids/isoflavonoids, and stilbenes (Dixon and Paiva 1995). PAL has been characterized in a number of plants and in all of the studied plants, the PAL proteins are encoded by a multigene family. Four PAL genes have been described in *Arabidopsis* (Raes et al. 2003), five in pine

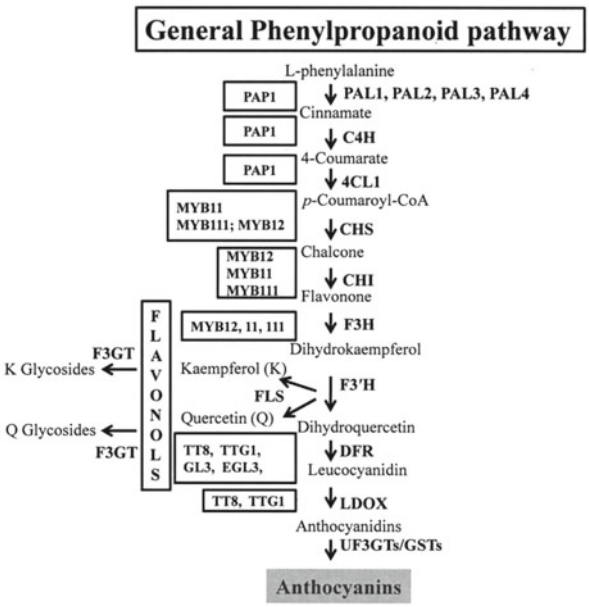

Fig. 3.1 The flavonoid biosynthesis pathway that leads to the production of anthocyanidins and flavonols in *Arabidopsis*; several transcription factors (MYB, bHLH, and WD-40) are indicated along the branches that are likely involved in the transcriptional regulation of the structural genes

(Butland et al. 1998), 16 in potato (Castillo Ruiz et al. 2005), five in tomato (Reichert et al. 2009), and four in *Fagopyrum* (Kim et al. 2011). These members are expressed in different developmental tissues in response to various environmental stimuli. For example, in *Arabidopsis*, expression of PAL1, PAL2, and PAL4 was found in stem, while PAL2 and PAL4 were expressed in seeds. PAL1 expression was found in vascular tissue (Raes et al. 2003; Rohde et al. 2004), while expression of PAL3 was found in stems (Mizutani et al. 1997; Raes et al. 2003). Isoforms of PAL1, PAL2, and PAL4 enzymes showed higher affinities for the substrate L-phenylalanine than PAL3 (Cochrane et al. 2004). In *Arabidopsis*, no phenotypic changes were observed in the mutant's *pal1* and *pal2*, however, expression of PAL1 was induced in the *pal2* mutant, whereas, PAL2 was induced in the *pal1* mutant, and by contrast, PAL4 is up-regulated in both mutants which suggests their involvement in lignification (Raes et al. 2003; Rohde et al. 2004; Huang et al. 2010). Induced expression of different PAL isoforms, including PAL1, PAL2, and PAL4 has been reported in response to different types of treatment such as sucrose, light, cold, temperature variations, gamma-ray irradiation, N and P deficiency (Solfanelli et al. 2006; Lillo et al. 2008; Olsen et al. 2008).

3.2 Cinnamate 4-Hydroxylase (EC 1.14.13.11)

Cinnamate 4-hydroxylase (C4H) is a second key enzyme in the phenylpropanoid pathway and catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid

including flavonoids, phytoalexins, lignin, pigments, and many defense molecules (Lu et al. 2006). C4H belongs to the CYP73 family, the large group of cytochrome P450 monooxygenases (P450s). It plays an important role in the biosynthesis of various compounds (e.g., fatty acids, phenylpropanoids, alkaloids, and terpenoids) and aids in the detoxification of herbicides and pesticides (Chapple 1998). C4H genes exist as a multigene family in various plant species, including *Populus tremuloides*, *P. trichocarpa* (Hotze et al. 1995; Lu et al. 2006), orange (Betz et al. 2001), pea (Whitbred and Schuler 2000) and periwinkle (Hotze et al. 1995). Only one C4H gene has been identified in *Arabidopsis* (Raes et al. 2003) and its expression was induced by wounding, fungal infection, light (Mizutani et al. 1997; Raes et al. 2003), nitrogen (N) and phosphorus (P) deficiency (Lillo et al. 2008). Expression studies showed that C4H is strongly and differentially expressed in *Populas* species (Lu et al. 2006), citrus (Betz et al. 2001), *Arabidopsis* roots (Mizutani et al. 1997; Park et al. 2010) and highly expressed in xylem cells during lignification (Ro et al. 2001; Lu et al. 2006).

3.3 4-Coumarate Coenzyme A Ligase (4CL: EC 6.2.1.12)

4-Coumaric acid: CoA ligase (4CL) plays an important role in the biosynthesis of lignin precursors such as hydroxycinnamate-CoA thio esters (Hamberger and Hahlbrock 2004). 4CL is expressed differentially and developmentally in different tissues and exists in multiple isoforms with different substrate specificities (Voo et al. 1995). In *Arabidopsis*, four 4CL isozymes (4CL1, 4CL2, 4CL3, and 4CL4) have been identified. 4CL1 and 4CL2 are known to be involved in the lignin biosynthesis, while 4CL3 participates in flavonoid and other nonlignin biosynthesis pathways (Ehlting et al. 1999; Cukovic et al. 2001). In *Populus trichocarpa*, 17 genes have been identified which showed sequence similarity with known 4CLs (Souza et al. 2008; Shi et al. 2010), however, *Populus tremuloides* 4CL1 was detected in developing tissues of xylem, whereas *Ptr*4CL2 could be involved in flavonoids biosynthesis (Hu et al. 1998). Five 4CL isoforms have been identified in rice and they are substrate-specific (Gui et al. 2011). Among them, 4CL3 showed the highest turnover rate and was the most abundantly expressed, followed by 4CL5 and 4CL1, whereas transcripts of 4CL2 was least expressed compared to other forms of 4CLs. Down-regulation of 4CL1 in *Arabidopsis* (Lee et al. 1997), 4CL1 in poplar (Hu et al. 1999; Sanchez et al. 2006; Voelker et al. 2010), and 4CL3 in rice (Gui et al. 2011) resulted in reduced lignin content.

3.4 Chalcone Synthase (EC 2.3.1.74)

CHS is the first committed enzyme in the flavonoid biosynthesis pathway and is responsible for the biosynthesis of various types of metabolites in different parts of

plant organs, such as in seeds, leaves, roots, trichomes, pods, and anthocyanins. CHS belongs to the plant polyketide synthase (PKS) family, including stilbene synthase (STS), acridone synthase, pyrone synthase, bibenzyl synthase, and *p-coumaroyl* triacetic acid synthase (Sanchez 2008). The monomeric size of these enzymes is of 42–45 kDa and these enzymes catalyze a series of reactions such as decarboxylation, condensation, and cyclization (Tropf et al. 1995) for the formation of flavonoids. Two structural domains have been found in CHS2 of alfalfa (Ferrer et al. 1999) and four amino acids (Cys164, Phe215, His303, and Asn336) are present in the upper domain of CHS. The lower domain of CHS is largely involved in chalcone formation (i.e., naringenin and resveratrol) (Jez et al. 2001a, b). The CHS proteins were detected in the cytosol and endoplasmic reticulum, however, not found in nuclei, plastids, mitochondria, golgi, or tonoplasts in buckwheat. Abundant amounts of CHS enzymes were accumulated parallely with the flavonoid products in the epidermal and cortex cells in *Arabidopsis* roots indicating that expression of CHS are organ-specific (Saslowsky and Winkel 2001). Multiple copies of the CHS gene have been detected in plants including morning glories (Durbin et al. 2000), Gerbera (Helariutta et al. 1996), leguminous plants (Ito et al. 1997) and *Cannabis sativa* (Sanchez 2008). A single copy of the gene has been found in *Arabidopsis*, parsley, and snapdragon. Transcripts of the CHS gene have been detected in flowers, and its expression could be induced by several agents such as light/UV, red, far-red, blue, and bacterial or fungal infection (Dao et al. 2011). A multiple CHS gene family is also found in pea and shows differential expression patterns in response to various external stimuli such as elicitor and UV irradiation (Ito et al. 1997). Expression of CHS was increased in response to N depletion, lower temperatures (Løvdal et al. 2010), P deficiency (Morcuende et al. 2007; Müller et al. 2007), silicon (Shetty et al. 2011), in virus-infected grape leaves (Gutha et al. 2010) and pathogen attack (Dao et al. 2011). Six CHS genes were identified in the "Tsuda" turnip and light-dependent expression patterns were observed in *Br*CHS1, 4, and 5, while the other three (*Br*CHS2, 3, and 6) did not respond to light (Wang et al. 2011a, b). They also observed that expression of *Br*CHS1, 4, and 5 was UV and light responsive and induced expression of these genes is in parallel with anthocyanin accumulation. Maximum expression of *Br*CHS4 was observed by blue plus UV-B co-irradiation, whereas lesser expression was detected by blue light (Wang et al. 2011a, b). Six isoforms of CHS genes (A, B, D, F, J, and H) have been identified in petunia, but only two genes (A and J) are flowering-specific. Induced expression of CHS short interfering RNAs (siRNAs) was also noted due to the reduced expression of CHS in petunia which resulted in the loss of flower pigmentation (De Paoli et al. 2009). In *Glycine max*, nine CHS genes have been identified and silencing of these CHS genes inhibited the flavonoid pathway in the seed coat (Tuteja et al. 2009). Loss of CHS activity affects pollen germination and pollen tubes growth in maize resulting in self-sterility and a mutation of CHS resulting in white anthers in petunia (Napoli et al. 1999). A role of calcium and calmodulin in controlling the UV-mediated induction of CHS expression has been proposed (Frohnmeyer et al. 1998).

3.5 Chalcone Isomerase (EC 5.5.1.6)

CHI is one of the most important intermediate enzymes in the flavonoid pathway and CHI substrates are synthesized by CHS. This step can occur spontaneously, CHI catalyzes it 10^7-fold more efficiently and therefore most plants do not accumulate chalcones and naringenin chalcone is rapidly isomerized to naringenin by CHI. Two types of CHI genes have been found in plants, type I and type II. Type I CHI catalyzes conversion of 6-hydrox-chalcone into (2*S*)-flavonoid or (2*S*)-5-desoxidation flavonoid. Type I CHI was found in most of plant species such as barley, rape, *Arabidopsis*, and rice (Druka et al. 2003). Type II CHI, which is mainly found in leguminous plants that act on both 6-hydrox-chalcone and 6-deoxidation-chalcone to convert into (2*S*)-flavonoid or (2*S*)-5-desoxidation flavonoid. The expression pattern of the CHI gene varies in plant species; its expression is tissue-specific and regulated developmentally (Muir et al. 2001). Overexpression of the CHI gene in plants led to increased production of flavonoids (Muir et al. 2001; Zhang et al. 2009; Park et al. 2011). In onion, mutation of the CHI gene resulted in high levels of chalcone accumulation and reduced amounts of flavonoid and ultimately generated a yellow corn (Kim et al. 2004). Decreased expression of CHI reduced the flavonoid accumulation in tobacco and maize (Nishihara et al. 2005; Bovy et al. 2002). CHI genes have been cloned from many plants including rice, barley (Druka et al. 2003), *Saussurea medusa* (Li et al. 2006), *Trigonella foenum-graecum* (Qin et al. 2011) and in peanut (Zhang et al. 2012).

3.6 Flavanone 3-Hydroxylase (EC 1.14.11.9)

F3H is one of the "core" enzymes that catalyzes the stereo-specific hydroxylation of (2*S*)-naringenin to form (2*R*, 3*R*)-dihydrokaempferol and therefore provides precursors for many classes of flavonoid compounds (Liu et al. 2002). Alterations of metabolic pathways have been noted depending upon the flow of precursors (Lo and Nicholson 1998; Liu et al. 2002). Several studies have shown that down-regulation of F3H in plant is accompanied by increased accumulation of isoflavonoids in soybean seeds (Yu et al. 2003). Seven copies of the F3H gene have been identified in wheat, barley, and rye (Khlestkina et al. 2011). F3H activity has been detected in young flower petals and its expression is associated with disease resistance in plant (Ardi et al. 1998; Cho et al. 2005; Giovanini et al. 2006). Activity of F3H was found to be higher in resistant cultivars than those of susceptible ones (Ardi et al. 1998). In Carnation cv. Eilot, antisense suppression of F3H reduced the levels of anthocyanin, while increased fragrant levels were detected in the RNAi plants than those of the control (Zuker et al. 2002).

3.7 Flavonoid-3′-O-Hydroxylase (EC 1.14.13.88)

Flavonoid-3′-*O*-hydroxylase (F3′H) is a cytochrome P450-dependent monooxygenase that requires NADPH as a cofactor and catalyses hydroxylation, which is an important structural feature in determining the color and stability of flavonoid compounds. The enzymatic activity of this enzyme was first demonstrated in microsomal preparations from cultured *Happloppapus gracilis* cells. F3′H was first isolated and characterized in petunia (Brugliera et al. 1999) and then from various plant species including *Arabidopsis* (Schoenbohm et al. 2000; Kitada et al. 2001). F3′H can act on a wide range of substrates: the flavonols, flavones, and flavanones, all of which are intermediates in the flavonoid biosynthetic pathway. It was shown that F3′H plays an important role in the flavonoid pathway branches leading to synthesis of flavonoids compounds in several plants including sorghum and maize (Boddu et al. 2004). F3′H catalyzes the conversion of naringenin to eriodictyol in maize, while in sorghum, the F3′H gene has been implicated in different subbranches of phlobaphene synthesis (Boddu et al. 2004). Sharma et al. (2011) have shown the role of F3′H1 in maize in the accumulation of dihydroquercetin. F3′5′H belongs to the CYP75 super family of P450 enzymes catalyze the hydroxylation of flavonoids (Winkel-Shirley 2001). F3′5′H genes have been isolated from several plants, including *Gentiana triflora*, *Eustoma grandiflorum*, *Eustoma rusellianum*, *Catharanthus roseus*, *Campanula medium*, *Vinca major*, and *Vitis vinifera*. The expression of F3′5′H has been detected in different parts of the grape (Bogs et al. 2006) and it plays an important roles in the accumulation of flavonoids in the berry skin (Castellarin et al. 2006). F3′5′H expression was also detected in *Dendrobium moniliforme* displaying various flower colors (Whang et al. 2011). The genes F3′H and F3′5′H mediate the addition of hydroxyl groups to the B ring of flavanones, flavones, dihydroflavonols, and flavonols, resulting in the formation of different colors (Kaltenbach et al. 1999). Bogs et al. (2006) reported that F3′H and F3′5′H influence the composition of the flavonoids affecting wine quality. Expression of F3′5′H has been shown to be controlled by a R2R3 MYB type transcription factor which induced the biosynthesis of anthocyanin in tomatoes (Butelli et al. 2008).

3.8 Flavonol Synthase (EC 1.14.11.23)

Flavonol synthase (FLS) is another key enzyme which plays crucial roles in the conversion of several precursors leading to different branches of the flavonoid biosynthesis. The biosynthesis of flavonols from dihydroflavonols is catalyzed by FLS, a soluble 2-oxoglutarate-dependent dioxygenase (2-ODD). It is a soluble enzyme that requires ascorbate for stabilization, 2-oxoglutarate as a cosubstrate, and Fe^{2+} as a cofactor (Turnbull et al. 2004). The first FLS gene was characterized in parsley cells and its activity has been detected in the extracts of a variety of plants, such as citrus, *Matthiola incana*, *Petunia hybrid*, *Dianthus caryophyllus*, and *Arabidopsis*

(Moriguchi et al. 2002; Preuß et al. 2009). FLS is encoded by a multicopy gene in plants, and is expressed in different parts of plant organs and varies from species to species (Pelletier et al. 1999; Preuß et al. 2009; Ferreyra et al. 2010; Kim et al. 2010a, b; Wellmann et al. 2002; Gupta et al. 2011). In *Arabidopsis*, besides, FLS1 (At5g08640), five more putative FLS genes (FLS2-FLS6) have been identified (Stracke et al. 2009). Expression of FLS1 has been shown to be induced by white light (Pelletier et al. 1997; Downey et al. 2004; Fujita et al. 2006), UV-B (Ferreyra et al. 2010), sugar (Weiss 2000; Gollop et al. 2002). FLS catalyzes the oxidation of dihydroflavonols to flavonols and competes at a crucial branch point with DFR acts on the common substrate (i.e., dihydroflavonols) in the anthocyanin pathway. Both enzymes FLS and DFR catalyze reactions and depending on the availability of precursors, increase the accumulation of flavonols and anthocyanidins, respectively (Fig. 3.1). Accumulation of anthocyanins has been detected in flowers of transgenic antisense FLS of petunia and tobacco (Nielsen et al. 2002; Davies et al. 2003; Nakatsuka et al. 2007). The induced expression of FLS by UV-B irradiation increased the accumulation of flavonols in soybean (Kim et al. 2008) while a mutation in the FLS gene was found to change the flower color from purple to magenta (Takahashi et al. 2007). Expression of FLS was also induced by various light intensities, pathogen infection and herbivore attack (Mellway et al. 2009; Ferreyra et al. 2010; Owens et al. 2008). Expression of FLS1 was found in every organ of *Fagopyrum tataricum*, and was expressed abundantly in leaves and flowers, moderately in stems, and to a lesser extents in flower buds and immature seeds (Li et al. 2012). In *Acacia*, maximum expression of FLS was found in flowers and it was found to be expressed in the leaves, branches, bark, and sapwood (Toh et al. 2013). In *Arabidopsis*, FLS1 was induced by white light, resulting in the accumulation of flavonols (Pelletier et al. 1997). Maximum expression of FLS was found in citrus leaves during the early developmental stage and increased in the peel during fruit maturation (Moriguchi et al. 2002). FLS1 expressions have been shown to be under the control of flavonol-specific transcription factors (TFs) MYB11, MYB12, and MYB111 in *Arabidopsis* (Stracke et al. 2007; Owens et al. 2008) and these TFs caused different spatial accumulation of specific flavonol derivatives in leaves, stems, inflorescences, siliques, and roots (Stracke et al. 2010). Overexpression of *Arabidopsis* MYB12 in tobacco increased flavonoid accumulation (Misra et al. 2010). In *Zea mays*, anthocyanin (C1/PL1 + R/B) and 3-deoxy flavonoid (P1) TFs influenced the expression of FLS1 resulting in higher accumulation of anthocyanin (Ferreyra et al. 2010).

3.9 Dihydroflavonol 4-Reductase (EC 1.1.1.219)

DFR diverts the substrate from flavonols formation to the anthocyanin and proanthocyanidin pathway. DFR which uses NADPH as a cofactor to reduce the precursor's dihydroflavonols can be used for anthocyanin and proanthocyanidin biosynthesis (Holton and Cornish 1995; Xie et al. 2004; Zhang et al. 2008). DFR

can accept wide range of substrate and it has been shown that substrate specificity of the DFR varied depending on the types of anthocyanins accumulated in each plant species. In *Zea mays*, dihydroquercetin is preferred for the DFR reaction. In some plants, DFR accepts dihydroflavonols as substrates, but it also reduces flavanone as flavanone 4-reductase to produce 3-deoxyanthocyanidin (Forkmann and Martens 2001; Martens et al. 2002; Fischer et al. 2003). In *Petunia hybrida* and *Cymbidium hybrida*, DFR is not able to efficiently convert DHK to leucopelargonidin, the precursor of pelargonidin-based anthocyanins (Johnson et al. 2001; Tanaka et al. 2005). DFR genes have been isolated and characterized in plants such as *Triticum aestivum* (Himi and Noda 2004), *Vitis vinifera* (Zhang et al. 2008), *Populus trichocarpa* (Huang et al. 2012), and *Ascocenda* spp. (Kunu et al. 2012). DFR exist as a single and multicopy gene in several plant species (Piero et al. 2006; Zhang et al. 2008), a single gene has been found in *Arabidopsis*, grape, tomato, rice, snapdragon, rose, barley, and buckwheat (Holton and Cornish 1995; Tanaka et al. 1995; Chen et al. 1998; Li et al. 2012), while multi copy gene have been found in *Vitis vinifera*, *Ipomoea purpurea*, *P. hybrid*, lotus, and *M. truncatula* (Inagaki et al. 1999; Xie et al. 2004; Shimada et al. 2005; Fujita et al. 2006). Increased flower pigmentation has been observed by transformation of petunia with a heterologous DFR (Tanaka et al. 1995). Expressions of DFR have been shown to be spatially and developmentally regulated, organ-specific, and induced the accumulation of anthocyanin in different plant tissues (Tanaka et al. 1995; Rosati et al. 1997; Farzad et al. 2003; Zhang et al. 2008). There are some factors which modulate the expression of the DFR including light (Hughes et al. 2005; Shahidul et al. 2005; Lightbourn et al. 2007), UV treatment (Himi and Noda 2004), sucrose (Solfanelli et al. 2006) and jasmonic acid (Shan et al. 2009). Expression of DFR has been observed in different parts of organ in several plants. In *Bromheadia finlaysoniana*, the DFR gene was expressed in all purple colored tissues including sepal, petal, column, and lip (Liew et al. 1998). In *Rosa hybrida*, the expression of DFR was found in petals, sepals, thorns, styles, and stamens (Tanaka et al. 1995). In *Foesythia* × *intermedia*, the accumulation level of DFR transcripts is mostly abundant in petals, sepals and it is absent in anthers (Rosati et al. 1997).

3.10 Leucoanthocyanidin Dioxygenase (LDOX: 1.14.11.19)

Leucoanthocyanidin dioxygenase (LDOX), also called 2-oxoglutarate iron-dependent dioxygenase (2-ODD) or anthocyanidin synthase (ANS), is involved in anthocyanin biosynthesis and catalyses the conversion of colorless leucoanthocyanidin to colored anthocyanidin (Abrahams et al. 2003; Lepiniec et al. 2006; Shikazono et al. 2003). Expression of the LDOX gene has been detected in different organs of Shiraz grapevine, such as leaves, roots, seeds, flowers, berry skin, and flesh (Boss et al. 1996a, b). Expression of LDOX was shown to be induced by light and sucrose (Gollop et al. 2001; Solfanelli et al. 2006), 6-benzylaminopurine (6-BA) in *coi1-1* mutant (Shan et al. 2009) and in *coi1-2 pap1-D* (Qi et al. 2011) plant. An

LDOX cDNA has been cloned from *Arabidopsis* and *transparent testa18* (Xie et al. 2003) and *transparent testa19* (Winkel-Shirley 2001), both being *ldox* mutants. In developing *Vitis vinifera* grapes, the expression of LDOX mRNA was noted before and after the ripening stage (Boss et al. 1996a, b).

4 Modification of Metabolites

Flavonoids are generally having a C6–C3–C6 skeleton structure that is very labile and can undergo several modifications resulting in a variety of chemical constituents (Hichri et al. 2011). Oxidation of the compounds in the central C heterocycle plays a major role for the determination of specific type of compounds including substitutions of the hydroxyl, methyl group on the A and B rings, additional modifications such as glycosylation (glucose, galactose, arabinose, and rhamnose), acylation (coumaric and caffeic acids), and polymerization (Sumner et al. 2003; Yonekura-Sakakibara et al. 2008). Such variation further increase from the nature of the sugar(s) attached to the compounds. The addition and substitutions of sugars are catalyzed by various enzymes such as glycosyltransferases, methyltransferases, and acyltransferases, which provide support to the compound and increase the structural stability of compounds such as anthocyanins (Winefield 2002).

4.1 Glycosylation

Glycosylation is one of the key modification processes required to produce a variety of flavonoid structures and colors (Gachon et al. 2005). In nature, glycoconjugates are formed by UDP-dependent glycosyltransferases (UGTs), a group of enzymes are encoded by a large multigene family in the plant kingdom (Mackenzie et al. 1997; Li et al. 2001). Glycosyltransferase family 1 comprises over 107 members in *Arabidopsis* (Ross et al. 2001) and approximately 150 members in *Medicago truncatula* (Modolo et al. 2007). Evidences indicate that a number of GTs varies among plant species. For example, 456 GTs had been identified in *Arabidopsis thaliana*, 570 in *Oryza sativa* ssp. *japonica*, 226 in *Homo sapiens*, 265 in *Caenorhabditis elegans*, and 149 in *Drosophila melanogaster* (Yonekura-Sakakibara and Hanada 2011). The diversity of GT in relation to the protein coding genes shows similarity across a wide range of organisms (Lairson et al. 2008); however, the GTs that are found in each organism differ within them and expression of these genes differs from each other reflecting their wide diversity in plant kingdom. In *A. thaliana*, more than 25 % of GTs belongs to the GT1 family, similarly in *O. sativa* more than 35 % of GTs are in the GT1 family, however, several other GTs families such as GT2, GT8, GT31, and GT47 comprise only 6–9 % of the genes. Four classes of UGTs have been found in the plant kingdom (Paquette et al. 2003; Morita et al. 2005; Sawada et al. 2005; Nagashima et al. 2004; Ogata et al. 2004; Tohge et al.

2005; Noguchi et al. 2008) and they are generally involved in glycosylation of natural plant products (Vogt and Jones 2000; Dangl and Jones 2001). However, substrate specificities of the large number of UGTs remain unidentified and these isoforms thus remain orphan glycosyltransferases. The most common sugar is glucose, but fructose, xylose, arabinose, rhamnose, galactose, as well as sophorose (2-*O*-b-D-XYLOSYL-D-glucose), gentobiose (6-*O*-b-D-glucosyl-D-glucose), rutinose (6-*O*-a-L-RHAMNOSYL-D-glucose), sambubiose (2-*O*-b-D-xylo-syl-D-glucose), xylosyl rutinose, and glycosyl rutinose may also be present (Clifford 2000; Shahidi and Naczk 1995). Such glycosylation contributes to the increased stability of anthocyanins, influences their color variation (Morita et al. 2005) and taste perception (Frydman et al. 2004). Flavonoids are usually glycosylated at their 3′ O and 7′ O positions in *Arabidopsis* (Veit and Pauli 1999; Bloor and Abrahams 2002; Jones et al. 2003; Tohge et al. 2005; Yonekura-Sakakibara et al. 2007, 2008). The 3′-O-glycosylation is considered to be the first step of conjugation followed by 7-O-glycosylation.

Several UGTs are involved in the glycosylation of flavonoids, among them the function of UGT78D1, UGT78D2, UGT78D3, UGT73C6, and UGT89C1 has been characterized (Jones et al. 2003; Tohge et al. 2005; Yonekura-Sakakibara et al. 2007, 2008). UGT75C1 is involved in 5-O-glycosylation, whereas three closely related homologous UGTs (UGT78D1, UGT78D2, and UGT78D3) are responsible for 3-O-glycosylation in *Arabidopsis* (Jones et al. 2003; Tohge et al. 2005). UGT73C6 contributes to 7-O-glucosylation in leaves, but only minute amounts of flavonols are 7-O-glucosylated. Instead, UGT89C1 plays an important role for 7-O-rhamnosylation is the major form of 7-O-conjugation (Yonekura-Sakakibara et al. 2007). UGT71C1 was found to be responsible for increased resistance to oxidative stress (Lim et al. 2005), UGT75C1 (At4g14090) has been described for 5-O glycosylation of anthocyanin (Tohge et al. 2005), while UGT84A2 plays an important role in the biosynthesis of sinapoyl malate (Sinlapadech et al. 2007). UGT72E2 plays an important role in monolignol-4-glycosylation (Lim et al. 2005; Lanot et al. 2006). Microarray studies have shown up-regulation of UGT73B3, UGT73B4, and UGT73B5 while UGT73C2 and UGT73C5 were down-regulated in the *sur2* mutant (Morant et al. 2010). Induced expressions of different isoforms of UGT73B were noted by abiotic stress such as oxidative stress, wounding, UV light and upon infection by *Pseudomonas syringae* (Morant et al. 2010). Similarly, Menadione treatment induced expression of UGT73B3, UGT73B4, and UGT73B5 in roots of *Arabidopsis* (Lehmann et al. 2009). Mutants of *ugt*73b3 and *ugt*73b5 showed reduced resistance to *P. syringae* (Langlois-Meurinne et al. 2005) indicating that glucosylation plays an important role in plant defense. Twofold-induced expression of UGT78D2 was observed in N and P depletion (Scheible et al. 2004; Morcuende et al. 2007) and a strong induction was noted by sucrose (Solfanelli et al. 2006).

GTs are also involved in the detoxification and compartmentation of endogenous compounds, xenobiotics, and detoxification of 2,4,6-trinitrotoluene TNT (Gandia-Herrero et al. 2008). Kim et al. (2010a, b) have shown that UGT73B2 plays a crucial role in glycosylation of flavonoids and modulate the response of plants to oxidative stress. Saint Paul et al. (2011) has shown that UGT76B1 conjugates isoleucic acid and plays an important role in plant defense and senescence in *Arabidopsis*.

Microarray studies have shown that the expression of UGT76B1 was induced by salicylic acid, methyl jasmonate (Zimmermann et al. 2005), wounding and is expressed constitutively in hydathodes and young tissues (Saint Paul et al. 2011), which provides resistance against the attack of herbivores or necrotrophs (Hugouvieux et al. 1998; Sprague et al. 2007). Overexpression of *Populus* UGT (*Pt*UGT72B1) in *Arabidopsis* displayed the highest trichlorophenol (TCP) conjugation which provides TCP resistance and degradation of TCP (Su et al. 2012) including 2,4,5-TCP by O-glucosylation (Brazier-Hicks and Edwards 2005; Messner et al. 2003; Brazier-Hicks et al. 2007).

4.2 Rhamnosylation

Rhamnosylation is an another important process of flavonols glycosylation in *Arabidopsis* and about 21 rhamnosylated compounds are detected in *Arabidopsis* (Yonekura-Sakakibara et al. 2008). Three UDP-rhamnose synthase genes (RHM1, RHM2, and RHM3) are present in *Arabidopsis*, have been shown to be involved in rhamnose-specific flavonol synthesis (Yonekura-Sakakibara et al. 2008). For example, it was shown that the RHM2/MUM4 plays an important role in the synthesis of pectinaceous rhamnogalacturonanI (Usadel et al. 2004; Western et al. 2004). Overexpression of the *At*RHM1 gene in *Arabidopsis* resulted in an increase of rhamnose content by as much as 40 % in the leaf cell wall compared to the wild type (Wang et al. 2009).

4.3 Acylation of Anthocyanins

Acylation is one of the most common modifications of plant secondary metabolites, in which aromatic and/or aliphatic groups are added to the resulting compounds and leads to an increased diversification of anthocyanins. About 65 % of characterized anthocyanins are acylated (Andersen and Jordheim 2006) and are catalyzed by acyltransferase enzymes such as At1g03495, At1g03940, and At3g29590. Generally, two types of acylation have been noted. Due to their structural diversity, they are named as aromatic acylation and/or aliphatic acylation. In the aromatic type, compounds are associated with hydroxycinnamoyl groups, such as *p*-coumaryl, caffeyl, feruryl, and sinapyl groups. In the aliphatic type, they are associated with malonyl, succinyl, acetyl, oxalyl, malyl, and tartaryl groups, among which the malonyl group is the most abundant. D'Auria et al. (2007) found that At3g29590 encodes the acyltransferase responsible for the synthesis of the major malonylated anthocyanidins in *Arabidopsis*. Malonylation of glycosides containing anthocyanidins might play a major role in increasing metabolite stability and solubility, which protect the compounds from the attack of enzyme known as glycosidase cleavage and compartmentation of organic compounds, either in the vacuole or the cell wall (Day and Saunders 2004; Dhaubhadel et al. 2008). Induced expression of these enzymes has

been observed in plants (Tohge et al. 2005; D'Auria et al. 2007; Luo et al. 2007). It has been reported that acylated anthocyanins increase the stability of color and resistance to discoloration (Cheynier et al. 2006). For example, in *Vitis vinifera*, five anthocyanidins have been found such as cyanidin, delphinidin, and its methylated derivatives, peonidin, petunidin, and malvidin. During berry development in the Norton cultivar, the accumulation of anthocyanin begins at véraison and the anthocyanin content increases until ripening (Ali et al. 2011).

5 Transcriptional Regulation

Flavonoid pathway is regulated by a class of transcription factors (TFs) belonging to the R_2R_3MYB family in plants including *Arabidopsis* (Mol and Koes 1998; Bailey et al. 2003; Heim et al. 2003). These R_2R_3MYB TFs act in a complex and coordinated manner which consists of MYB, bHLH, and WD40 repeat proteins (Broun 2005). The first group includes MYB-related TFs, such as production of anthocyanin pigments1 (PAP1), PAP2, MYB113, MYB114, MYB11, MYB111, and MYB12, controlling the "early" steps of flavonoid pathway genes. Overexpression of any one of these TFs results in increased accumulation of anthocyanin (Borevitz et al. 2000; Gonzalez et al. 2008). Among them, MYB12 has been identified as a regulator of flavonols synthesis in *Arabidopsis* (Stracke et al. 2007). Seedlings of the triple mutant of *myb11 myb12 myb111* do not produce flavonols, while no change was found in the accumulation of anthocyanins. Most of the genes in the flavonoid pathways were down-regulated in the *myb11 myb12 myb111* triple knockout mutant (Stracke et al. 2007). The second group named as bHLH factors includes transparent testa8 (TT8), glabrous3 (GL3), and enhancer of glabra3 (EGL3), controlling the "late" flavonoid pathway genes including DFR, UF3GT, and LDOX in *Arabidopsis* (Stracke et al. 2007). MYB and bHLH proteins interact with the WD-40 repeat containing protein (Transparent testa glabrous1, TTG1) to form a transcriptional complex that activates anthocyanin biosynthetic pathway genes, including ANS, DFR, F3′H, and LDOX (Tohge et al. 2005; Gonzalez et al. 2008). GL3, EGL3, and TT8 are three homologs of bHLH that interact with TTG1, and were found to be associated in a transcriptional complex and involved in the regulation of anthocyanidins biosynthesis and mucilage synthesis in *Arabidopsis* (Usadel et al. 2004; Western et al. 2004; Gonzalez et al. 2008). Transcripts of GL3 transcripts were increased sixfold in response to N depletion in leaves of *Arabidopsis* (Lillo et al. 2008). TT8, when associated with PAP1 or PAP2, is involved in the control of flavonoids pigmentation and plays a key role in regulating DFR (Zimmermann et al. 2004). Induced expression of TT8 results in higher anthocyanidins accumulation in the leaves of *Arabidopsis* (Zimmermann et al. 2004). Recently, Maier et al. (2013) demonstrated that when PAP1 and PAP2 are associated with CONSTITUTIVELY PHOTOMORPHOGENIC1/SUPPRESSOR OF PHYA-105 (COP1/SPA) complex, an anthocyanin accumulation is induced. MYBL2, an R3-MYB-related protein and lateral organ boundary domain (LBD) gene family, LBD37, LBD38, and LBD39

have recently been identified as negative regulators of anthocyanin biosynthesis (Dubos et al. 2008; Matsui et al. 2008; Rubin et al. 2009). Among them, PAP1 is a master regulator of the anthocyanin synthesis pathway (Borevitz et al. 2000; Tohge et al. 2005). The regulation of PAP1 is highly controlled by environmental factors, however, the *pap1-D Arabidopsis* plants overexpress PAP1 and produced higher amounts of anthocyanin in most of the tissues (Borevitz et al. 2000); when growth conditions were changed, anthocyanin levels and composition were dramatically altered in leaves of *pap1-D* plants (Tohge et al. 2005). Similarly, overexpression of the PAP1 gene induced the expression of PAL1 and several other anthocyanin pathway genes increased the anthocyanin accumulation (Tohge et al. 2005; Lillo et al. 2008). There are several studies that have examined factors influencing the regulation of "early" and "late" flavonoid pathway genes. For example, N deficiency induced the expression of MYB12 and bHLH TFs in *Arabidopsis* resulting in the production of anthocyanin and flavonols (Lea et al. 2007). These authors found that MYB type TFs especially PAP1 appeared to be partnered with the bHLH and GL3 triggering anthocyanin accumulation. The availability of N and P was also shown to influence the transcription levels of PAP1 and PAP2 (Scheible et al. 2004; Morcuende et al. 2007) which were then shown to quickly decrease after NO_3^- addition to N-depleted *Arabidopsis* seedlings (Scheible et al. 2004). Nitrogen deficiency resulted in an increase in MYB12, bHLH, activation of acyl-coenzyme A: diacylglycerol acyltransferase1 (DGAT1) and triacylglycerol accumulation in *Arabidopsis* seedlings (Lea et al. 2007; Yang et al. 2011a, b). However, nitrogen deficiency is associated with decreased leaf area, chlorophyll content, and photosynthesis resulting in lower dry matter accumulation in plants (Zhao et al. 2005), affects the properties of thylakoid membranes (Malavolta et al. 2004), causes severe changes in carbon, nitrogen, and amino acids and proteins metabolism (Wang et al. 2003; Scheible et al. 2004). The roles of MYB TFs in controlling the flavonoid biosynthesis genes have been studied widely in grapes. These are MYBA1 and MYBA2 genes (Kobayashi et al. 2004; Lijavetzky et al. 2006; Walker et al. 2006, 2007; This et al. 2007), which regulate the last biosynthetic step of anthocyanin synthesis, a glycosylation reaction mediated by the UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT) enzyme (Kobayashi et al. 2002). MYB5a (Deluc et al. 2006), MYB5b (Deluc et al. 2008), MYBPA1 (Bogs et al. 2007), and MYBPA2 (Terrier et al. 2009) appear to regulate general branches of the pathway.

6 Functions

6.1 Radical Scavenging Power

Adverse conditions can lead to production of ROS in plants. ROS includes free radicals such as superoxide anion ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), and non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Free radicals are

molecules, usually of oxygen, by losing an electron. Free radicals induce oxidative stress in various cell components leading to certain diseases. Plant-derived flavonoids have become an alternative source of antioxidants to prevent oxidative stress in cells and can protect cell from injury by reacting with the free radicals in various ways. One of them is the direct reaction of flavonoids with free radicals by which flavonoids are oxidized by radicals. As a consequence more stable and less reactive radicals are formed indicating that flavonoids scavenge ROS (Agati et al. 2013). Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical, according to the following reaction (Pietta 2000).

$$FOH + \mathrm{R}' \rightarrow \mathrm{FO}' + RH$$

where FOH is flavonoid, R′ is free radical, and FO′ is less reactive free radicals.

6.2 Antioxidative Effects

One of the most important properties of every group of flavonoid is their capacity to act as antioxidants. Biochemical studies demonstrated that catechins, particularly epigallocatechin-3-gallate (EGCg), help to prevent oxidation of low-density lipoprotein (Miyazawa 2000). The flavones and catechins have been thought to be the most powerful flavonoids for protecting the body against ROS (Koga and Meydani 2001). Cells are continuously attacked by ROS and free radicals, which increase lipid peroxidation resulting in cellular membrane damage. Such damage disturbs the normal physiology of cells, including osmotic pressure, damage nucleic acid (RNA and DNA) and eventually cell death. ROS and free radicals can attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage. Flavonoids have the ability to scavenge ROS in a wide range of biological systems which has resulted in suggestions that they may also have a role as dietary antioxidants which benefit health (Pietta 2000; McPhail et al. 2003; Benmalek et al. 2013).

6.3 Antiviral Activity of Flavonoids

Antiviral activities of various flavonoids have been reported against some viruses including human cytomegalovirus (HCMV), HSV-1, HSV-2, and some types of human adenoviruses (Chiang et al. 2003; Lyu et al. 2005; Evers et al. 2005). There are some other reports indicating that flavonoids are responsible for inhibiting the human immunodeficiency virus (HIV). Flavonoids (2-styrylchromones) are considered to be a new class of antirhinovirus flavonoids with activity against both rhinovirus groups A and B (Desideri et al. 2000, 2003).

6.4 Neuroprotective Properties of Flavonoids

Several studies have reported that flavonoids such as quercetin aglycone, quercetin-3-O-rutinoside, or quercetin-3-*O*-galactoside from plants, e.g., *Fagopyrum esculentum*, *Abelmoschus manihot* inhibit brain damage and neurological deficits in rodents following cerebral ischemia (Chen et al. 2007; Khan et al. 2009; Lapi et al. 2012; Lee et al. 2011; Keddy et al. 2012). Epigallocatechin has been shown to be neuroprotective (Kang et al. 2010) and prevents aging-related oxidative injury in the brain (Li et al. 2010), including epigallocatechin gallate (EGCG) (Weinreb et al. 2009; Mandel et al. 2008; Nath et al. 2012). Catechin has also been shown to improve blood flow by causing cerebral vasodilatation and also acts as a neuroprotective agent (Drouin et al. 2011; Nath et al. 2012).

6.5 Anti-inflammatory Properties of Flavonoids

Flavonoids have been shown to have anti-inflammatory properties such as inhibition of pro-inflammatory enzymes (cyclooxygenase-2, lipoxygenase and inducible NO synthase, inhibition of NF-κB and activating protein-1), activation of antioxidative defense mechanism, mitogen-activated protein kinase (MAPK), protein kinase C, and nuclear factor erythroid 2-related factor 2 (Middleton et al. 2000; Yoon and Baek 2005; Santangelo et al. 2007).

7 Conclusions and Future Perspectives

This chapter describes the importance of flavonoids and their biosynthesis in plants. A series of structural genes and transcriptional factors controlling the flavonoids biosynthesis in plants have been discussed. Flavonoids are gaining interest due to their various pharmacological activities, dietary intake, and antioxidant activity which lead to its beneficial effects in majority of the human diseases, such as anti-inflammatory, anticancer, hepatoprotective, antidiarrheal, and for its antimicrobial properties. Tremendous progress has been made with respect to the functional analysis of specific gene responsible for flavonoid biosynthesis. Despite advancements, it is still unclear how these structural genes and transcriptional factors orchestrate rapid, coordinated induction of phenylpropanoid defenses in response to microbial attack. Further research is also needed to understand the mechanism of bioactivity of different flavonoid compounds and signaling events leading to enhanced tolerance of pathogen attack in relation to metabolites biosynthesis at the molecular level and this will be an important field of future research.

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Chapter 4
Major Phytohormones Under Abiotic Stress

Iwona Morkunas, Van Chung Mai, Agnieszka Waśkiewicz, Magda Formela, and Piotr Goliński

1 Introduction

"Stress" in plants can be defined as any external factor that negatively influences plant growth, productivity, reproductive capacity or survival (Rhodes and Nadolska-Orczyk 2002). The abiotic environmental stress factors which most commonly influence plant performance include deficiencies or excesses of water (drought and flooding), excessively low or high temperature, deficiencies or excesses of several nutrients, high salinity, or extremes of irradiance (Waskiewicz et al. 2013b). Abiotic stresses may also include mechanical stresses (e.g., wounding), and stresses associated with toxic, manmade chemicals, including gaseous pollutants (e.g., ozone), heavy metals and herbicides.

Under abiotic stress, plants integrate multiple external stress cues to bring responses and establish a mechanism to mitigate the stress by triggering a cascade of events leading to enhanced tolerance. Responses to stress are complicated, integrated circuits involving multiple pathways and specific cellular compartments, and the interaction of additional cofactors and/or signaling molecules coordinates a

I. Morkunas • M. Formela
Department of Plant Physiology, Poznań University of Life Sciences, Wołyńska 35, 60-637 Poznań, Poland

V.C. Mai
Department of Plant Physiology, Poznań University of Life Sciences, Wołyńska 35, 60-637 Poznań, Poland

Department of Plant Physiology, Vinh University, Le Duan 182, Vinh, Vietnam

A. Waśkiewicz • P. Goliński (✉)
Department of Chemistry, Poznań University of Life Sciences, Wojska Polskiego 75, 60-625 Poznań, Poland
e-mail: piotrg@up.poznan.pl

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specific response to a given stimulus (Dombrowski 2003). A stress signal is first perceived by the receptors present on the membrane of the plant cells (Tuteja and Sopory 2008). The signal information is then transduced downstream, resulting in the activation of various stress-responsive genes. The products of these stress-inducible genes ultimately lead to a plant response or tolerance to stress, and help the plant to withdraw and survive the unfavorable conditions (Gilroy and Trewavas 2001). Often, under natural conditions, many stress factors occur simultaneously or sequentially. Moreover, as reported by PłaŻek and Żur (2003), plants exposed to one stress may become more tolerant to another. This phenomenon, called cross-tolerance, has been known for many years (Itai et al. 1973). Tolerance is associated with minimizing the negative effects of stress, i.e., maintenance of development processes at the same level despite the impact of the stress factor.

Abiotic stress conditions lead to biosynthesis of signaling molecule(s), including phytohormones, which have important functions as primary messengers in signal transduction, regulating cell metabolism. Therefore, these participate in the regulation of numerous genes, which leads to a specific metabolic effect. Phytohormones are endogenous bioactive substances formed in plants, which are critical for plant growth and development. Auxins (IAA), gibberellins (GA), cytokinins (CKs), abscisic acid (ABA), and ethylene (ET) were for long time recognized as the five major classes of hormones in plants. Recently, jasmonic acid (JA), salicylic acid (SA), and brassinosteroids (BRs), which function in plant metabolism and stress responses, have also been classified as phytohormones. Additionally, new classes of plant growth regulators are emerging, such as polyamines (PAs). Phytohormones are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses (Tuteja and Sopory 2008; Santner and Estelle 2009). The adaptive responses imposed by salt, cold, heat, drought, oxidative stress, heavy metals, and wounding are mainly controlled by phytohormones at extremely low concentrations via signaling pathways. Substantial progress has been made in understanding individual aspects of phytohormone perception, signal transduction, and homeostasis. They often rapidly alter gene expression by inducing or preventing the degradation of transcriptional regulators (Santner and Estelle 2010).

Significant progress has been made in identifying the key components of ABA in regulation of many processes in plants, including abiotic stress tolerance (Hubbard et al. 2010), and the role of SA, JA, and ET in plant responses to biotic stresses (Bari and Jones 2009). Recent studies indicate that IAA, GA, CK, BRs, and PAs are also implicated in plant defense signaling pathways. However, the physiological, biochemical, and molecular mechanisms induced by phytohormones through which plants integrate adaptive responses under abiotic stress are largely unknown. This section updates the current knowledge (since the year 2000) on the mechanisms responsible for the perception and signal transduction of phytohormones, and also provides a further understanding of the complexity of signal cross-talk and controlling downstream abiotic stress responses.

2 Phytohormones in Plant Response to Abiotic Stresses

2.1 Signaling Modules in Abiotic Stress Responses

2.1.1 Stress Signaling Perception

Plants perceive environmental signals via specific receptors, which then trigger a cascade of events leading to modification of cellular or metabolic activity, including regulation of the expression of specific genes. Ion channels, intracellular signaling proteins, and second messengers play a key role in these signal transduction cascades (Rhodes and Nadolska-Orczyk 2002). Receptor-coupled protein phosphorylation is a common form of signal initiation (Xiong and Zhu 2001).

The external stress signal is perceived at the membrane level by the membrane receptors, including receptor-like kinase (RLK) or histidine kinase (HK) (Tuteja 2007). Some RLKs have been implicated in abiotic stress responses. One examples of an RLK receptor is *NtC7* in tobacco; this putative membrane-localized receptor-like protein may play an important role in osmotic stress tolerance (Tamura et al. 2003).

The two-component HK was initially found in plants due to its role in perception of various environmental signals. When the extracellular sensor domain perceives a signal, the cytoplasmic histidine residue is autophosphorylated and the phosphoryl moiety is then passed to an aspartate receiver in a response regulator, which may constitute part of the sensor protein or a separate protein. These two-component sensors may couple with a downstream mitogen-activated protein kinase (MAPK) cascade or directly phosphorylate specific targets to initiate cellular responses (Xiong and Zhu 2001). They have also been implicated to function in the perception of environmental stress signals such as low temperature and osmotic stress in plants.

2.1.2 Stress Signaling Transduction

Ca^{2+}-Releasing

Early events in the response of plant cells to many environmental stimuli are known to involve membrane depolarization and elevations of cytosolic Ca^{2+}. Membrane fluidity and reorganization of the cytoskeleton are essential for cold-induced cytosolic Ca^{2+} oscillations in alfalfa and *Brassica* (Orvar et al. 2000; Sangwan et al. 2001). The cyclic ADP-ribose-gated (cADPR-gated) Ca^{2+} channels are involved in ABA-induced expression of cold-regulated genes of *B. napus* (Sangwan et al. 2001). Inositol 1,4,5-trisphosphate (IP_3)-gated Ca^{2+} channels have been implicated in dehydration and salt stress-induced cytosolic Ca^{2+} elevations (Takahashi et al. 2001). Calcium may in turn regulate a host of enzyme activities via calcium-dependent protein kinases and calmodulin. The changes in the cytoplasmic calcium concentrations lead to perception of stresses such as cold, heat, water stress, and salinity stress

(Tuteja and Sopory 2008). Recently, using various activators and inhibitors, it was found that calcium may be involved even in long-term processing of signals in plants in response to abiotic stresses (Verdus et al. 2007).

The stress signal then transduces inside the nucleus to induce multiple stress-responsive genes, the products of which ultimately lead to plant adaptation to stress tolerance directly or indirectly (Mahajan and Tuteja 2005). Overall, the stress response could be a coordinated action of many genes, which may cross-talk with each other (Tuteja 2007). The stress-induced gene products are also involved in the generation of regulatory molecules such as phytohormones ABA, ET, SA, JA, etc., which can initiate the second round of signaling.

Protein Kinases and Phosphatases in Intracellular Signaling Transduction

Upon receiving a signal from membrane receptors, cells often utilize multiple phosphoprotein cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are perhaps the most common intracellular signaling modes (Xiong and Zhu 2001). They regulate a wide range of cellular processes such as enzyme activation, protein localization and degradation. In plants, many protein kinases and phosphatases are thought to be involved in that function.

Most plant protein kinases are serine/threonine kinases that play major roles in protein phosphorelay. An ABA-activated protein kinase (AAPK), homologous to serine/threonine protein kinase from *Vicia faba*, was found to be specifically expressed in guard cells (Li et al. 2000). AAPK blocked ABA-induced stomatal closure by eliminating ABA activation of plasma membrane anion channels.

In a stress signaling cascade, inactivation of phosphoproteins is usually accomplished by dephosphorylation (Xiong and Zhu 2001). Among four major subgroups of protein phosphatases (PP1, PP2A, PP2B, and PP2C), the serine/threonine phosphatase PP2A is a component of the stress signaling pathway, and participates in the control of abiotic stress responses (País et al. 2009). In response to water deficit, *OsPP2A-1-5* in rice was up-regulated in response to high salinity in leaves (Yu et al. 2005), whereas *TaPP2Ac-1* exhibited enhanced drought tolerance in wheat (Xu et al. 2007).

Additional evidence is that PP2A may regulate the MAPK cascade in the regulation of cell survival (Junttila et al. 2008). In plants, the MAPK pathways are intracellular signal modules that mediate signal transduction, and are involved in the regulation of development, growth, programmed cell death and in responses to a range of environmental stimuli including cold, heat, drought, UV, reactive oxygen species (ROS), and pathogen attack (Colcombet and Hirt 2008). MAPK cascades have been shown to participate in SA, ABA, auxin and CKs signal transduction. A salicylic acid-induced protein kinase (SIPK) belonging to the MAPK family was found to be early activated after osmotic stress (Mikolajczyk et al. 2000). In *Arabidopsis*, the transcription of an *MAPK* gene, *ATMPK3*, is induced by drought, low temperature, and salinity. *ATMPK3* is further enhanced by ectopically expressed *ANP1*, an *MAPKKK* (Kovtun et al. 2000). Furthermore, overexpression of *NPK1*, an

ANP1 ortholog in tobacco, increases tolerance to salt and other stresses, and then activates the expression of *GST6* and *HSP* genes (Kovtun et al. 2000). Salt stress induces the expression and activity of *AtMEKK1*, which is also activated by cold, low humidity, osmotic stress, and wounding (Ichimura et al. 2000). Activation in activity and expression of *AtMEKK1* may form an *MAPK* cascade.

2.2 Abscisic Acid

2.2.1 Abscisic Acid Signaling

ABA is an important phytohormone and plays a critical role in response to various stress signals (Tuteja 2007). As a result, ABA has become the most studied stress-responsive hormone (Peleg and Blumwald 2011). The main stress function of ABA seems to involve the regulation of water balance and osmotic stress tolerance in plants. The most studies concern the participation of ABA in plant response to abiotic stress such as drought, salt, and cold stress (Xiong 2007). These different stresses share some common features and all induce dehydration stress to the plant cells, activate ABA generation to various extents, and induce a common set of genes involved in ABA biosynthesis. Several ABA biosynthesis genes have been cloned, which include *zeaxanthin epoxidase* (*ZEP*, known as *ABA1* in *Arabidopsis*), *9-cis-epoxycarotenoid dioxygenase* (*NCED*), *ABA aldehyde oxidase* (*AAO*) and *ABA3*, also known as *LOS5* (Xiong et al. 2001, 2002). These abiotic stress-induced genes, together with *molybdenum cofactor sulfurase* (*MCSU*), appear to be regulated through a calcium-dependent phosphorylation pathway (Zhu 2002; Xiong et al. 2002).

ABA Perception and Transduction

ABA perception and signal transduction have been extensively studied. Studies on endogenous ABA, or treatment with ABA or its analogs, have suggested multiple ABA receptors at various locations including cytosol and plasma membrane. The flowering time control protein FCA (Razem et al. 2006), G-protein-coupled receptor 2 (GCR2) (Liu et al. 2007), GCR-type G-protein 1 (GTG1) and GTG2 (Pandey et al. 2009), Mg-chelatase H subunit (ChlH) (Shen et al. 2006), and cytosol/nucleus-localized Pyrabactin Resistant (PYR)/PYR-Like (PYL)/regulatory component of ABA receptor 1 (RCAR) (Guo et al. 2011) were identified as ABA receptors. One of them, PYR/PYL/RCAR, was found to directly bind and regulate the activity of a long-known central regulator of ABA signaling, the A-group protein phosphatase 2C (PP2C). Together with the sucrose non-fermenting-related kinase 2 (*SnRK2*) subfamily, a central signaling complex (ABA-PYR-PP2Cs-SnRK2s) is responsible for ABA signal perception and transduction (Guo et al. 2011).

The activated *SnRK2s* are able to phosphorylate different downstream targets to trigger various ABA-induced physiological responses (Guo et al. 2011). Up to date,

two classes of *SnRK2* downstream targets have been identified. The first class can bind to the promoter of ABA-responsive genes including *ABA-responsive element 1* (*ABRE1*) and phosphorylates *ABA-responsive element-binding protein 1* (*AREB1*); the phosphorylated *AREB1* can then activate the expression of ABA-responsive genes (Furihata et al. 2006; Fujii et al. 2009). The second class of *SnRK2* targets includes the SLAC1 (slow anion channel 1) and the guard cell inward K^+ channel (KAT), both of which are known to mediate ABA-regulated stomatal closure (Pilot et al. 2001; Vahisalu et al. 2008). The *SnRK2* phosphorylation activates SLAC1 and deactivates KAT, and consequently leads to depolarization of the plasma membrane and activation of the outward K^+ channel, which causes a loss of turgor in guard cells and hence stomatal closure. This contributes to transduction of the ABA signal.

Gene Regulation by ABA

ABA synthesis is one of the fastest responses of plants to abiotic stress, triggering ABA-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki 2006). Genes associated with ABA biosynthesis, receptors and signal relays have been characterized mainly in *Arabidopsis* and other plants (Cutler et al. 2010). Dehydration and salt stress activate ABA-dependent gene expression systems involving *ABFs* (*ABRE-binding factor*)*/AREBs* (*ABA-responsive element-binding protein*), *MYC* (*myelocytomatosis*)*/MYB* (*myeloblastosis*) transcription factors, and *NAC* (*NAM—no apical meristem*, *ATAF—Arabidopsis transcription activation factor* and *CUC—cup-shaped cotyledon*) complex (Agarwal and Jha 2010).

Stress-inducible *AREB1* and *AREB2* function as transcriptional activators in the ABA-inducible expression of *RD29B* (Uno et al. 2000). Four *ABFs* (*ABF1*, *ABF2*, *ABF3*, and *ABF4*), similar to *AREB1* and *AREB2* reported from *Arabidopsis*, act as positive regulators of ABA signaling. *ABF1* expression is induced by cold, *ABF2* and *ABF3* by high salt and *ABF4* by cold, drought, and high salt (Choi et al. 2000). The constitutive overexpression of stress-responsive *ABF3* or *ABF4*/*AREB2* resulted in ABA hypersensitivity as well as reduced transpiration rates and enhanced drought tolerance (Kang et al. 2002). *ABF2*/*AREB1* is an essential component of glucose signaling, and its overexpression increased tolerance to multiple stresses (Fujita et al. 2005). Furthermore, key regulators of ABA-mediated gene expression are *AREBs*/*ABFs* with *ABI5* (*ABA insensitive* 5) as a typical representative. *ABI5* expression is higher in mature seeds and young seedlings exposed to ABA or dehydration stress and also its expression is promoted by multiple *ABI* gene products (Brocard et al. 2002). *OsABI5* from rice showed transcript up-regulation by ABA and high salinity and down-regulation by drought and cold. Its overexpression enhanced salinity tolerance (Zou et al. 2008). ABA and coordinated action of different hormonal signaling pathways control regulation of stress-responsive gene expression, accumulation of osmocompatible solutes, and synthesis of dehydrins and late embryogenesis abundant (LEA) proteins under environmental stress (Zhu 2002). Some wheat *LEA* genes, *WDHN13*, *WRAB17*, *WRAB18*, and *WRAB19*, showed high resistance to cold and drought (Kobayashi et al. 2008).

MYC/*MYB* transcription factors participate in the ABA-dependent pathway for the up-regulation of the abiotic stress-responsive genes (Agarwal and Jha 2010). The DNA-binding domain of plant MYB proteins usually consists of two imperfect repeats of about 50 residues and the differential binding ability suggests that these genes may regulate different sets of downstream genes. For example, in soybean (*Glycine max*) 156 *GmMYB* genes were identified, of which the expression of 43 genes changed on treatment with ABA, salt, drought and/or cold stress (Liao et al. 2008). Overexpression of *AtMYC2* and *AtMYB2* in *Arabidopsis* induced ABA-responsive stress genes. The transgenic showed an ABA-hypersensitive phenotype and increased osmotic stress tolerance (Abe et al. 2003). In contrast, *OsMYB3R-2* transgenic plants showed enhanced tolerance to freezing, dehydration and salt stress and decreased sensitivity to ABA (Dai et al. 2007).

The NAC family of plant-specific transcription factors is one of the largest in the plant genome, but a few *NAC* genes were found to be involved in response to various environmental stresses such as *ANAC019*, *ANAC055*, and *ANAC072* from *Arabidopsis* (Tran et al. 2004), and *BnNAC* from *Brassica* (Hegedus et al. 2003). In soybean 101 NAC domain containing proteins, identified as functionally non-redundant, were involved in response to abiotic stresses and in cell death events, whereas *GmNAC2*, *GmNAC3*, and *GmNAC4* were strongly induced by osmotic stress (Pinheiro et al. 2009). Soybean *NACs* such as *GmNAC3* and *GmNAC4* were also induced by ABA, JA, and salinity but differed in their response to cold. A rice *NAC* gene, *ONAC045* was induced by drought, high salt, low temperature, and ABA treatment in leaves and roots (Zheng et al. 2009).

These major transcription factors show differential transcript regulation in response to different stresses and their overexpression resulted in up-regulation of a large number of genes directly or indirectly linked with stress tolerance in plants (Agarwal and Jha 2010).

2.2.2 ABA in Plant Response to Abiotic Stresses

Perhaps the best known and also the most studied process that ABA is involved in is plant response to abiotic stress such as drought, salt, and cold stress (Xiong 2007). These different stresses share some common features in that they all induce dehydration stress to the plant cells. Accordingly, all these abiotic stresses activate ABA biosynthesis to various extents and induce a common set of stress-responsive genes.

ABA and Drought Stress

Drought stress is often caused by prolonged water deficit in the soil that cannot provide for plant transpiration demand. Plants have some ways to deal with drought challenges: to reduce water consumption, to increase water uptake, and to mitigate the negative impacts of water deficit (Xiong 2007). First, guard cell stomatal pores

are closed upon drought stress and thus the transpirational water loss is minimized. This is a relatively quick response. Second, an array of stress-responsive genes is activated. The products of these genes function directly or indirectly in drought tolerance. ABA is either required or is involved in all these processes.

Under drought stress, plants synthesize ABA, which in turn induces rapid closure of stoma (Zhang et al. 2008b). Stomatal closure takes place to minimize the water loss by transpiration, and ABA plays a fundamental role in this process by inducing stomatal closure via the efflux of potassium and anions from guard cells and the removal of osmolytes (Schroeder et al. 2001). Thus, stomatal resistance is used as a reference to compare the intensity of water deficit in different species and growth conditions (Medrano et al. 2002). Interestingly, stomatal closure under drought is a response to increasing levels of endogenous ABA synthesized in the roots as a result of water deprivation in the soil (Kim et al. 2010). Hence, decrease of stomatal conductance under water stress is a wide-ranging response in plants. Stomatal conductance in kidney beam diminishes rapidly after 2 days of drought, but it recovers in well-watered plants after 2 days of re-watering (Miyashita et al. 2005). In *Brachiaria* plants, stomatal conductance significantly decreased after 6 days of water deprivation (Carmona et al. 2003).

Although drought alone can activate these stress-responsive genes, ABA can synergistically enhance their expression. The promoter of a drought-, high salinity-, and cold-inducible gene contains two major elements, *ABRE* and *DRE*/*CRT* (C-RepeaT), both of which are involved in stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki 2005). In response to abiotic stress, *ABRE* is a major *cis*-acting element that functions in ABA-dependent and ABA-independent gene expression. Two *ABRE* motifs are important *cis*-acting elements controlling ABA-responsive expression of the *Arabidopsis RD29B* gene (Uno et al. 2000). Two basic leucine zipper (bZIP) transcription factors, *AREB*/*ABF*, can bind to *ABRE*, thereby activating ABA-dependent gene expression. The *AREB*/*ABF* proteins require an ABA-mediated signal for their activation, as indicated by their reduced activity in the ABA-deficient *aba2* and ABA-insensitive *abi1* mutants and their enhanced activity in the ABA-hypersensitive *era1* mutant of *Arabidopsis* (Uno et al. 2000). This phenomenon is very likely due to the ABA-dependent phosphorylation of the AREB/ABF proteins.

Induction of the *responsive to dehydration* genes (*RDs*) is mediated by ABA and requires protein biosynthesis for ABA-dependent expression. An *MYC* transcription factor in *Arabidopsis*, *RD22BP1*, and an *MYB* transcription factor, *AtMYB2*, were shown to bind *cis*-elements in the *RD22* promoter and co-operatively activate *RD22* (Abe et al. 2003). Recently, a drought-inducible *RD26* gene encoding an *NAC* transcription factor was identified (Fujita et al. 2004). Expression of this *RD26 NAC* transcription factor gene is induced by drought, high salinity, ABA, and JA treatments. *RD26* protein is localized in the nucleus and has transcriptional activity. An *RD26*-overexpressing transgenic plant was hypersensitive to ABA, and an *RD26-dominant* repressor was insensitive to ABA.

ABA and Salt Stress

ABA is also the major internal signal enabling plants to survive adverse environmental conditions such as salt stress (Waskiewicz et al. 2013a). Salt stress signaling through Ca^{2+} and ABA mediates the expression of *LEA*-type genes including the *DRE*/*CRT* class of stress-responsive genes *COR*. The activation of *LEA*-type genes may actually represent damage repair pathways (Xiong et al. 2002). Salt and osmotic stress regulation of *LEA* gene expression is mediated by both ABA-dependent and -independent signaling pathways. Both the pathways use Ca^{2+} signaling to induce *LEA* gene expression during salinity.

Exposure of plants to salinity is known to induce a proportional increase in ABA concentration. Increases of the endogenous ABA concentration in leaf tissue for salt-stressed *Zea mays* (Cramer and Quarrie 2002) and *Phaseolus vulgaris* (Cabot et al. 2009) strongly correlated with growth inhibition. ABA, as a signal for stomatal closure, induces rapid depolymerization of cortical actin filaments and slower formation of a new type of actin which is randomly oriented throughout the cell (Hwang and Lee 2001). This change in actin organization appears to be important in stomatal closing movement, since actin antagonists alter the normal stomatal responses to ABA. The generic stress hormone ABA is up-regulated by salinity and induces genes involved in salt and osmotic alleviation, e.g., the tissue distribution and regulation of *AtNHX1* expression by ABA and salt stress in *Arabidopsis* (Shi and Zhu 2002), or the *MAPK4-like*, *TIP1* and *GLP1* genes induced much faster in response to ABA treatment in wheat (Keskin et al. 2010). ABA regulates the expression of some of the transporters involved in salt uptake and compartmentalization. ABA affected the expression of two genes, *HVP1* and *HVP10*, for vacuolar H^+-inorganic pyrophosphatase and one, *HvVHA-A*, for the catalytic subunit of vacuolar H^+-ATPase in barley response to salt stress (Fukuda and Tanaka 2006).

The protein SOS2 (salt overly sensitive 2), a serine-threonine protein kinase necessary for Na^+ and K^+ ion homeostasis and salt tolerance in *Arabidopsis*, could interact with ABI2, a 2C type phosphatase that negatively regulates ABA signaling (Ohta et al. 2003). Other 2C type phosphatases, PP2CA, which may act as a negative regulator of several ABA responses, also interact with K^+ channels (Chérel et al. 2002). While K^+ channels in guard cells play critical roles in stomatal opening and closing, disturbed K^+ homeostasis in roots and other tissues and cell types may contribute to salt sensitivity (Rus et al. 2004). It is thus likely that ABA may play a role in regulating the ion transporter activities under salt stress.

ABA and Cold Stress

Low temperature is one of the major abiotic stresses limiting the productivity and the geographical distribution of many important crops. Tolerance of many plants to low temperatures increases, this phenomenon is known as cold acclimation (Heidarvand and Amiri 2010). Following perception of the cold stress signal,

transcriptional cascades are the next players which operate through ABA-dependent and ABA-independent pathways.

In the ABA-dependent pathways, there is a transient rise in endogenous ABA content when plants are exposed to cold stress. The ABREs confer ABA responsiveness to many genes when more than one copy is present. The class of *bZIP* transcription factors, *AREBs* or *ABFs*, can bind to ABRE and activate ABA-dependent gene expression. The *ABF* genes are themselves induced by ABA and show differential regulation by various environmental stresses; *ABF1* is induced by cold, *ABF2* and *ABF3* by high salt concentration, and *ABF4* by cold, high salt concentration and drought (Choi et al. 2000). The differential regulation of *ABF* expression suggests that separate *ABFs* are likely to function in these signal transduction cascades through common *ABREs*. Genetic analysis of ABA-deficient mutants showed that ABA plays a pivotal role in osmotic stress-regulated gene expression.

2.3 Ethylene

2.3.1 Ethylene Signaling

Ethylene (ET) is perceived by a family of five membrane-localized receptors: ETR1, ETR2, ERS1, ERS2, and EIN4 (O'Malley et al. 2005). Each ET receptor has a similar overall modular structure, with transmembrane domains containing the ET binding site near the N-terminus. Although similar, the ET receptors can be divided into two subfamilies based on phylogenetic analysis and some shared structural features, subfamily 1 being composed of ETR1 and ERS1 and subfamily 2 being composed of ETR2, ERS2, and EIN4 (Chang and Stadler 2001; Schaller and Kieber 2002).

A key question is how the receptors transmit information to downstream signaling components in the pathway. The initial discovery that the receptors contain an HK domain and receiver domains, motifs known to participate in His-Asp phosphorelays, might be relevant in ET signaling (Hall et al. 2007). The next immediate element in the signaling pathway appeared to be the Raf-like kinase *CTR1*. *CTR1* is a negative regulator of ET signaling and shows similarity to the Raf family of serine/threonine protein kinases in its C-terminal half (Huang et al. 2003). *CTR1* interacts with the HK domain and receiver domains of the receptor. The kinase domain of *CTR1* actively represses ET responses. Binding of ET by the receptor induces a conformational change in *CTR1* that reduces its kinase activity, thereby relieving repression of the ET response pathway (Hall et al. 2007). In the absence of ET, the receptor maintains *CTR1* in an active conformation so that the kinase domain of *CTR1* actively represses ET responses (Huang et al. 2003). Binding of ET by the receptor induces a conformational change in the receptor, possibly involving a change in the receptor's kinase activity, and this is transmitted to *CTR1*. The conformational change in *CTR1* reduces its own kinase activity, thereby relieving repression of the ET response pathway.

Many ET responses involve changes in gene expression. Up to date, different members of plant *ERF* (*ethylene response factor*) genes have been found to be mainly involved in the response to abiotic stresses (Zhang et al. 2008a). Transcription factors encoded by genes in the *dehydration-responsive element* (*DRE*)*-binding* (*DREB*) subfamily play an important role in the resistance of plants to abiotic stresses by recognizing *DRE*. *ERF* and *DREB* subfamily transcription factors have been identified in various plant species, e.g., *Arabidopsis* (Oñate-Sánchez and Singh 2002), and rice (Cao et al. 2006). The roles of *ERF* and *DREB* proteins in the plant response to biotic and abiotic stresses have also been extensively documented (Agarwal et al. 2006). Both *DREB1* and *DREB2* factors are induced by water stress or cold. Their transcripts accumulate at high levels shortly after initiation of the stress treatment. It was shown that *DREB1* genes are induced by low temperature, whereas the *DREB2* homologs are induced by drought and high salt stresses (Kizis et al. 2001). Environmental stresses including drought, desiccation, and low temperature significantly increased the expression level of the putative repressor *LeERF3b*, but markedly reduced the expression level of the putative activator *Pti4* (Chen et al. 2008). Tobacco plants expressing *JERF3* showed enhanced adaptation to drought, freezing, and osmotic stress during germination and seedling development (Wu et al. 2008).

2.3.2 ET in Plant Response to Abiotic Stresses

ET and Oxidative Stress

There are several reports that demonstrate a functional link between ET and hydrogen peroxide (H_2O_2) synthesis, signaling in ozone-exposed tomato leaves (Moeder et al. 2002), and H_2O_2- or ET-treated stomatal guard cells (Desikan et al. 2005, 2006). The *Arabidopsis* ET receptor (*AtETR1*) could act as a central node mediating cross-talk between ET and H_2O_2 signaling in stomatal guard cells (Desikan et al. 2005), and *Arabidopsis* NADPH oxidase (*AtrbohF*) was identified as a key mediator of the stomatal response to ET (Desikan et al. 2006). ET synthesis and recognition are required for the burst of H_2O_2 production that regulates the spread of cell death (Moeder et al. 2002). Although correlations between ET synthesis, ROS accumulation and tissue damage have been reported previously, the functional interaction between ET biosynthesis and ROS detoxification, which might be important for alleviating cell damage and subsequent stress tolerance, has not yet been explored.

Ozone has been previously observed as an air pollutant and now is recognized as an abiotic elicitor for activation of an oxidative burst, which evokes a local cell death response similar to that caused by the hypersensitive response (Overmyer et al. 2000). ROS generated by ozone result from its reactions with water and other cellular components when it enters from the stomata (Wang et al. 2002). ET synthesis is one of the earliest responses to ozone stress (Overmyer et al. 2000). An ozone-sensitive mutant, *rcd1* (radical-induced cell death 1), has been shown to have a higher susceptibility to the oxidative burst. Compared with wild-type plants, *rcd1* is

more susceptible to O_2^- than H_2O_2 and shows prolonged lesions on leaves even after ozone is removed, suggesting a defect in restraining the toxicity of ROS. ET production in *rcd1* is higher than that of the wild type and continues even after ozone is removed. In contrast, ET synthesis returns to the basal level in the wild type when ozone treatment is ended. The prolonged cell death response observed even after ozone treatment is removed in *rcd1* can be suppressed by norbornadiene (an ET receptor antagonist), application of methyl jasmonate (MeJA), or by mutations in *EIN2*, suggesting that ET signaling is required for cell death and is antagonized by the JA pathway. The other implication is that *RCD1* may function upstream of the ET receptor and acts to confine ET production once it is initiated. Therefore, it is possible that the hypersensitivity of *rcd1* to ozone stress may be a consequence of defective feedback regulation of ET synthesis or elevated ET sensitivity.

To a lesser extent than ozone, UV also causes an oxidative burst. Treatment of *Arabidopsis* plants with UV-B light resulted in the increased expression of *PR-1* and *PDF1.2* (Wang et al. 2002). ROS are required for this altered gene expression because pretreatment of plants with ascorbic acid blocks the induction of *PDF1.2* by UV-B. Induction of *PDF1.2* is also inhibited in *etr1-1* and *jar1* mutants, suggesting that ROS lie upstream of the ET and JA pathways. Both ET and JA are required for the maximal induction of *PDF1.2*, as evidenced by application of these two growth regulators separately or together, and by examining the signaling defective *jar1* and *etr1-1* mutants. Interestingly, induction of *PR-1* is dependent on ET, but not on JA, and shows faster kinetics than that of *PDF1.2*, suggesting that ET is an early signal required to activate the SA pathway upon UV-B treatment. These results suggest that ET potentiates the response to both SA (*PR-1* induction) and JA (*PDF1.2* induction).

ET and Salt Stress

A type II ET receptor homolog gene, *NTHK1* (*Nicotiana tabacum histidine kinase 1*, functions as ET receptor), was introduced into *Arabidopsis* and it was found that the resulting transgenic plants, with *NTHK1* mRNA and protein expression, were salt-sensitive, as could be seen from the severe characteristics of plagiotropic, high electrolyte leakage, and reduced root growth under salt stress (Cao et al. 2007). *NTHK1* enhances expression of salt-responsive genes such as *AtERF4*, indicating its role in the salt-stress response. *AtERF4* has been found to be a transcriptional repressor conferring ET insensitivity in its transgenic *Arabidopsis* plants, and the *AtERF4*-overexpressing plants are hypersensitive to NaCl (Yang et al. 2005). Moreover, an NAC-type transcription factor gene, *AtNAC2*, was salt-inducible and involved in lateral root development, and the salt induction of the *AtNAC2* gene was reduced in intensity in *NTHK1*-transgenic plants and the ET-response mutants of *etr1-1* and *ein2-1* (He et al. 2005). In addition to salt stress, the ET receptor ETR1 may also play roles in H_2O_2 signaling in stomatal closure (Desikan et al. 2005). These results suggest that ET signaling can regulate the salt-stress response by controlling expression of multiple genes.

ET and Flood Stress

Flood stress subjects plants to oxygen depletion that consists of hypoxia (deficiency of oxygen) and anoxia (absence of oxygen). Capacity to survive the oxygen deprivation depends on a number of developmental, morphological, and metabolic adaptations in plants. A majority of these morphological and metabolic adaptations are strictly regulated by the plant hormonal system.

ET is the first phytohormone which has been studied under conditions of oxygen deficiency. Yemelyanov and Shishova (2012) noted in their review that an increase in ET production under the lack of oxygen has been observed in a wide variety of cultivated plants, including beans, radish, tomato, sunflower, chrysanthemum, corn, and wheat. Higher plants synthesize ET from L-methionine via *S*-adenosyl methionine (SAM) and ACC. Hypoxia-induced accumulation of *ACC* and activation of *ACC synthase* (*ACS*) genes have been shown in *Arabidopsis* (Muhlenbock et al. 2007) and some other plants. The reaction of ACC oxidation by ACC oxidase (ACO) requires O_2 and is blocked by oxygen depletion. Down-regulation of some *ACS* genes, such as *OsACS5*, in flooded rice revealed by expression analysis (Van der Straeten et al. 2001), and hypoxia-induced stimulation demonstrated for expression of *ACS2*, *ACS6*, *ACS7*, and *ACS9* in *Arabidopsis* (Peng et al. 2005), as well as *RpACS1* and *RpACO1* in *Rumex* plants (Rieu et al. 2005), are recent examples of ET function in flood stress responses.

ET promotes fast apoplastic acidification in flooded plants, which is important for growth promotion (Vreeburg et al. 2005). In addition, promotion of shoot extension by ET is linked to cell wall loosening. Loosening of the cell wall is provided by ET-dependent stimulation of pectinase (Bragina et al. 2003). These enzymes are involved in aerenchyma formation. Aerenchyma is a special pneumatic tissue providing air transport and storage facility for plants under oxygen deficiency (Yemelyanov and Shishova 2012). Spaces within the aerenchymatous organ appear either by cell separation at the middle lamella (schizogeny) or by cell death and decomposition of the cell wall (lysigeny) in which ET plays a role.

2.4 Auxin

2.4.1 Auxin Signaling

There are two proteins which may function as auxin receptors. The first protein, TRANSPORT-INHIBITOR-RESISTANT1 (TIR1), is accepted as an auxin receptor by the scientific community and functions together with at least three other related F-box protein/receptors to mediate the auxin response (Parry and Estelle 2006). The second protein, AUXIN-BINDING PROTEIN1 (ABP1), contains the C-terminus upon binding auxin (Scherer 2011). TIR1 and ABP1 as two interacting receptors are enhanced by the fact that ABP1 can perceive apoplastic auxin concentration, while TIR1 perceives cytosolic concentration.

The receptor TIR1 and its paralogs, Auxin signaling F-Box 1 (AFB1), AFB2, and AFB3 are the F-box subunits of SCF (Skp1-Cul1-F-box) E3-ubiquitin ligase complex (Iglesias et al. 2010). Auxin binding to $SCF^{TIR1/AFB}$ results in the targeted ubiquitination and degradation of Aux/IAA proteins (Dharmasiri et al. 2005). Aux/IAA degradation promotes activation of ARF transcription factors and the consequent expression of auxin-responsive genes (Hagen and Guilfoyle 2002). TIR1 predetermines the transcriptional corepressors (the IAA proteins) to proteolysis by ubiquitination through its E3 ligase activity (Mockaitis and Estelle 2008). Thus, the receptor TIR1 is close to proteolytic regulation of central negative transcriptional co-regulators in the nucleus.

ABP1 can sense the transported auxin concentration in the apoplast and regulates the auxin efflux carrier component (PIN) activity (Robert et al. 2010). In turn, the intracellular auxin concentration regulates gene activity, including *PIN* genes. Auxin transport changes induced by auxin occur quickly, probably by inhibiting the endocytosis of plasma membrane-bound PIN proteins (Paciorek et al. 2005). Most recently, this group showed that the block of endocytosis by auxin was not dependent on TIR1 or on protein synthesis (Robert et al. 2010). The authors point out that TIR1-induced gene regulation and protein biosynthesis of PIN proteins cannot be a mechanism for this effect and that ABP1, rather, must be the relevant auxin receptor here. Beyond the cellular level, the complex network of vascular bundles in the whole plant could be the morphological basis for an interlocking network of auxin transport and gene regulation throughout the plant body.

Auxin-Responsive Gene Expression

Auxin functions to some extent by regulating a group of primary responsive genes: *Aux/IAA*, *Gretchen Hagen 3* (*GH3*), and *small auxin-up RNAs* (*SAURs*) (Hagen and Guilfoyle 2002). Members of the *Aux/IAA* gene family have been studied in regulation of auxin responses (Overvoorde et al. 2005). Several *GH3* genes have been studied using mutants with altered gene expression (Park et al. 2007). Although none of the *SAUR* genes are as yet functionally characterized, the *SAUR* proteins have been shown to bind to calcium/calmodulin (Galon et al. 2010), suggesting the role the involvement of CAMTA1 (CAMTA-binding motifs encompass the recently identified Ca^{2+}-responsive *cis*-elements) in auxin signaling.

A typical example of auxin-responsive genes involved in a stress response is in rice plants. Their expression profile was investigated by microarray analysis under desiccation, cold, and salt stress (Jain and Khurana 2009). At least 154 auxin-induced and 50 auxin-repressed probe sets were differentially expressed, under one or more of the stress conditions analyzed. Moreover, 41 members of auxin-related gene families were found to be differentially expressed under at least one abiotic stress condition. Among these, 18 (two *GH3*, seven *Aux/IAA*, seven *SAUR*, and two *ARF*) were up-regulated and 18 (one *GH3*, five *Aux/IAA*, eight *SAUR*, and four *ARF*) were down-regulated under one or more abiotic stress conditions. However, five genes (*OsGH3-2*, *OsIAA4*, *OsSAUR22*, *OsSAUR48*, and *OsSAUR54*) were

up-regulated under one or more abiotic stress conditions and down-regulated under other stress conditions.

It was indicated that the expression of *Aux/IAA* and *ARF* gene family members was altered during cold acclimation in *Arabidopsis* (Hannah et al. 2005). Molecular genetic analysis of the auxin and ABA response pathways provided evidence for auxin–ABA interaction (Brady et al. 2003). The role of *IBR5*, a dual-specificity phosphatase-like protein, supported the link between auxin and ABA signaling pathways (Monroe-Augustus et al. 2003). Promoters of the auxin-responsive genes and members of auxin-related gene families differentially expressed under various abiotic stress conditions were analyzed to identify *cis*-acting regulatory elements linked to specific abiotic stress conditions. Although no specific *cis*-acting regulatory elements could be linked to a specific stress condition analyzed, several ABA and other stress-responsive elements were identified. The presence of these elements further confirms the stress responsiveness of auxin-responsive genes (Jain and Khurana 2009).

2.4.2 Auxin in Plant Responses to Abiotic Stresses

Auxin and Cold Stress

Among the abiotic stresses, low temperature is one of the major stresses in limiting the plant development and crop productivity (Rahman 2013). The cold response in plants involves perception and relaying of the signal through a transcriptional cascade composed of different transduction components resulting in altered transcription of several genes.

The response of auxin mutants to cold-stress-induced inhibition of root growth and gravity response, expression analysis of the auxin responsive marker *IAA2-GUS* and the direct auxin transport assay confirmed that cold stress primarily targets intracellular auxin transport (Shibasaki et al. 2009). Cold stress selectively inhibits the intracellular trafficking of a subset of proteins that include auxin efflux carriers. Moreover, cold stress also blocks the asymmetric redistribution and intracellular cycling of PIN3 that facilitates the plant response to gravity. For shootward transport of auxin, recent molecular and cellular findings suggest that the polar deployment of PIN2 and the constitutive cycling of this protein from membrane to endosome are required for its functionality (Paciorek et al. 2005). The reduced intracellular cycling affects the functionality of PINs, resulting in reduced shootward transport of auxin and diminishing the root's capability to form an auxin gradient (Shibasaki et al. 2009). Cold stress-induced change in plant growth and development is tightly linked to the intracellular auxin gradient, which is regulated by the polar deployment and intracellular trafficking of auxin carriers (Rahman 2013).

Some components of the cold signaling pathway are linked to auxin. SIZ1, a central regulatory component of the cold signaling pathway, has been shown to negatively regulate phosphate-starvation-induced root architecture remodeling through the control of auxin patterning (Miura et al. 2011). Another downstream component of the cold signaling pathway in *Arabidopsis* is *AtNUP160*, which plays

a critical role in the nucleocytoplasmic transport of mRNAs under cold stress (Dong et al. 2006). This *AtNUP160*/*SAR1* has also been shown to play an important role in auxin signaling (Parry et al. 2006).

Auxin and Drought Stress

Drought stress is one of the major abiotic stresses that restrict plant growth and development. Many phytohormones, such as ABA, SA and JA, are known to respond to drought stress; however, comparatively little insight has been obtained regarding the auxin transport response to drought stress. There is some evidence for the involvement of an auxin transport efflux carrier in the drought stress response. Drought stress treatments significantly reduced concentrations of IAA in rice grains during the grain filling stage (Yang et al. 2001). As the primary mediators of auxin transport in plants, PIN proteins were presumed to participate in the drought stress response either directly or indirectly. Also in rice plants, the gene *OsPIN3t* encodes a member of the auxin efflux carrier protein family (Zhang et al. 2012). *OsPIN3t* plays a key role in rice shoot and root development and is involved in drought stress responses. *Phototropin 1* is an *Arabidopsis* ortholog of the Ser/Thr protein kinase PINOID, which catalyzes PIN phosphorylation, contributes crucially to the regulation of apical-basal PIN polarity (Kleine-Vehn et al. 2009), and can improve drought tolerance at the seedling stage (Galen et al. 2007). Taken together, these results suggest that auxin transport is involved in regulation of the response to water stress in plants.

Auxin and Salinity Stress

Seed germination is the first developmental process that is critical for plant establishment and propagation in nature. A recent study discovered that the membrane-bound NAC transcription factor *NTM2* mediates the signaling crosstalk between auxin and salt stress via *IAA30* gene during *Arabidopsis* seed germination (Jung and Park 2011). Germination of the NTM2-deficient *ntm2-1* mutant seeds exhibited enhanced resistance to high salinity. However, the salt resistance was reduced in the *ntm2-1* mutant overexpressing the *IAA30* gene, which was induced by high salinity in an NTM2-dependent manner. NTM2 is a molecular link that incorporates the auxin signal into salt stress signaling, providing a role of auxin in modulating seed germination under high salinity.

2.5 Cytokinins

2.5.1 Cytokinin Signaling

Cytokinins (CKs) play a key role in various processes that regulate plant growth and development via a complex network of signaling (Nishiyama et al. 2011). CKs have

been recognized as an important signal that regulates the protective responses in plants to abiotic stresses. The first step in CK perception and signal transduction is accomplished by cytokinin receptors (CRs), hybrid-type histidine kinases, membrane proteins with a cytokinin-binding extracellular domain (de la Peña et al. 2008). CRs belong to a multigenic family, and three different CRs, Arabidopsis Histidine Kinase 2 (AHK2), AHK3, and AHK4 (also known as CRE1-Cytokinin response 1) have been identified in *Arabidopsis* (Kakimoto 2003). Orthologs have been identified in maize (Yonekura-Sakakibara et al. 2004) and rice (Ito and Kurata 2006), and several functional roles of CRs have been elucidated. In legumes, some members of the CR multigenic family are mainly essential for nodulation. However, two new CRs, MsHK1 from *Medicago sativa*, and LaHK1 from *Lupinus albus*, were involved in the stress response (de la Peña et al. 2008). Expression of the *MsHK1* gene increased under osmotic stress, and both genes were induced following dark stress, indicating that CRs are likely to play a significant role in the response to stress.

CRs initiate and propagate CK signaling by means of phosphorylation and phosphotransfer to downstream proteins (Aoyama and Oka 2003). Typically in *Arabidopsis*, the signal is then transferred via histidine-containing phosphotransfer factors, AHPs, via the C-terminal receiver domains of the sensor histidine kinases. AHPs transmit the signal from the receptor, which is presumably localized in the plasma membrane, to transcription-factor-type *Arabidopsis* response regulators (*ARRs*), which are mostly found in the nucleus (Hwang and Sheen 2001). Analysis of the *Arabidopsis* genome sequence reveals the existence of 22 predicted *ARR* genes necessary for signal-accepting activity (Schaller et al. 2002). The *ARRs* are divided into two major classes, A and B type, on the basis of their structure. The 11 type-A *ARRs* consist mainly of the receiver domain with a short extension at the N- and C-terminal ends. By contrast, the 11 type-B *ARRs* contain a C-terminal output domain in addition to the receiver domain. The genes coding for the two types of *ARR* respond differently to CKs. A-type *ARR* genes are rapidly induced in the presence of CKs and fulfill the criteria of a primary response gene (D'Agostino et al. 2000), suggesting that they are likely to be mediators of CK responses within the cell.

2.5.2 CKs in Plant Response to Abiotic Stresses

CKs and Drought Stress

Scarcity of water is a severe environmental constraint on plant productivity. Drought stresses reduces leaf size, stem elongation (growth) and root proliferation, disturbs plant water relations and reduces water-use efficiency (Farooq et al. 2009). Plants display a variety of physiological and biochemical responses at cellular and whole-organism levels toward drought stress, in which phytohormones such as CKs, SA, auxins, GA, and ABA modulate those responses.

CKs are an important signal class traveling from roots to shoots. There have been few reports providing information on the CK content of xylem sap and how that content changes under drought conditions. In grapevines, a reduction in zeatin (Z)

and zeatin riboside (ZR) was found in plants that had been subjected to partial root-zone drying (PRD) (Stoll et al. 2000). In tomato, Z, ZR, and zeatin nucleotide (ZN) were measured; PRD reduced the ZN content of the xylem sap, but the magnitude of that change and the contribution of ZN to the total CK content were not shown (Kudoyarova et al. 2007). In at least two studies on sunflower xylem sap, combined Z and ZR and combined isopentenyladenine and isopentenyladenosine concentrations in xylem sap decreased under drought-stress conditions (Hansen and Dörffling 2003). In a recent study on maize, a decrease was observed in Z and ZR concentrations in xylem sap from roots of drought-stressed plants as compared to well-watered controls (Alvarez et al. 2008). Surprisingly, high concentration of the aromatic CK 6-benzylaminopurine (BAP) was found in maize xylem sap, the concentration of which increased significantly as a result of water stress.

Increased CK contents following over-expression of the CK biosynthetic gene *isopentenyl transferase* (*IPT*) driven by senescence-inducible promoter *SAG12* positively correlated with elevated flooding tolerance (Zhang et al. 2000; Huynh et al. 2005). It was found that the expression of *IPT* under control of the *senescence-associated receptor kinase* (*SARK*), a drought/maturation-induced promoter, resulted in a remarkable tolerance to extreme drought conditions in tobacco (Rivero et al. 2007). This together with the reported role of CKs in sink–source polarization during mild water stress (Cowan et al. 2005) indicated that CKs play an important role in plant responses to drought. Furthermore, during water stress, the *SARK* promoter linked to the *IPT* gene in rice and displayed increased expression of brassinosteroid-related genes and repression of jasmonate-related genes (Peleg et al. 2011). Changes in hormone homeostasis were associated with resource mobilization during stress.

CKs and Salt Stress

A recent microarray analysis of *Arabidopsis* CK receptor mutants clearly showed that CK-mediated signaling can also be involved in stress responses. Knockout lines of two out of three CK receptors were strongly tolerant of drought and salt stress due to up-regulation of many stress-inducible genes (Tran et al. 2007). Alteration in the CK content in plants exposed to various stresses has been frequently reported. For example, *trans*-zeatin and *trans*-zeatin riboside contents decreased rapidly in the elongation zone of barley leaves after salinity stress induction (Fricke et al. 2006).

Several components of the CK signaling pathway have been shown to be involved in the regulation of stress responses, including several members of *ARR* receptors (Ha et al. 2012). The functional analyses of the *arr1 arr12* double mutant indicated that the type-B *ARR1* and *ARR12* act as negative regulators during a salt stress response. These two proteins redundantly regulate sodium accumulation through *AtHKT1;1*, which encodes a high affinity potassium transporter responsible for removing sodium ions from the root xylem (Mason et al. 2010). On the other hand, there was some evidence of the positive role of the response regulator (RR) of CKs in the plant response to salt stress. *OsRR* genes were shown to be up-regulated in

rice seedlings exposed to a high concentration of salt (Jain et al. 2006). In developing kernels where the role of CKs in response to water stress was previously studied (Brugiere et al. 2003), only specific genes for de novo biosynthesis (e.g., *IPT2*), degradation (e.g., cytokinin oxidase genes such as *CKX1* and *CKX4*), and signal response (e.g., *RR3*) were activated. These genes may play an important role in mediating the input of CKs into the salt stress response pathway.

CKs and Cold Stress

Cold stress appears to rapidly up-regulate the expression of multiple type A *ARRs* and conversely to down-regulate the expression of all three CK receptors (Argueso et al. 2009). Multiple mutant analyses have suggested a complex function for the type-A CK component (Wohlbach et al. 2008). Individual mutations in *arr5*, *arr6*, or *arr7* resulted in enhanced cold tolerance (Jeon et al. 2010). *AHK2* and *AHK3* were found to be primarily involved in mediating cold to express A-type *ARRs* despite CK deficiency (decreased CK levels). Although there are no reports linking CKs to a rapid response to cold stress, these results may suggest a negative role for CKs in the response to cold stress.

2.6 Jasmonic Acid

2.6.1 Jasmonic Acid Signaling

Perception and Transduction

Abiotic stresses (as well as biotic stresses) generate signals/elicitors that activate a phosphorylation cascade that regulates jasmonic acid (JA) biosynthesis and signaling (Kazan and Manners 2008). Following synthesis, JAs are perceived by receptor proteins, and this presumably activates a signal transduction pathway that culminates in the transcriptional activation or repression of a large number of JA-responsive genes.

It has been revealed that CORONATINE INSENSITIVE 1 (COI1) is involved in jasmonate perception and signaling (Thines et al. 2007; Paschold et al. 2008). COI1 is an F-box protein that functions as the substrate-recruiting module of the SCF protein ubiquitin E3 ligase complex. At first, COI1 was suggested to bind directly to JA-Ile and COR, and serves as a receptor for JA (Yan et al. 2009). A later study discovered that COI1 mediates JA signaling by promoting hormone-dependent ubiquitination and degradation of transcriptional repressor JAZ (Jasmonate ZIM-domain) proteins, and the complex of both COI1 and JAZ was identified as the true JA receptor (Sheard et al. 2010). In this complex, COI1 might act as part of an SCF E3 ubiquitin ligase to mediate JA signaling, whereas JAZ proteins bind and repress the transcription factors that modulate transcription of JA-responsive genes.

Genes Regulated by JA in Abiotic Stress Response

The salinity response in plants involves the expression of several genes. Transcript levels of the *arginine decarboxylase 2* (*ADC2*) genes increased in plants directly exposed to salinity stress. Arginine decarboxylase enzyme catalyzes the first step in the conversion of arginine to putrescine in the polyamine biosynthesis pathway. Two independent mutants of *ADC2* in *Arabidopsis* resulted in decreased polyamine content and consequently decreased salt tolerance of a mutant relative to respective wild types (Kasinathan and Wingler 2004). The decreased salt tolerance of the *ADC2* mutant, *spe2-1*, was reflected in loss of chlorophyll from leaves. The expression level of *ADC2* was also elevated in the JA treatment in barley plants. Walia et al. (2007) reported that *ADC2* is JA induced and involved in salt tolerance but is not induced independently by salt stress to significant levels; this fact caused it a good candidate gene for further characterization.

Another gene that came to the fore from searches for potential JA-regulated salt tolerance candidates was an apoplastic *invertase*. An apoplastic *invertase* gene in tobacco resulted in increased levels of sucrose and hexoses in leaves (Fukushima et al. 2001). The transgenic plants were able to better withstand high levels of salt stress compared with wild type. The increased levels of sucrose in the leaves prevented inhibition of photosynthesis in the transgenic plants under salt stress. This observation is striking, considering that JA-pre-treated barley plants in our experiment also maintained higher net photosynthetic levels compared with plants exposed to salt stress alone.

2.6.2 JA in Plant Response to Abiotic Stresses

JA and Drought Stress

The participation of JA in response to drought has been reported in several species. Sorbitol treatment enhanced octadecanoid and JA contents, and this threshold was necessary and sufficient to initiate JA-responsive gene expression (Kramell et al. 2000). Under water stress, endogenous JA content was able to elicit betaine accumulation in pear leaves (Gao et al. 2004). Studies in contrasting environments showed different basal JA contents and patterns of response to water stress in a population of *Pinus pinaster*, perhaps as an adaptation to diverse ecological conditions (Pedranzani et al. 2007). Moreover, in maize developing kernels, expression patterns of some genes in several stress response-associated pathways, including ABA and JA, were examined, and these specific genes were positively responsive to drought stress (Luo et al. 2010).

JA has been shown to increase in spear tips of *Asparagus officinalis* (Gapper et al. 2002) and in *Carica papaya* (Mahouachi et al. 2007) exposed to drought. In addition, MeJA increases in *Cistus albidus* also subjected to drought (Jubany-Marí et al. 2010). Exogenous application of JA or MeJA increased antioxidative ability of plants under water stress (Bandurska et al. 2003). Along the same line, other studies

also showed that JAs play an important role in signaling in drought-induced antioxidant responses, including ascorbate metabolism (Ai et al. 2008). Certainly, MeJA promoted increased production of several antioxidative enzymes, including glutathione reductase (GR), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX), and it has been suggested that this increase may be due to up-regulation of genes controlling the synthesis of these enzymes, or activation of diverse constitutive genes (Norastehnia and Asghari 2006).

Although JA and MeJA were previously thought to be key regulators of jasmonate responses, it has been demonstrated that it is the isoleucine conjugate of jasmonic acid (JA-Ile), the active form of JA, that acts in the signal transduction pathway (Staswick 2008). However, no studies have been reported to date on the variations in endogenous concentrations of JA-Ile in plants subjected to water stress. Furthermore, a rapid increase in endogenous JA levels resulting from environmental stimuli leads to a concomitant increase in JA-Ile (Wasternack and Kombrink 2010), and it has not been ruled out that this could occur in conditions of water stress.

JA and Salinity and Heavy Metal Stress

Research has been concentrated on the role of JA and its metabolites in the defense response against abiotic stresses such as salinity, and heavy metal toxicity. Salt stress increased the JA levels in roots of rice and in leaves of *Iris hexagona* (Wang et al. 2001). Endogenous JA generally increased in response to salinity; therefore, high levels of JA in salt-tolerant plants accumulated after salt treatments may be an effective protection against high salt concentration. Investigating the changes of endogenous JA levels in rice plants under various salt stresses, Kang et al. (2005) discovered that the concentrations of JA in a salt-sensitive cultivar were lower than those in a salt-tolerant cultivar. Especially, considering the concentration of JA in the shoots of salt-sensitive plants, endogenous JA was rapidly decreased following the increase of NaCl concentration in treatment. Similarly, tomato cultivars differing in salt tolerance differed in basal JA content (Pedranzani et al. 2003). Steady-state amounts of JA and related compounds were higher in the salt-tolerant cultivar compared to the salt-sensitive cultivar. A wounding-JA salinity interaction in tissues, where salt stress induced wound-related genes through activation of the octadecanoid pathway, was reported in tomato (Dombrowski 2003).

Exogenous application of JA to salt-stressed rice seedlings improved recovery, suggesting a role for JA during the response to salinity stress (Kang et al. 2005). In barley, induction of genes involved in JA biosynthesis known as JA-responsive genes was reported as a key feature of the response to salinity (Walia et al. 2007). Expression profiling after a short-term exposure to salinity stress indicated a considerable overlap between genes regulated by salinity stress and JA application. It was suggested that three JA-regulated genes, *ADC*, *ribulose 1,5-bisphosphate carboxylase/oxygenase activase* (*RA*), and *apoplastic invertase*, were possibly involved in salinity tolerance mediated by JA (Walia et al. 2007).

Phytohormones are involved in many physiological and developmental processes, and play a crucial role in the adaptation to abiotic stress, as shown by the regulation of hormone synthesis in the presence of heavy metals (Peleg and Blumwald 2011). For example, copper (Cu) and cadmium (Cd) induce the rapid accumulation of JA in *Phaseolus coccineus*, and Cu has also been shown to have this effect in *Arabidopsis* plants (Maksymiec et al. 2005). The dynamics of JA accumulation showed a biphasic character in both plants. The first phase was a rapid increase of JA level occurring after exposure to Cu or Cd for several hours, followed by its rapid decrease. In the next phase, again, increase—but slow—of JA level occurred. Additionally, the most recent investigations indicate that Cu or Cd ions can induce some JA-responsive events, such as the *vegetative-storage protein 2* (*VSP2*) transcripts in *A. thaliana* (Mira et al. 2002) and *MAPK* in rice (Agrawal et al. 2003). In a global transcriptome analysis of the response to boron (B) toxicity using microarrays, it was found that high concentrations of boric acid treatment effected in up-regulation of JA-biosynthetic and JA-induced genes in barley leaves. Induction of JA-related genes (e.g., *GST*, *PR*) was found to be an important late response to B toxicity (Öz et al. 2009).

2.7 Salicylic Acid

2.7.1 Salicylic Acid Signaling

Perception and Transduction

Salicylic acid (SA) is synthesized through two distinct and compartmentalized pathways that employ different precursors: the phenylpropanoid pathway in the cytoplasm starting from phenylalanine, and the isochorismate pathway that takes place in the chloroplast (Rivas-San Vicente and Plasencia 2011). The scientific community has made an important effort to find the SA receptor. *NPR1* is the only known gene that, when mutated, renders plants insensitive to SA (Canet et al. 2010). *NPR1* has been shown to accumulate in the cytosol and migrate to the nucleus upon SA perception. This evidence indicates the essential role it plays in SA perception.

SA seems to be pivotal in the induction of different signal-transduction pathways. For example, in tobacco culture, SA also mediates alternative signal transduction pathways leading to induction of the *pathogenesis-related acidic β-1,3-glucanase* (*PR-N*) gene (Chen et al. 2002). ROS elevation and external Ca^{2+} influx are components likely associated with the SA activation mechanism. Unlike tobacco, in the aluminum (Al)-responsive oxidative burst in *Arabidopsis* cell suspension culture, SA signaling is activated downstream of ROS (Kunihiro et al. 2011). The increased expression of *ICS1* (*isochorismate synthase 1*) was involved in SA biosynthesis and *NPR1* (*nonexpressor of PR-1*) for SA perception and transduction. *NPR1* transferred SA signaling to induce expression of the respiratory burst oxidase homologs (*Atrbohs*) coding for plant NADPH oxidase, *AtrbohD*. A loop of SA signaling and

SA-dependent expression of the *AtrbohD* gene leading to prolonged ROS production and cell death developed in Al-exposed *Arabidopsis* cells.

SA has been recognized as a regulatory signal mediating plant responses to abiotic stresses such as drought, chilling, heat, heavy metal tolerance, and osmotic stress (Rivas-San Vicente and Plasencia 2011). However, the transduction pathways leading to gene expression induced by SA have not all been defined.

Abiotic Stress-Responsive Proteins and Genes Induced by SA

The abiotic stress tolerance induced by SA may have various causes. Several other genes may be induced by abiotic stress factors, while several "stress-related" compounds, such as ABA and SA, may also induce their expression (Salzman et al. 2005).

Many evidences support the important but not the only involvement of heat shock proteins (HSPs) in thermotolerance in plants (Clarke et al. 2004). The synthesis of these proteins is induced during heat acclimation, and it is proposed that they act as molecular chaperones to protect proteins against irreversible heat-induced damage. Heat shock was found to induce SA-regulated *pathogenesis-related 1* (*PR1*) transcripts and the ability of the nonexpressor of PR1 protein (*npr1-1*) mutant, which is involved in SA-signal transduction, to recover from heat stress was impaired (Clarke et al. 2009). Correspondingly, the constitutive expresser of PR1 protein in the *cpr5-1* mutant displayed an enhanced basal thermotolerant phenotype. Although the deduced *CPR5* sequence revealed no significant homology to any other genes, it has features of a signal transduction protein with a putative nuclear localization signal and five putative transmembrane domains (Clarke et al. 2000). The exogenous application of SA at an optimal concentration induced the synthesis of HSP70 and HSP17.6, which belongs to the class I cytosolic family of small-plant HSPs and has protein-refolding activity, parallel with an increase in the heat tolerance of pea (Pan et al. 2006).

Osmotin is a stress-responsive multifunctional 24-kDa protein and provides osmotolerance to plants (Husaini and Abdin 2008), and there is a direct correlation between overexpression of osmotin and the physiological parameters associated with tolerance against salt or water deficit stress (Goel et al. 2010). Osmotin could be involved in osmotic adjustment of cells by facilitating the accumulation or compartmentation of solutes (Barthakur et al. 2001). Osmotin is also known to protect the native structure of proteins during stress and repair of denatured proteins. It has been shown that overexpression of the *osmotin* gene in potato provides tolerance to salinity stress. The expression of an *osmotin* gene isolated from a cDNA library constructed from petal protoplast cultures of *Petunia hybrida* was strongly induced in leaves that were exposed to certain pathogens, or upon wounding in the damaged leaves (Kim et al. 2002). Moreover, its transcript levels increased in response to octadecanoid pathway intermediates and treatment with aspirin or SA, indicating that this *osmotin* gene is also involved in stress signal transduction.

Another osmoprotectant, glycine-betaine (GB, a compatible osmotic solute), is also accumulated by plants in response to high levels of NaCl, drought, cold stress, or ABA treatment, as shown in barley (Jagendorf and Takabe 2001). Additional inducers of GB accumulation have been detected in barley seedlings, including other inorganic salts, oxidants, and organic compounds. The same concentrations of SA that induced GB accumulation increased the level of lipid peroxidation.

Dehydrins, also known as LEA proteins, are the most commonly observed proteins to accumulate in plants in response not only to certain abiotic stresses such as drought, temperature stress, salinity, or wounding, but also to SA (Shen et al. 2004). Transcripts of the *BcDh2* dehydrin-like gene isolated from *Boea crassifolia* accumulated to a great extent when the plants were exposed to drought, salinity and moderate heat shock or hormone treatment.

Investigations on expression of the *SbPRP* gene, encoding a soybean proline-rich protein, showed that it accumulates in the leaves and epicotyls of soybean seedlings, but not in the cotyledons, hypocotyls or roots (He et al. 2002). In addition, *SbPRP* gene transcription was regulated by circadian rhythm, salt stress, drought stress and plant hormones such as SA. These results indicate that the *SbPRP* gene might play a role in plant responses to multiple internal and external factors. Another water stress-induced gene from *Brassica oleracea* (*BoWS*), encoding a 95-amino-acid protein, was up-regulated by SA, ABA, and mannitol, indicating that this gene is closely related to water-deficit stress in this species (Li et al. 2004).

2.7.2 SA in Plant Response to Abiotic Stresses

SA and Drought Stress

The endogenous SA content in leaves of *Phillyrea* plants increased progressively during drought (Munne-Bosch and Penuelas 2003). During recovery, the SA levels decreased but remained slightly higher than those observed before drought. Water deficit increased the SA content in the roots of barley plants, whereas the SA content in the leaves did not change (Bandurska and Stroinski 2005).

Application of exogenous SA improves the plant performance under water, as reported by several authors. Low concentrations of exogenous SA provided tolerance against the damaging effects of drought in tomato and bean plants, whereas higher concentrations did not show the same positive results (Senaratna et al. 2000). Enhanced tolerance to drought and dry matter accumulation was also observed in plants of wheat raised from grains soaked in acetyl SA aqueous solution (Hamada and Al-Hakimi 2001). SA is also involved in the promotion of drought-induced leaf senescence in *Salvia officinalis* plants grown under drought in Mediterranean field conditions (Abreu and Munne-Bosch 2008). In addition, SA applied exogenously was effective in providing resistance to the plants against the excessive water stress in cell suspensions from the fully turgid leaves of *Sporobolus stapfianus* (Ghasempour et al. 2001). Exogenous application of SA and GB enhanced the yield of sunflower

hybrids under different degrees of water stress. Under stress, diameter of the head (inflorescence), number of achenes and seed oil content were reduced. However, applications of SA and GB improved these parameters (Hussain et al. 2008).

At the molecular level, the constitutive or conditional enhanced expression of the *activated disease resistance 1* (*ADR1*) gene conferred significant drought tolerance in *Arabidopsis* (Chini et al. 2004). However, the northern analysis of abiotic marker genes revealed that the *dehydration-responsive element* (*DRE*)*B2A* was expressed in *adr1* plant lines. Furthermore, *DREB2A* expression was SA-dependent. In *adr1*/*ADR1 nahG* (*naphthalene hydroxylase G*), *adr1*/*ADR1 eds* (*enhanced disease susceptibility*)1 and *adr1*/*ADR1 abi1* double mutants, drought tolerance was significantly reduced. The SA-dependent suppression of some abiotic stress-signaling pathways may explain why the *adr1* mutants exhibit increased sensitivity to dehydration stress.

SA and High/Low Temperature Stress

There is some evidence that SA may be involved in heat shock responses in plants. Heat acclimation was also followed by a transient increase in the endogenous SA level in *Pisum sativum*, whereas inhibitors of SA biosynthesis reduced the tolerance of the plants to high temperature (Pan et al. 2006). The level of endogenous SA was shown to increase slightly after the first hour of heat stress in creeping bentgrass (Larkindale and Huang 2005). Not only exogenous SA application modulates stress effects, but also abiotic stress factors may alter the endogenous SA levels in plant cells. Experiments on grapevine also showed a sharp increase in the endogenous SA level at the beginning of heat acclimation, whereas exogenous SA also induced a level of thermotolerance similar to that of heat acclimation (Wang and Li 2006). This induction of thermotolerance was related to changes in the antioxidant enzyme activities.

Plants may also tolerate elevated temperatures without heat acclimation. This phenomenon is called basal thermotolerance (Clarke et al. 2004). In *Arabidopsis* seedlings, endogenous SA correlated with basal thermotolerance, but SA only partially induced expression of *HSP* genes. In other cases, plants subjected to mild heat stress may transiently acquire tolerance to previously lethal high temperatures (i.e., heat acclimatization or acquired thermotolerance) and SA is essential for acquired thermotolerance. Screening *Arabidopsis* mutants and *NahG* transgenic plants for their basal and acquired thermotolerance showed that SA plays a role in the development of acquired thermotolerance (Larkindale et al. 2005). Heat caused increased levels of thiobarbituric acid reactive substance (TBARS—an indicator of oxidative damage to membranes) and reduced survival. SA, together with Ca^{2+} messenger, ABA, and ET, protect plants against heat stress-induced oxidative damage (Larkindale and Knight 2002).

In chilling-resistant *Arabidopsis*, SA was shown to accumulate during cold treatment at 5 °C (Scott et al. 2004). *Arabidopsis* plants have restricted growth at

this temperature, and a higher level of SA might mediate this growth inhibition because SA-deficient *NahG* plants showed a higher growth rate than wild-type plants. SA treatments increased the chilling tolerance of the aerial portion of maize, cucumber, and rice seedlings, but not their radicles (Kang and Saltveit 2002). The SA-induced chilling tolerance in the aerial portions of maize and cucumber plants appeared to be associated with an increase in activity of antioxidative enzymes such as GR and GPX.

SA and Salinity and Heavy Metal Stress

Plants show a complex molecular response to salt stress, and it has been shown that SA could provide protection against this stress (Hamada and Al-Hakimi 2001). The salt stress leads to oxidative stress and severe impairment of plant survival. SA is involved in the plant response to salt stress by playing a role in the ROS-mediated damage caused by high salt and osmotic conditions. Evidence for a role of SA in the oxidative damage generated by NaCl and osmotic stress was obtained in a study on *Arabidopsis* seedlings (Borsani et al. 2001). Not only was the endogenous level of SA increased but also the activity of the SA biosynthesis enzyme benzoic acid 2-hydroxylase (BA2H) was induced under salt stress in rice seedlings (Sawada et al. 2006). The effect of salinity on the endogenous concentration of various phytohormones was investigated in *Iris hexagona* (Wang et al. 2001), and the levels of ABA and JA were generally found to increase, although, interestingly enough, SA declined in response to salinity.

Environmental pollution by heavy metals has received increasing attention over the last few decades. The accumulation of heavy metals in soils may be toxic to plants, and at toxic concentrations they interfere with numerous physiological processes (El-Tayeb et al. 2006). The SA level in plants increases or exogenous application of SA could ameliorate the adverse effects of heavy metal toxicity on plants. In one of the first works demonstrating the protective effect of SA against an abiotic stress factor, SA treatment induced tolerance against Cu toxicity in sunflower plants (El-Tayeb et al. 2006). Pretreatment of barley seedlings with SA prevented the lipid peroxidation caused by Cd and increased shoot and root (Metwally et al. 2003). This protection was not, however, the consequence of up-regulation of antioxidant activity. On the other hand, antioxidant enzyme activities were found to increase in Cd-stressed seedlings, but pretreatment with SA suppressed this effect. Cd treatment also increased the endogenous-free SA content in maize (Pál et al. 2005). In *Cassia* plants, SA enhanced Al tolerance by increasing the citrate efflux of the roots and thus inhibiting Al uptake (Yang et al. 2003). Al was also shown to increase the SA concentration of the roots. SA was a strong predictor of nickel (Ni) hyperaccumulation in the six diverse *Thlaspi* species investigated (Freeman et al. 2005). Elevation of free SA levels in *Arabidopsis* enhances the specific activity of serine acetyltransferase, leading to elevated glutathione and increased Ni resistance.

2.8 Gibberellins

Gibberellins (GAs) are a group of plant tetracyclic diterpenes that play roles in growth and development. Over 136 naturally occurring GAs are known, but GA_3, commonly known as gibberellic acid, is the most important GA in plants. This group of phytohormones is produced in roots and younger leaves and in seeds of higher plants. They control seed germination, leaf expansion, stem elongation and flowering. GAs have also been implicated in physiological responses in plants and alter plant metabolism under stress (Magome et al. 2004; Javid et al. 2011). The GA biosynthetic pathway has been described by a combination of biochemical and genetic approaches (Weiss and Ori 2007). Research conducted on *Arabidopsis* seeds revealed that exogenous GA_3 was able to reverse the inhibitory effect of salt, oxidative, and heat stresses through increasing content of endogenous SA. Moreover, priming with GA_3 was very effective in enhancing SA concentration in wheat when under salt stress (Iqbal et al. 2011). Interestingly, GAs and SA may play an important role in plant responses to abiotic stress. Hamayun et al. (2010) observed the role of GA_3 in salinity alleviation of soybean. They found that GA_3 application significantly promoted plant length and plant biomass, while they were markedly hindered by NaCl-induced salt stress. In contrast, they showed that endogenous SA content decreased under the influence of elevated GA_3, whereas it increased in NaCl-treated plants. In another study, it was demonstrated that exogenous GA in tomato may benefit plant growth and yield at low to moderate salinity (Maggio et al. 2010).

Results presented by Li et al. (2011) indicated that GA_3 application could decrease excess accumulation of ROS under suboptimal temperature in cucumber hypocotyls. These effects were correlated with the increasing activities of antioxidant enzymes, e.g., superoxide dismutase (SOD), catalase (CAT), APX, and GPX. In addition, it has been suggested that abiotic stress inhibits growth by means of a reduction in bioactive GA level, with consequent accumulation of DELLAs (Magome et al. 2004).

2.9 Brassinosteroids

Brassinosteroids (BRs) are plant growth regulators of steroidal nature that are synthesized by plants and affect many aspects of plant growth (Ahmad et al. 2011) and development, including cell elongation, photomorphogenesis, xylem differentiation, and seed germination (Sasse 2003), as well as adaptation to abiotic and biotic environmental stresses (Krishna 2003; Divi and Krishna 2009; Divi et al. 2010). A remarkable feature of BRs is their potential to increase resistance in plants to a wide spectrum of stresses, such as low and high temperatures, drought, high salt, and pathogen attack (Krishna 2003). Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage (Bajguz and Hayat 2009). It is known from the results reported by a few research

groups that BRs are involved in tolerance of plants to abiotic stress, e.g., heat, cold, drought and salinity. Dhaubhadel et al. (2002) reported increased accumulation of HSP in 24-epibrassinolide (EBR)-treated *Brassica napus* seedlings. Furthermore, other results showed that treatment with EBR increases the basic thermotolerance of *Brassica napus* and tomato seedlings (Kagale et al. 2007).

The effect of BRs on the inhibition of germination and seedling growth of rice (*Oryza sativa*) induced by salinity stress was studied by Anuradha and Rao (2001). Supplementation of the saline solution with BRs reduced the inhibitory effect of salinity on seed germination. In addition, BRs considerably restored pigment levels and increased the nitrate reductase activity. The activation of seedling growth by BRs under salinity stress was associated with enhanced levels of nucleic acids and soluble proteins. However, damage imposed by salt stress on nuclei and chloroplasts was significantly reduced by BR treatment (Krishna 2003; Javid et al. 2011). In another study, Kagale et al. (2007) reported that EBR, a BR, helps to overcome salt stress-induced inhibition of seed germination in *B. napus*. The results suggest that BRs can alleviate the inhibitory effects of salinity on germination, seedling growth, and plant yield. Moreover, positive correlations have been observed between BR levels and tolerance to cold stress and photo-oxidation in *Cucumis sativus* (Xia et al. 2009). Those results also showed that BR treatment induced the expression of regulatory genes *MAPK1*, *MAPK3*, and *RBOH* and genes related to antioxidative defense (Xia et al. 2009; Ahmad et al. 2011).

ROS play a particular signaling role in plant responses to abiotic stress. Unfortunately, little is known about cross-talk between oxidative stress and levels of BRs in plants. Núñez et al. (2003) revealed that when rice seedlings treated with BRs were subjected to saline stress, the activities of CAT, SOD, and GR increased and there was a slight increase in APX contents. Moreover, the results of studies conducted by Vardhini and Rao (2003) showed that during osmotic stress BRs increased the activity of CAT and reduced the activities of peroxidase and ascorbic acid oxidase in *Sorghum vulgare*.

Anuradha and Rao (2007) studied the effect of EBR and 28-homobrassinolide on seed germination and seedling growth of radish (*Raphanus sativus* L.) and activities of antioxidant cadmium (Cd) toxicity. Their results revealed that BRs strongly protect radish plants by increasing antioxidant enzyme activities (e.g., SOD, CAT, APX, and GPX), limiting ROS levels, and improving tolerance. There is also a rich literature on participation of the effects of BRs on heavy metal stress during seed germination and seedling growth.

The results of Villiers et al. (2012) revealed the existence of a transcriptional Cd-BR cross-talk and linked for the first time the BR signaling pathway with the Cd-induced response in *Arabidopsis*. These experiments also demonstrate that a modulation of the BR content in *Arabidopsis* seedlings affects their response to Cd. Moreover, earlier studies showed that BRs also protected against Cd stress in *Brassica juncea*, *Raphanus sativus*, and tomato cultivars (Hayat et al. 2007, 2010; Hasan et al. 2011), and reduced Ni toxicity in *Triticum aestivum* (Yusuf et al. 2011).

BRs regulate the stress response by a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein

synthesis, and the production of various chemical defense compounds (Bajguz and Hayat 2009; Javid et al. 2011). Since BRs interact with other phytohormones, it is likely that the stress tolerance conferring ability of BRs exhibits interactions with other stress hormones. There are a few reports indicating interactions of BRs with other stress-related hormones and their signaling pathways in conferring stress tolerance (Divi et al. 2010). Previous reports indicated interactions of BR with IAA (Hardtke et al. 2007), GAs (Shimada et al. 2006), ABA (Steber and McCourt 2001; Zhang et al. 2009b), ET (Arteca and Arteca 2001), and JA (Ren et al. 2009), but the relationship of BRs with these hormones has been documented primarily in plant growth regulatory processes.

2.10 Polyamines

Polyamines (PAs), including triamine spermidine (Spd), tetraamine spermine (Spm), and their precursor diamine putrescine (Put), are small aliphatic amines ubiquitous in all plant species (Kubiś 2006). These compounds are regarded as a new class of phytohormones. The concentrations of PAs in the plant (10^{-9}–10^{-5} M) are much higher than those of other endogenous phytohormones (10^{-13}–10^{-7} M). The total PA concentrations and the ratios between individual polyamines vary significantly depending on plant species, organ, tissue, and also developmental stage (Kuznetsov et al. 2006).

The biosynthesis of PAs is initiated with the formation of putresine. Put biosynthesis in plants occurs through two distinct pathways: directly from ornithine by ornithine decarboxylase and indirectly from arginine by arginine decarboxylase through agmatine by two enzymes: agmatine iminohydrolase and *N*-carbamoylputrescine aminohydrolase. Spd and Spm are formed by addition of aminopropyl groups to Put. These reactions are catalyzed by spermidine synthase and spermine synthase for Spd and Spm, respectively. The aminopropyl groups are produced from decarboxylation of SAM catalyzed by SAM decarboxylase. The formation of Put from arginine is usually associated with the plant responses to different stresses (Kuznetsov and Shevyakova 2007).

PAs play a pivotal role in the regulation of plant growth and developmental and physiological processes such as regulation of cell proliferation, somatic embryogenesis, differentiation and morphogenesis, dormancy breaking of tubers and in seed germination, development of flowers and fruits and senescence (Takahashi and Kakehi 2010; Alcazar et al. 2010). The participation of PAs in antioxidant activity, scavenging of free radicals and plant stress tolerance under various stress conditions has been widely reported (Groppa and Benavides 2008; Alcazar et al. 2010). These compounds play important roles in modulating the defense response of plants to diverse environmental stresses, such as salinity (Duan et al. 2008; Yamamoto et al. 2011; Hu et al. 2012), metal toxicity (Groppa et al. 2003; Zhao and Yang 2008), drought (Yamaguchi et al. 2007; Amooaghaie 2011), chilling (Groppa and Benavides 2008; Zhang et al. 2009a), high temperature (Todorova et al. 2007;

Cvikrova et al. 2012), and oxidative stress (Rider et al. 2007). The effect of various stress conditions on endogenous PA concentrations depends on plant species and cultivars, plant organ and developmental stage of tissues, and duration and intensity of stress (Jang et al. 2012).

Many authors have reported changes in PA contents under salt stress conditions. Mutlu and Bozcuk (2007) observed the effects of various concentration levels of NaCl on the endogenous levels of free, bound and total PAs in root tissues of salt-tolerant and salt-sensitive cultivars of sunflower. Changes in total PA levels in tested cultivars showed that only total Spm increased, whereas the levels of total Put in roots decreased in relation to the increase in salt stress. The increase of Spm under salt stress suggests its possible role in combating the adverse effect of salinity. Similar results were obtained in studies conducted by Yamamoto et al. (2011) concerning the physiological and biochemical responses to salt stress of rice seedlings. The decrease of Put and Spm and the increase of Spm were observed in leaf blades under salinity. It is worth to underline opinion that Put is responsible for salt-stress tolerance (Liu et al. 2006b) but on the other hand it is also suggested that Spm rather than putrescine is involved in this favorable response (Rodríguez et al. 2009). In plants, arginine decarboxylase is considered to be the rate-limiting step for PA biosynthesis under abiotic stress, leading to an increase in Put biosynthesis.

Heat stress induced an increase in levels of free Spd and Spm and a decrease in Put content in leaves and roots of tobacco (Cvikrova et al. 2012), whereas under cold stress the concentration of Put and Spd exhibited an increase and Spm and cadaverine levels showed only slight changes (Kocsy et al. 2011). Choudhary et al. (2010) tested changes induced by metal stress in PA contents of radish seedlings. Treatment with Cr^{6+} cation significantly reduced Put level with higher Spd and Spm content in comparison to controls.

Classical approaches using exogenous polyamine application and/or inhibitors of enzymes involved in PA biosynthesis hence pointed to a possible role of these compounds in plant adaptation to several environmental stresses. High cellular levels of PAs, particularly Spd and Spm, are positively correlated with plant tolerance to a wide array of environmental stresses (Alcazar et al. 2006). There is a significant evidence suggesting that exogenous application of PAs stabilizes plant cell membranes and protects them from damage under stress conditions (Chattopadhayay et al. 2002; Verma and Mishra 2005). Among the three major PAs, Spd is in many cases the most closely associated with stress tolerance in plants. The effect of exogenous Spd on PA content, metabolism, photosynthesis and the xanthophyll cycle in various plants under salt stress has been investigated. Duan et al. (2008) found that exogenous Spd application to salinity-stressed roots of cucumber markedly inhibited the accumulation of free Put and further promoted the increase of free Spd and Spm, soluble conjugated and insoluble bound Put, Spd and Spm, particularly Spd. In addition, under short-term salinity, exogenous Spd elevated the activities of antioxidant enzymes, suppressed free radical production and membrane damage, and thereby mitigated the oxidative stress. Similar results were obtained by Hu et al. (2012) in studies concerning tomato exposed to salinity–alkalinity mixed stress. Exogenous application of Spd to seeds can easily improve the saline–alkaline

tolerance of tomato seedlings. In other research, effects of Spd on photosynthesis and the xanthophyll cycle were demonstrated in cucumber seedlings under salinity (Shu et al. 2012). Cucumber seedlings with Spd application grew better than without Spd under salt stress and the content of free Put and Spd was significantly decreased, while Spm level increased. Also in cucumber, exogenous application of Spd differentially influenced the enzymes of antioxidative system in leaves under water-stressed conditions (Kubiś 2008). An increase of GPX activity, and, to a lesser degree, a reduction of SOD and CAT activities were observed in Spd-treated plants in comparison to untreated stressed plants. H_2O_2 and superoxide radical contents were also reduced in stressed plants after Spd pretreatment. Apart from application of only Spd, in other studies the effects of adding two PAs were observed. Two exogenous PAs, Spd and Put, can reduce the effects of salinity on growth and development of pomegranate (Amri et al. 2011). The application of Spd and Put suppressed the H_2O_2 production in cucumber leaves caused by chilling (Zhang et al. 2009a). Chattopadhayay et al. (2002) studied two other exogenous PAs, Spd and Spm, on salinity-stressed rice and demonstrated the inhibitory effect of salinity stress and its reversal by adding PAs. The same PAs (Spd and Spm) were added to *Malus hupehensis* under heavy metal stress and it was found that both can alleviate the lipid peroxidation caused by $CdCl_2$ (Zhao and Yang 2008). Amooaghaie (2011) tested the addition of Spm, Spd and Put to water-stressed soybean seedlings and found that PAs are involved in the stress adaptive response.

2.11 Interactions Among Phytohormone Signaling Pathways Under Abiotic Stresses

Phytohormones are crucial for adaptation of plants to abiotic stress conditions (Jaillais and Chory 2010). The perception of environmental factors triggers the activation of signal transduction cascades that interact with the baseline pathways transduced by phytohormones (Harrison 2012). ABA accumulation is one of the fastest responses (Yamaguchi-Shinozaki and Shinozaki 2006), and other phytohormones can play direct or indirect roles in the response of plants to abiotic stress (Mahouachi et al. 2007; Argueso et al. 2009; Brossa et al. 2011). The overlap between hormone-regulated processes during the adaptive responses of plants to environmental stresses suggests the existence of a complex network with extensive cross-talk between the different hormone signaling pathways (Peleg and Blumwald 2011).

Among the phytohormonal cross-talks, the interaction of ABA and ET in the abiotic stress response has been the most studied (Xiong 2007). Under drought and salt stress, ET production increases because of the activation of biosynthetic genes and enzymes (Liu and Zhang 2004). Increased accumulation of ET under abiotic stress may inhibit plant growth. It was thought that ABA may restrict the production of ET and thus could promote growth under abiotic stresses (Sharp 2002). On the other hand, ET may promote ABA biosynthesis under drought

stress. At the molecular level, interaction between ABA and ET under abiotic stress is suggested by the fact that certain transcription factors responsible for the activation of ABA/stress-responsive genes and ET-responsive genes are of a similar class and may be subject to similar regulations. The *ERF*/*EREBP* and *CBF*/*DREB* of ET responsive transcription factors may cross-activate stress genes (Fujimoto et al. 2000). Some ERF proteins act as transcription repressors regulating ET and ABA responses (Yang et al. 2005; Song et al. 2005). Accordingly, regulating these ERF transcriptional regulators may result in altered drought stress sensitivity (Song et al. 2005). The antagonism between ET and ABA was also found in other stress response processes. It was noted that the *ABA-induced* genes *ABI1* and *ABI2* were highly up-regulated by ET (De Paepe et al. 2004). ET inhibits ABA-induced stomatal closure and reduces the induction of the ABA-induced gene *RAB18* (Tanaka et al. 2005). These negative regulators of ABA signaling may thus reduce ABA responses.

Regarding the function in plant defense against environmental factors, JA may interact with ABA synthesis under water stress conditions (Bandurska et al. 2003) and JA and ABA could regulate stomatal closure (Acharya and Assmann 2009). The molecular levels in crosstalk between JA and ABA signal transduction pathways have recently received attention. Lackman et al. (2011) found that JA signaling involves the ABA receptor PYL4 regulating metabolic reprogramming in *Arabidopsis* and tobacco cells. It has also been shown that both the JA and the ABA pathways can recruit the TOPLESS co-repressor proteins through interaction with specific adaptor proteins (Pauwels et al. 2010). Cross-talk between the JA and ABA signaling pathways can also occur through *MYC2* and, actually, this transcription factor was originally described as an activator of ABA signaling (Abe et al. 2003) before being found to play a critical role in JA signaling (Lorenzo et al. 2004).

Several interesting papers have recently mentioned the interaction between ABA and SA in responses to abiotic stresses, but the relationship has not yet been completely elucidated (Yasuda et al. 2008). In wheat, SA treatment caused ABA accumulation and increased the level of resistance to salinity (Shakirova et al. 2003). Furthermore, pre-treatment with SA protected barley plants against the damaging influence of water deficit and also increased the ABA content in the leaves (Bandurska and Stroinski 2005). Treatment of pea plants with an ABA biosynthesis inhibitor resulted in disappearance of the SA peak during heat acclimation (Liu et al. 2006a). The effect of the ABA inhibitor on SA levels implies that heat acclimation causes a rapid rise in ABA prior to a peak in the SA content. Rapid ABA elevation corresponding to heat acclimation should precede the SA content. When plants treated with ABA for 2 days were exposed to chilling, the SA levels decreased, in contrast to their unchilled counterparts (Pál et al. 2011). A possible reason for this is that ABA-treated plants do not require further enhancement of the SA-related pathway. Although the exact mechanism of the cross-talk between the ABA and SA signaling pathways is unclear, it is also possible that ABA inhibits the activity of SA-glucosyl transferase, thus increasing the level of free SA. An overlap may exist between the ABA-induced cold acclimation and the SA-related stress response.

ABA is a known antagonist of BR signaling. Expression of the BR-enhanced expression (BEE) proteins was repressed by ABA. BEEs are members of the *basic helix-loop-helix* (*bHLH*) transcription factors required for the BR response in *Arabidopsis* (Friedrichsen et al. 2002). Stimulation of proline synthesis by ABA and salt stress was correlated with increase in expression of Delta-1-pyrroline-5-carboxylate synthetase 1 (P5CS1), the rate-limiting enzyme in proline biosynthesis. Both ABA and salt induction of *P5CS1* transcription were inhibited by BRs in light-grown *Arabidopsis* plants. Thus, it was suggested that BRs might negatively regulate proline accumulation, which is a common salt and ABA response pathway (Abraham et al. 2003). Expression of the *12-oxo-phytodienoic acid reductase 3* (*OPR3*) gene, encoding an enzyme functioning in JA biosynthesis, was induced by BR treatment. This indicates a potential link between BR action and JA biosynthesis (Mussig et al. 2000). It was shown that exogenous application of BRs modified the activities of antioxidant enzymes and cellular levels of nonenzymatic antioxidants in plants under stress conditions (Núñez et al. 2003).

Despite the well-known physiological functions of ET production and stress signaling via ROS during stresses, the action of ET in conjunction with ROS has been little elucidated. The relationship between ET production and ROS accumulation during the response of transgenic tobacco lines to abiotic stress indicated that these plants exhibited significantly reduced H_2O_2-induced gene-specific expression of ACS members, which were regulated in a time-dependent manner following salt stress (Wi et al. 2010). This stress tolerance of H_2O_2-treated transgenic plants resulted from reduced ET biosynthesis, which decreased ROS accumulation via increased gene expression and activity of ROS-detoxifying enzymes, including SOD and CAT. Therefore, it is suggested that ET plays a potentially critical role as an amplifier for ROS accumulation, implying a synergistic effect between biosynthesis of ROS and ET (Wi et al. 2010). ET has an important role in triggering programmed cell death in plant cells (Poór and Tari 2011). A simultaneous increase in ET production and H_2O_2 accumulation was observed in Cd-induced cell death (Yakimova et al. 2006). Moreover, ET and H_2O_2 can act as self-amplifying signal molecules in feed-forward loop regulation (Wi et al. 2010). If ET were to exceed a survivable threshold, subsequent endogenous ROS levels would determine the severity of tissue damage by increasing further ET biosynthesis.

Analysis of auxin signaling in *Arabidopsis* plants showed that transcripts of several auxin receptors and Aux/IAA transcriptional repressors were reduced in response to apoplastic ROS (Blomster et al. 2011). The ROS-derived changes in the expression of auxin signaling genes partially overlapped with abiotic stress, pathogen responses, and SA signaling. Several mechanisms known to suppress auxin signaling during biotic stress were excluded, indicating that ROS regulated auxin responses via a novel mechanism.

Molecular studies revealed that the cross-talk between phytohormones represents a precisely coordinated web of nodes and lines (Eyidogan et al. 2012). Considering the cross-talk among different hormone signaling pathways, the roles of hormone signaling in regulating expression of the genome seem very complex.

3 Conclusion and Future Perspective

The multiple stress responses via different phytohormones are important mechanisms by which plants cope in adverse environmental conditions. Perception, signal transduction, and changes in gene expression are the main aspects in the substantial process of phytohormonal signaling pathways in plant response and/or tolerance. Many novel lines of evidence have implicated the key roles of ABA, ET, CKs, IAA, JA, and SA in plant signaling pathways in defense against abiotic stresses. However, the defensive mechanisms induced by some phytohormones, such as GA, BRs, and PAs, through which plants integrate adaptive responses, are not well known. Different signal transduction pathways act independently and also have a significant cross-talk with each other in response or tolerance of one or more abiotic stresses.

There are still major challenges concerning interactions between different phytohormones under various environmental conditions. Further integration of molecular, biochemical, and physiological studies need to address how phytohormone signaling and changes in gene expression are integrated into phenotype and specific traits. Understanding phytohormone signaling in detail will provide the necessary tools for improving agricultural practice and production.

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Chapter 5
Nitric Oxide: Role in Plants Under Abiotic Stress

Andrzej Bajguz

1 Introduction

Nitric oxide (NO) is a gaseous compound previously investigated as an air pollutant and metabolic product of certain bacteria. Formerly, ethylene was the only gaseous signalling molecule in plants. NO emission from plants was first observed by Klepper in 1975, much earlier than in animals (Klepper 1978, 1979). In 1992, *Science Magazine* declared NO as the "Molecule of the Year" (Koshland 1992). In 1998, The Nobel Prize in Physiology or Medicine was awarded jointly to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad "for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system" (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1998/). Nowadays, NO has emerged in plant signal transduction pathways, where NO can interact with other signalling molecules such as cyclic nucleotides (cAMP, cGMP), cytosolic calcium, H_2O_2, brassinosteroids, abscisic, jasmonic and salicylic acids (Yamasaki 2005; Arasimowicz and Floryszak-Wieczorek 2007).

Environmental stresses limit plant growth and crop production. Understanding the mechanisms of plant signal transduction is important to improve production efficiency. Plants cope with stresses by activating signal pathways that control and coordinate the physiological and biochemical responses necessary for their adaptation. Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific genes. They are involved in the sequence of stress responses, e.g. protection of membranes and proteins, and free radical and toxic compound scavenging. Oxidative stress can cause disruptions in the redox homeostasis by increasing the rate of reactive oxygen species (ROS) generation.

A. Bajguz (✉)
Institute of Biology, Department of Plant Biochemistry and Toxicology, University of Bialystok, Swierkowa 20 B, 15-950 Bialystok, Poland
e-mail: abajguz@uwb.edu.pl

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Table 5.1 Role of NO in different plant processes

Processes	
Growth and development	Germination
	Root organogenesis
	Stomatal movement
	Senescence and programmed cell death
	Cell wall lignification
	Nodule metabolism
Metabolism of subcellular compartments	Chloroplasts: chlorophyll biosynthesis, photophosphorylation
	Mitochondria: cytochrome *c* oxidase, alternative oxidase
	Peroxisomes: catalase regulation
	Cytosol: aconitase modulation
Biochemical interactions	Protein nitration
	Ferritin (iron homeostasis)
	Haemoglobins (NO levels modulation)
	ROS, GSH, ethylene, MAPKs, Ca^{2+}, ABA
Abiotic stress	Wounding, salinity, high temperature, drought, hypoxia
Biotic stress	Hypersensitive reaction, systemic-acquired resistance

The enhanced oxidative stress resistance in plants is correlated with an increase in the synthesis of stress proteins, up-regulation of many antioxidants (Smirnoff 1995; Fujita et al. 2006; Zaninotto et al. 2006; Neill et al. 2008; Pareek et al. 2010).

In recent years, nitric oxide (NO) can mediate many physiological and developmental processes including seed germination, floral transition and stomatal movement. NO decreases the expansion of leaves, growth of shoots and roots and changes viscosity of thylakoid. It was also implicated in the DNA damage response (Hayat et al. 2010). Table 5.1 shows different processes in plants that can be regulated by NO. Moreover, signalling interactions between abscisic acid (ABA), H_2O_2 and NO have been presented in Fig. 5.1. ABA activates generation of H_2O_2 by NAD(P)H oxidase. It may include the ABA receptor(s), Ca^{2+}/calmodulin, the OST1 protein kinase and other unidentified components. On the other hand, H_2O_2 induces generation of NO by nitrate reductase and NOS-like enzyme(s) via a yet to be fully characterized signalling pathway. It may include the OXI1 protein kinase which is required for full activation of the mitogen-activated protein kinases (MAPKs). However, NO induces stomatal closure via steps that require Ca^{2+}, cGMP and MAPKs (Garcia-Mata and Lamattina 2002, 2003; Neill et al. 2003a, b, 2008; Delledonne 2005; Wang et al. 2012a, b).

2 Nitric Oxide and Reactive Nitrogen Species

Nitric oxide is one of the smallest diatomic molecules, with high diffusivity (4.8×10^{-5} cm^2 s^{-1} in H_2O), exhibiting hydrophobic properties. Thus, NO may not only easily migrate in the hydrophilic regions of the cell (cytoplasm) but also freely

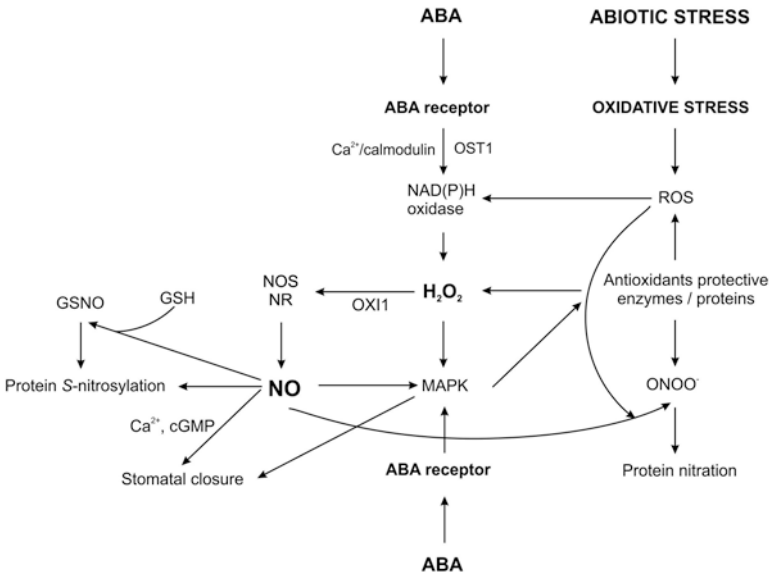

Fig. 5.1 Abiotic stress and signalling interactions between abscisic acid (ABA), H_2O_2 and nitric oxide (NO)

diffuse through the lipid phase of membranes (Arasimowicz and Floryszak-Wieczorek 2007). The half-life of NO in biological tissues is estimated to be below 6 s. This short half-life reflects the highly reactive nature of NO, which reacts: (1) directly with metal complexes and other radicals and (2) indirectly as a reactive nitrogen species (RNS) with DNA, proteins and lipids (Fig. 5.2) (Bethke et al. 2004; Sánchez-Calvo et al. 2013).

NO^+ mediates electrophoretic attack on reactive sulfur, oxygen, nitrogen and aromatic carbon centers, with thiols being the most reactive group. Overproduction of these species under stress conditions can raise the process of nitration in proteins (3-nitrotyrosine), nucleic acids (8-nitroguanine) and fatty acids. It acts as a prooxidant to stimulate superoxide generation by NADPH cytochrome P450 reductase and NO synthases. However, the process of protein and lipid nitrations has not been fully investigated in plants. Nitration is considered as potential marker of nitrosative stress (Schopfer et al. 2003; Wang et al. 2005; Chaki et al. 2009a, b). The presence of an unpaired electron in π orbital makes NO a radical. There are three interconvertible forms of NO: (1) the highly reactive, uncharged free radical ($NO^{\bullet}$) with an unpaired electron, (2) the nitrosonium cation (NO^+) and (3) nitroxyl anion (NO^-) (Table 5.2) (Floryszak-Wieczorek et al. 2006; Ederli et al. 2009). $NO^{\bullet}$ rapidly reacts with O_2 to form NO_2 and breaks down into nitrite and nitrate in an aqueous solution. The ion peroxynitrite ($OONO^-$) is synthesized when NO reacts with superoxide (O^{2-}) or H_2O_2. $NO^{\bullet}$ reacts with iron and iron-containing proteins, thus forming iron-nitrosyl complexes. NO is a perfect plant signalling molecule because it has highly promiscuous reactivity and can readily cross cell membranes (Wang et al. 2005; Corpas et al. 2007, 2001; Moreau et al. 2009; Baudouin 2011; Gupta et al. 2011).

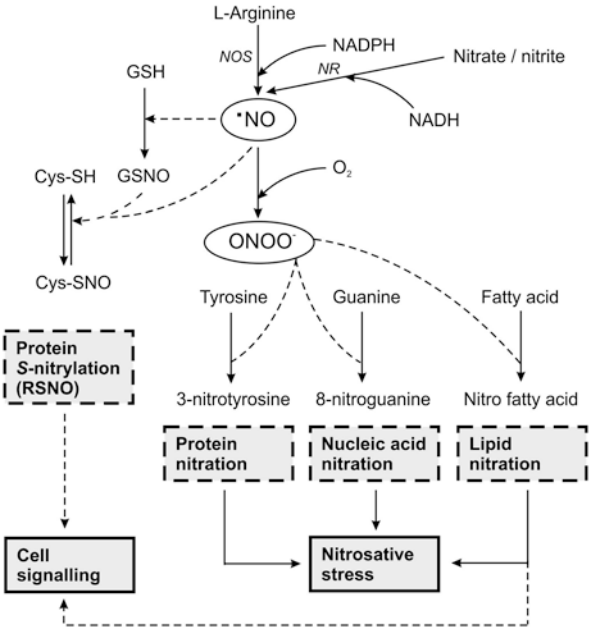

Fig. 5.2 Role of nitric oxide and other reactive nitrogen species involved in cell signalling

Table 5.2 Reactive nitrogen species

Radicals	Non-radicals
Nitric oxide ($NO^{\bullet}$)	Nitroxyl anion (NO^{-})
Nitrogen dioxide ($NO_2^{\bullet}$)	Nitrosonium cation (NO^{+})
	Nitrous acid (HNO_2)
	Dinitrogen trioxide (N_2O_3)
	Dinitrogen tetroxide (N_2O_4)
	Peroxynitrite ($ONOO^{-}$)
	Peroxynitrous acid (ONOOH)
	Alkyl peroxynitrite (ROONO)

NO reacts with proteins in different ways: (1) by metals present in the protein giving metal nitrosyl; (2) by sulfhydryl groups to render a process of *S*-nitrosylation and (3) by adding a nitro ($–NO_2$) group in nitration process (Fig. 5.2). Cysteine, methionine, tryptophan and tyrosine can be nitrate. NO also reacts with superoxide radicals (O_2^{-}) to generate peroxynitrite ($ONOO^{-}$). The process of *S*-nitrosylation of glutathione to form the *S*-nitrosoglutathione (GSNO) functions as NO source. Under abiotic stresses, L-arginine-dependent NO synthase (NOS) and/or nitrate reductase (NR) generated NO which can react with reduced glutathione (GSH) in the presence of O_2 to GSNO. This metabolite is converted by GSNO reductase (GSNOR) into oxidized glutathione (GSSG) and NH_3 (Crawford 2006; Corpas et al. 2004, 2009; Chaki et al. 2009a, b; Heikal et al. 2009; Molassiotis and Fotopoulos 2011; Yu et al. 2012).

3 Sources of Nitric Oxide

The concentration of NO is regulated by its synthesis and removal. There are many possible sources of NO (Fig. 5.3).

The reductive pathways that lead to NO production depend on nitrite which is primarily produced from nitrate by nitrate reductase (NR). Reductive formation of NO is assumed to depend on NR activity. Moreover, NO can be generated non-enzymatically as a by-product of denitrification, fixation of nitrogen and respiration. Superoxide anions, glutathione, transition metals and non-symbiotic haemoglobins are responsible for quick NO removal from the solution. The enzyme responsible for NO generation in animals is NO synthase (NOS). In plants, the activity of NOS-like enzymes has been detected. They appear to produce NO by two different pathways (Fig. 5.3) (Dordas et al. 2003, 2004; Corpas et al. 2004, 2009; Chaki et al. 2009a, b; Molassiotis and Fotopoulos 2011):

1. The L-arginine-dependent pathway uses NAD(P)H and O_2 as cosubstrates and is catalyzed by nitric oxide synthase according to the following reaction:

$$\text{L-arginine} + \text{NAD}(\text{P})\text{H} + \text{H}^+ + \text{O}_2 \rightarrow \text{L-citruline} + \text{NAD}(\text{P})^+ + \text{NO}$$

2. The nitrite-dependent pathway uses NADH or "electrons" as reductants and is catalyzed by a different enzymes according to the following reaction:

$$\text{NO}_2^- + \text{e}^- + 2\text{H}^+ \rightarrow 2\text{NO} + \text{H}_2\text{O}$$

In mammals, NO is produced mainly by the enzyme NOS (EC 1.14.13.39), which catalyzes the conversion of L-arginine to L-citruline and NO in the presence of O_2. There are three different known NOS isoforms, two constitutive (neuronal [nNOS] and endothelial [eNOS]) and one inducible (iNOS). In plants, the role of NOS-like enzymes in NO production was never confirmed. NO production from L-arginine or polyamine is an oxidative route. Polyamine synthesis depends on the availability of arginine which is a substrate for the enzyme arginine decarboxylase (ADC).

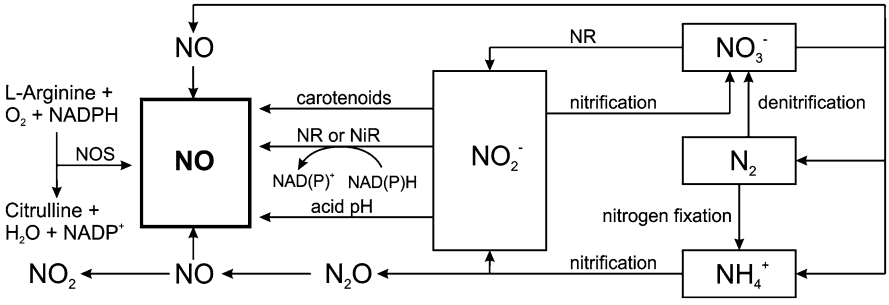

Fig. 5.3 Enzymatic and non-enzymatic sources of nitric oxide

Arginine leads to the biosynthesis of spermine and spermidine. A decrease in arginase activity resulted in increased production of NO, while up-regulation of arginase reduced the release of NO. It suggests that polyamine synthesis is involved in the production of NO (Rockel et al. 2002).

In plants, reduction of nitrite to NO was originally thought to be only catalyzed by nitrate reductases (NR) (Fig. 5.3). Under normal growth conditions, the percentage of in vivo activity of NR involved in nitrite reduction is estimated to constitute only about 1 % of the nitrate-reducing capacity (Modolo et al. 2005, 2006). NR is involved in the regulation of stomatal opening, activation of antioxidant enzymes, induction of crassulacean acid metabolism upon environmental stresses. Furthermore, nitrite reduction to NO can also be catalyzed by the peroxisomal enzyme xanthine oxidoreductase (XOR). In pea (*Pisum sativum*) leaves, the activity of XOD is associated with peroxisomes. It suggests the possibility of an interaction between the production of ROS and RNS. XOR can produce the free radicals $O_2^{\bullet-}$ and $NO^{\bullet}$ during its catalytic reaction, depending on whether oxygen tensions are high and low, respectively. This property of producing these radicals confers a key role of XOR as a source of signal molecules in plants. Another Ni-NOR enzyme can generate NO from nitrite in tobacco roots (Zhang et al. 1998; Godber et al. 2000; Harrison 2002; Li et al. 2004).

In plants, NO can also be generated by non-enzymatic mechanisms. The nitrification/denitrification cycle releases NO into the atmosphere as a by-product of N_2O oxidation (Fig. 5.3). It is known that the non-enzymatic reduction of nitrite can lead to the formation of NO. This reaction is favored at acidic pH when nitrite can dismutate NO and nitrate. Nitrite can also be chemically reduced by ascorbic acid at pH 3–6 to yield NO and dehydroascorbic acid. This reaction could occur under microlocalized pH conditions in the chloroplast and apoplastic space where ascorbic acid is known to be present. In barley aleurone layer cells, NO can also be synthesized by the reduction of nitrite by ascorbate at acidic pH. Another non-enzymatic mechanism proposed for NO formation is the light-mediated reduction of NO_2 by carotenoids (Wojtaszek 2000; Neill et al. 2003a, b; Bethke et al. 2004). In companion cells of *Vicia faba*, generation of NO was induced by salicylic acid (JA) and H_2O_2. However, in phloem, synthesis of NO was only dependent on Ca^{2+} and activity of NOS (Gaupels et al. 2008).

Several types of symbiotic and non-symbiotic haemoglobins have been found in plants. Non-symbiotic haemoglobins are divided into: class-1 proteins (Hb1) and class-2 proteins (Hb2), which have high or low affinity with oxygen, respectively. Hb1 proteins are induced by hypoxia and protect plants in low oxygen environments. Hypoxia and anoxia also increase emission of NO. It has been proposed that Hb1 proteins modulate the level of NO in plants. Hb1 reacts with NO to produce nitrate and methemoglobin in the presence of oxygen (Fig. 5.4). Methemoglobin (Fe^{3+}) must be reduced back to hemoglobin (Fe^{2+}) to react again with NO. NADPH alone can reduce Hb1 (Fe^{3+}) to Hb1 (Fe^{2+}), indicating that Hb1 by itself can catalytically degrade NO to nitrate. Furthermore, *S*-nitrosylation of Hb1 was found, indicating that Hb1 can scavenge NO through the production of *S*-nitrosohemoglobin. *S*-nitrosylation is a key mechanism for NO signalling in animals but its role in plant

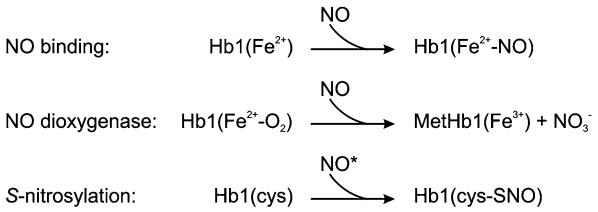

Fig. 5.4 Reactions of NO with hemoglobin. Figure shows NO binding to deoxyhemoglobin, NO reacting with oxyhemoglobin and NO• nitrosylating a cysteine to form an *S*-nitrosothiol. Note that NO• refers to oxidized forms of NO or *S*-nitrosoglutathione (GSNO)

signalling is still unknown. The Hb2 has a lower affinity for oxygen and are better candidates to produce NO from nitrite at low oxygen levels (Arredondo-Peter et al. 1997; Taylor et al. 1994; Trevaskis et al. 1997; Dordas et al. 2003, 2004; Perazzolli et al. 2004; Molassiotis and Fotopoulos 2011; Yu et al. 2012).

4 Reactive Nitrogen Species and Abiotic Stress

Nitric oxide plays a double role as an antioxidant and an anti-stress compound against various abiotic stresses (Table 5.3). Increased NO• production in response to certain abiotic stresses has been reported in different plant species. NO also acts as a messenger following pathogen invasion and during stimulation of hypersensitivity response (Murgia et al. 2004; Qiao and Fan 2008).

4.1 Drought

Exogenously applied sodium nitroprusside (SNP), an NO donor, can enhance plant tolerance to drought stress by stomatal closure (García-Mata and Lamattina 2001). NO induced stomatal closure by modulating intracellular Ca^{2+} in *Vicia faba* guard cells. NO selectively activates intracellular Ca^{2+} channels in guard cells through a cGMP/cADPR-dependent signalling pathway (García-Mata and Lamattina 2003). In *Arabidopsis thaliana* guard cells, NR-mediated NO synthesis was associated with ABA and was required for ABA-induced stomatal closure (Desikan et al. 2002, 2004). ABA is synthesized following turgor loss and stimulates NO synthesis in guard cells. However, the effect of dehydration on NO generation has not been fully analyzed yet. In response to drought stress, an increase in NOS-like activity was observed in wheat seedlings, and ABA accumulation was inhibited by NOS inhibitors (L-NNA). ROS and NO induced the biosynthesis of ABA to maintain the water in leaves (Zhao et al. 2001). It was shown that ABA can enhance the activity of NADPH oxidase under water stress (Jiang and Zhang 2002; Lu et al. 2009). NO is

Table 5.3 Nitric oxide as regulatory mediator of physiological responses to abiotic stresses (examples)

Type of abiotic stress	NO-mediated effect	Plant species of induced NO	References
Drought/osmotic stress	Involving in ABA signalling, stomatal closure induction of ABA synthesis, late embryogenesis abundant (LEA) expression	*Nicotiana tabacum* *Pisum sativum*	Gould et al. (2003) Leshem and Haramaty (1996)
Heavy metal toxicity	Increased the root elongation, reduced the NOS activity, reduced NO level	*Hibiscus moscheutos*	Tian et al. (2007)
Herbicide	Promoted the activity of antioxidant enzymes	*Scenedesmus obliquus* *Chlamydomonas reinhardtii*	Mallick et al. (2000) Sakihama et al. (2002)
High temperature	Increased tolerance of seedlings, rapid NO release	*Medicago sativa* *Nicotiana tabacum*	Leshem et al. (1998) Gould et al. (2003)
Low temperature	Decline the ROS level	*Scenedesmus obliquus*	Mallick et al. (2000)
Salinity	Increased osmotic tolerance; induced the expression of Na^+/H^+ antiporter gene	*Nicotiana tabacum* *Zea mays*	Gould et al. (2003) Zhang et al. (2006)
UV-B radiation	Induced the expression of chalcone synthase gene	*Arabidopsis thaliana*	Mackerness et al. (2001)
Wounding	NO burst result in cell death	*Arabidopsis thaliana* *Taxus brevifolia*	Garces et al. (2001) Pedroso et al. (2000)

involved in the ABA-induced up-regulation in the expression and the activities of antioxidant enzymes. ABA-induced NO generation, which acts downstream of H_2O_2 production, activates an MAPK, resulting in the induction of antioxidant defence systems in the ABA signalling in leaves of maize plants (Zhang et al. 2007).

It is known that NO enhanced the content of ABA in wheat root tip under osmotic stress. 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-1-oxy-3-oxide (c-PTIO) or NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) inhibited the NO induction. However, NO induction was enhanced by ROS (Zhao et al. 2001; Xing et al. 2004). NO alleviates the ROS-mediated cytotoxic process in potato leaves (Beligni and Lamattina 1999). ROS-mediated damages (cell death, ion leakage and DNA fragmentation) caused by drought stress have been inhibited by exogenous NO application (Beligni and Lamattina 1999, 2000, 2001; Tun et al. 2001; Carimi et al. 2005; Hao et al. 2008).

In maize leaves exposed to water stress and treated with NOS and NR inhibitors, the synthesis of NO has been blocked. It suggests that NO is produced from NOS and NR. Water stress also induced increases in activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and proline accumulation. Exogenous NO increased the activities of water stress-induced subcellular antioxidant enzymes, which decreased the accumulation of H_2O_2. These results also suggest that NOS and NR are involved in water stress-induced NO production and NOS is the major source of NO (Sang et al. 2008; Zhao et al. 2008; Xiong et al. 2012).

Hao et al. (2008) also showed that NO is involved in the signalling of drought-induced protective responses in maize seedlings. Both NOS activity and the rate of NO release increased substantially under stress. It indicates that production of NO under drought stress may be generated from NOS-like activity. Maize leaves treated with SNP alleviated water loss and oxidative damage. This effect has been counteracted in plants treated with c-PTIO. Moreover, treatment of (NG-nitro-L-arginine methyl ester) L-NAME led to a higher membrane permeability, higher transpiration rate and lower activity of SOD.

4.2 Extreme Temperature

Temperature stress limits plant growth and development. High temperature stress leads to lipid peroxidation, membrane injury, metabolite degradation, inactivation of enzymes, pigment bleaching and DNA strands disruption in plants. Similarly, cold stress causes changes in biochemical and physiological processes and ROS-homoeostasis (Suzuki and Mittler 2006; Zhao et al. 2009).

High temperature treatment of lucerne and alfalfa resulted in an increase of NO synthesis, whereas, the application of exogenous NO induced cold tolerance in tomato, wheat and maize. It is possible that this effect was related to the antioxidative action of NO by the intensification of peroxidative metabolism in cold and heat stress (Neill et al. 2002b, 2003a, b). The application of SNP and *S*-nitroso-*N*-*acetylpenicillamine* (SNAP) alleviated the heat stress-induced ion leakage increase, growth suppression and cell viability decrease in reed callus (Song et al. 2006). It was also shown that the content of H_2O_2 and MDA was decreased but activities of SOD, catalase, APX and peroxidase, and NO content were increased. These results suggest that NO can effectively protect plants from oxidative stress by activating antioxidant enzymes. Zhao et al. (2009) presents that cold acclimation induced an increase in endogenous NO production in wild type of *Arabidopsis thaliana* and mutant *Atnoa1*/*rif1* (for NO associated 1/resistant to inhibition by fosmidomycin 1) leaves. Endogenous NO level in *nia1nia2* (NR-defective double mutant) leaves was lower than that in wild type. Cold acclimation stimulated NR activity and induced up-regulation of NIA1 gene expression. In contrast, cold acclimation reduced quantity of NOA1/RIF1 protein and inhibited NOS activity. These results indicated that up-regulation of NR-dependent NO synthesis underpins cold acclimation-induced

NO production. Uchida et al. (2002) reported that H_2O_2 and NO can increase both salt and heat tolerance in rice seedlings. It resulted in higher quantum yield for photosynthesis II than in non-treated control under salinity and heat stress. NO application resulted in enhancement of antioxidant enzyme activities and expression of genes encoding sucrose-phosphate synthase, D-pyrroline-5-carboxylate synthase and small HSP 26.

4.3 Heavy Metals

Plants have a remarkable ability to take up and accumulate heavy metals from their external, for example aquatic environment. Metal contamination of the aquatic environment occurs as a result of human activities and affects organisms at the biochemical, cellular, community and population level. High concentration of heavy metals exerts toxic effect on the metabolic pathways of plants (Ahmad et al. 2011, 2012). Toxicity mechanisms include the blocking of functional groups of important molecules, e.g. enzymes, polynucleotides, transport systems for essential nutrients and ions, displacement and/or substitution of essential ions from cellular sites, denaturation and inactivation of enzymes and disruption of cell and organellar membrane integrity (Hall 2002).

Nitric oxide has been reported to regulate toxic metal response in plants. NO enhanced the activity of antioxidant enzymes (Gill et al. 2013). Treatment of SNP caused the reduction in copper toxicity and NH_4^+ accumulation in rice leaves (Yu et al. 2005). The protective effect of SNP can be reversed by cPTIO. It suggests that the protective effect of SNP is attributed to NO released. These results also suggest that reduction of Cu-induced toxicity and NH_4^+ accumulation by SNP is mediated through its ability to scavenge active oxygen species. Ye et al. (2013) showed that Cd induced caspase-3-like activity and was inhibited in the presence of cPTIO. It suggests that NO mediated the activation of caspase-3-like protease under heavy metal stress. Pretreatment with cPTIO effectively inhibited Cd-induced MAPK activation, indicating that NO also affected the MAPK pathway. Zhang et al. (2008a) reported that pretreatment of SNP caused increase in proline level in *Chlamydomonas reinhardtii* cells treated with copper. This accumulation was inhibited by c-PTIO application. Copper-treated algae show that NO has able to stimulate the activity of Δ′-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11) which is the key enzyme of proline biosynthesis. These results indicate that copper-responsive proline synthesis is related to generation of NO in *Chlamydomonas reinhardtii*.

In the roots of *Pisum sativum* and *Brassica juncea*, treated with 100 μM Cu, Zn and Cd, there is time-dependent endogenous $NO^{\bullet}$ production (Bartha et al. 2005). In contrast, the concentration of 50 μM Cd (a toxic concentration) caused inhibition of growth and oxidative damage (Sandalio et al. 2002; Romero-Puertas et al. 2002, 2004), as well as reduction in $NO^{\bullet}$ content (Rodríguez-Serrano et al. 2006; Barroso et al. 2006). In soybean plants exposed to 200 mM $CdCl_2$, similar effect on growth was observed, but the application of NO increases the level of heme oxygenase-1

expression and protects it against oxidative damage (Noriega et al. 2007). In contrast, pretreatment of seedlings with 100 mM SNP protected sunflower leaves against Cd-induced oxidative stress (Laspina et al. 2005). A similar effect has been found in *Lupinus* roots treated with 50 mM Cd^{2+} (Kopyra and Gwóźdź 2003). It was suggested that the protective effect of NO could consist of stimulation of SOD activity to counteract the overproduction of superoxide radicals, thus preventing the formation of peroxynitrite from NO and O_2^-. Similar properties of NO were also found in plants under aluminum, cadmium and copper stress (Tian et al. 2007; Singh et al. 2008; Li et al. 2012; Qiu et al. 2013). On the other hand, *Cassia tora* plants pretreated for 12 h with 0.4 mM SNP and then treated with 10 mM aluminum for 24 h showed significantly greater root elongation and decrease in accumulation of aluminum in root apexes compared to control plants (Wang and Yang 2005). Application of SNP resulted in enhancement of SOD, CAT, APX activities and protein content in plants exposed to aluminum (Zhang et al. 2008b).

Hu et al. (2007) also found that pretreatment with NO improved seed germination in wheat and alleviated oxidative stress caused by Cu toxicity. The activity of SOD and catalase (CAT) has been enhanced but the activity of lipoxygenase and MDA content was decreased. NO is mediated through the modulation in the activities of antioxidant enzymes involved in H_2O_2 detoxification and in the maintenance of cellular redox couples (Tewari et al. 2008).

4.4 Salinity

Salinity is one of the major abiotic stresses affecting plant productivity (Ahmad and Sharma 2008; Ahmad et al. 2010, 2013). It has a negative effect on plant growth, ion balance and water relations, leading to nutrition disorder and oxidative stress (Hasegawa and Bressan 2000; Munns and Tester 2008). Application of $NO^{\bullet}$ donors in callus of *Phragmites communis* exposed to 200 mM NaCl revealed that $NO^{\bullet}$ affected the K^+/Na^+ ratio by increasing a plasma membrane H^+-ATPase activity (Zhao et al. 2004). Also, treatment of $NO^{\bullet}$ donors enhanced maize tolerance to salinity by elevating the activities of proton-pump and Na^+/H^+ antiport of the tonoplast (Zhang et al. 2006). The concentration of 200 mM NaCl inhibited germination of *Lupinus luteus*, but preincubation of seeds with SNP restored this process (Kopyra and Gwóźdź 2003). Treatment of 200 mM NaCl caused a 40 % reduction in leaf fresh weight and induced oxidative stress in olive (*Olea europaea*) plants (Valderrama et al. 2006). It was shown that salt stress caused an increase in the L-arginine-dependent production of NO and total *S*-nitrosothiols (SNO).

Rice (*Oryza sativa*) treated with 1 M SNP or 10 M H_2O_2 and then exposed to salt stress showed increased tolerance which is induced by both antioxidant enzymes and stress-related genes (Uchida et al. 2002). Similar results have been observed in orange (*Citrus aurantium* L.) trees. It suggests that the induction of antioxidant enzymes as a consequence of SNP pretreatment provided a resistance to salt stress (Tanou et al. 2009). Liu et al. (2007) showed that glucose-6-phosphate

dehydrogenase enzyme played an important role in NR-dependent NO production and in establishing the tolerance to red kidney bean root to salinity. Furthermore, NO decreases membrane permeability, rate of ROS production, MDA, H_2O_2 and intercellular CO_2 concentration under salt stress by inducing CAT, peroxidase (POD), SOD, APX activities and proline accumulation (Kopyra and Gwóźdź 2003; Fan et al. 2007; Shi et al. 2007; Yu-qing et al. 2007; López-Carrión et al. 2008; Sheokand et al. 2008; Guo et al. 2009; Li et al. 2012; Lin et al. 2012; Wang et al. 2012a, b).

In *Arabidopsis thaliana*, the effect of NaCl on wild type and *Atnoa1* mutant (with defect in vivo NOS activity) has been studied (Zhao et al. 2007). *Atnoa1* mutant plants displayed a greater Na^+ to K^+ ratio in shoots than wild-type plants due to enhanced accumulation of Na1 and reduced accumulation of K^+ when exposed to NaCl. Germination of *Atnoa1* seeds was more sensitive to NaCl than that of wild-type seeds. *Atnoa1* plants had higher levels of hydrogen peroxide than wild-type plants under control and salt stress. It suggests that *Atnoa1* is more vulnerable to salt and oxidative stress than wild-type plants. Treatments of wild-type plants with NOS inhibitor (L-NNA) and c-PTIO reduced the content of NO and enhanced NaCl-induced increase in Na^+ to K^+ ratio. These results further confirmed the counteracting effect of NO on ionic toxicity and oxidative damage induced by salt stress. It is an evidence that NO is an important molecule involved in plant tolerance of salt stress.

4.5 UV-B Radiation and Ozone

The negative effects of UV-B (280–320 nm) radiation result in deformed morphological parameters of plants. Exposure to UV-B not only decreases plant height, leaf area and plant dry weight but also increases auxiliary branching and leaf curling. Mechanisms of plant response to UV-B stress are connected with changes in gene expression. These include down-regulation of photosynthetic genes and up-regulation of genes for flavonoid biosynthesis and antioxidant enzymes. In addition, the expression of a number of *pathogenesis-related* (*PR*) genes, the acidic *PR* genes and the defencin gene, *PDF1.2*, have also been shown to increase in response to UV-B exposure (Greenberg et al. 1997; Frohnmeyer and Staiger 2003).

Mackerness et al. (2001) reported that *Arabidopsis* plants generated both NO and ROS when exposed to UV-B radiation. The expression of *chalcone synthase* (*CHS*) gene was only induced by NO. The NOS activity of maize hypocotyls was significantly increased by UV-B induction. It suggests that NO acts as a second messenger and carry out antioxidant responses to UVB radiation. Maize hypocotyl cells treated with SNP displayed the decrease of glucosidase activity and increase of protein content (Zhang et al. 2003; An et al. 2005). Wang et al. (2006) reported that NO generated from NOS-like activity appeared to act in the same direction or synergistically with ROS to induce ethyl synthesis in defence response under UV-B radiation in maize leaves. Tossi et al. (2009a, 2012) showed that ROS widely occurred in

chloroplasts and mesophyll cells of maize exposed to UV-B. Pretreatment with apocynin and coinciding NO accumulation prevented this damage. Tossi et al. (2009b) also suggested that UV-B perception cause plants to produce high level of ABA, activated NADPH oxidase and H_2O_2 generation. UV-B also induced stomatal closure, which was mediated by NO and H_2O_2. The generation of NO was caused by NOS-like activity (He et al. 2005). However, generation of NO by NR has been demonstrated in guard cells (Bright et al. 2006). c-PTIO arrested the protective effects against UV-B-induced oxidative damage mediated by NO (Shi et al. 2005). Treatment of thylakoid membrane with H_2O_2 showed an enhancement in carbonyl contents. In the presence of NO under UV-B radiation, the content of H_2O_2 has been suppressed through increasing activities of CAT, SOD and APX.

Ozone can induce photochemical reactions which may involve oxides of nitrogen (NO_x) and volatile hydrocarbons. Ozone causes varying symptoms including chlorosis and necrosis. For example, *Arabidopsis thaliana* exposed to ozone induced the activity of NOS and this preceded salicylic acid accumulation and death of cells. NO treatment causes increase the level of ozone-induced ethylene production and leaf injury (Rao and Davis 2001) and flavonol production (Xu et al. 2012). In tobacco (*Nicotiana tabacum* L. cv BelW3) plants, fumigated with ozone, accumulation of H_2O_2 in mitochondria was discovered, as well as NO and ethylene accumulation in leaf tissues. NO generation was produced by alternative oxidase (AOX). It was also demonstrated that ozone induced up-regulation of AOX in NO- and ethylene-dependent pathways. However, only NO is indispensable for the activation of AOX gene expression (Ederli et al. 2006).

4.6 Wounding

Wounding is a common stress which affects plant growth and metabolism. This stress is often caused by different stress factors, e.g. herbivores and insects feeding during which the generation and increased accumulation of NO and H_2O_2 are frequently observed (Leon et al. 2001; Schilmiller and Howe 2005).

Although wounding per se does not induce the synthesis of NO, treatment with NO donors inhibited generation of H_2O_2 (Orozco-Cardenas and Ryan 2002). It also inhibited the expression of specific genes related to wounding. This suggests that NO produced during pathogenesis might inhibit H_2O_2 synthesis and the activation of specific wound-induced signalling pathways. Neither wound-induced NO burst, nor NO-induced elevation of endogenous salicylic acid (SA) levels could be demonstrated here. Moreover, H_2O_2 accumulation and expression of the proteinase inhibitors *Inh1*, *Inh2*, cathepsin D inhibitor (*CDI*) and metallocarboxypeptidase inhibitor (*CPI*) have been inhibited by SNP-derived NO, but not the expression of oxide synthase (*AOS*) or lipoxygenase (*LOX2*). Thus the authors suggest that NO is inhibiting signalling downstream from jasmonic acid (JA), but still upstream from ROS generation (Leon et al. 2001). Nevertheless, the accumulation of one signalling molecule alone is not sufficient to induce any physiological changes in

Arabidopsis (Durner et al. 1998; Durner and Klessig 1999; Huang et al. 2004). In SA-deficient *NahG* plants, NO treatments led to elevated JA levels along with the induction of PDF1.2 and JIP, which were non-responsive in wild-type plants. In tobacco leaves treated with NO an increase of total SA levels and the induction of Pr-1 and Pal expression was demonstrated (Durner et al. 1998). Astonishingly, the induction of Pr-1 was shown to be SA-dependent, whereas Pal expression was not. Nevertheless, SA does not always play a role in NO-induced gene expression. The *Ipomoelin* gene (*IPO*) in sweet potato was shown to be enhanced by methyl jasmonate (MeJA) and mechanical wounding (Imanishi et al. 1997).

In *Arabidopsis thaliana*, mechanical wounding caused an increase in NO level, which was involved in JA-associated defence response (Huang et al. 2004). In pea (*Pisum sativum*) seedlings, an accumulation of NO shared with an increase in the content of SNO, as well as induction of NOS and GSNO activity (Corpas et al. 2008). In sunflower (*Helianthus annuus*) hypocotyls, mechanical wounding apparently did not affect the NO content. However, it leads to the accumulation of SNO due to a down-regulation of GSNOR activity, while nitration of tyrosine increases. Consequently, a process of nitrosative stress is induced, and SNO seem to be a new wound signal in plants (Chaki et al. 2011).

5 Interactions Between Nitric Oxide and Plant Hormones Under Abiotic Stress

Although there is an ever-increasing number of NO responses in plants, relatively little knowledge has been gathered on the relation between NO and all groups of phytohormones in plants under abiotic stress. Interaction between nitric oxide and ethylene in the induction of AOX in ozone-treated tobacco plants has been described in this chapter. Moreover, NO has a protective effect on plants exposed to salinity. NO-induced ethylene stimulates the alternative respiratory pathway (Wang et al. 2010a, b). Many studies indicate that there is a crucial "ABA–H_2O_2–NO–MAPK–antioxidant survival cycle." It indicates that NO is an important molecule in the plant tolerance to oxidative stress which caused increased activity of antioxidant enzymes. Furthermore, under drought stress ABA may involve NO which induced stomatal closure to reduce transpirational water loss (Fig. 5.1) (Garcia-Mata and Lamattina 2002, 2003; Neill et al. 2003a, b, 2008; Wang et al. 2012a, b).

NO is involved in the mechanism of salt tolerance generated by SA in tomato plants (Gémes et al. 2011). Thus, salt stress enhanced the content of NO in roots. However, tomato treated with SA changes that response and prevents accumulation of NO. In *Arabidopsis* root, SA promoted the biosynthesis of NO by NOS-dependent pathways (Zottini et al. 2007). NO and ROS are both required in SA-induced stomatal closure. SA activates peroxidase to produce extracellular ROS, which affects on production of NO in guard cells, inactivates K^+ channels and causes stomatal closure (Khokon et al. 2011).

Polyamine (PA) increases were found in several species such as rice, sorghum, maize, tomato and cucumber under salt, osmotic and copper stress (Flores and Galston 1984; Prakash and Prathapsenan 1988; Erdei et al. 1996; Willidiano et al. 1996; Santa-Cruz et al. 1997; Bouchereau et al. 1999; Xu et al. 2011; Fan et al. 2013). It is known that NO as well as PAs are associated with various abiotic stresses. In heat-tolerant cotton and rice, substantial increases in free and conjugated PAs and long-chained PA, as well as greater accumulation of polyamine oxidases and PA-biosynthesizing ADC, were observed during heat stress (Kuehn et al. 1990; Roy and Ghosh 1996). An increase in NO production was observed in alfalfa during short-term heat stress conditions, and exogenous NO was shown to mediate chilling resistance in tomato, wheat and corn. NO generated during heat and chilling conditions might be partly due to accumulated PAs. Increased NO biosynthesis during osmotic stress has been also reported (Erdei et al. 1996; Leshem and Haramaty 1996; Neill et al. 2002a, b). In response to drought stress, an increase in NOS-like activity was observed in wheat seedlings, and ABA accumulation was inhibited by NOS inhibitors (Zhao et al. 2001). An accumulation of putrescine levels is a common result in plants under osmotic stress. Synthesis of other PAs from putrescine is the key protective factor for the stressed cells (Bouchereau et al. 1999; Filippou et al. 2013).

The role of ABA in brassinosteroid (BR)-induced stress tolerance was investigated in leaves of maize (*Zea mays*) plants, as was the relationship between BR, NO and ABA under water stress induced by polyethylene glycol (PEG) (Zhang et al. 2011). BR treatment increased the content of ABA and up-regulated the expression of the ABA biosynthetic gene *vp14* in maize leaves, which was blocked by pretreatments with the NO scavenger cPTIO and the nitric oxide synthase inhibitor L-NAME. Moreover, BR treatment induced an increase in the generation of NO in mesophyll cells of maize leaves, while treatment with SNP up-regulated the content of ABA and the expression of *vp14* in maize leaves. These results suggest that the BR-induced increase in the biosynthesis of ABA in maize leaves exposed to water stress is, at least in part, due to the production of NO induced by BR. However, it is still unknown how NO regulates BR-induced ABA biosynthesis.

The role of NO in H_2O_2-dependent induction of abiotic stress tolerance by BRs in cucumber was reported by Cui et al. (2011). It has been shown that BR can induce NO production through both NOS-like and nitrate/nitrite enzymatic-dependent routes in an ROS-dependent manner. NO is involved in BR-induced stress tolerance most likely by mediating induction of antioxidant genes, which in turn lead to increased activities of antioxidant enzymes, i.e. APX, catalase, glutathione reductase and SOD.

6 Conclusions and Future Perspective

Nitric oxide (NO) is a reactive molecule, which plays a key role in many physiological and developmental processes in plants. It is probably the inorganic molecule with the best-characterized influence on many processes in plants. NO can provoke

both beneficial and harmful effects within cells, depending on its localization and concentration. NO belongs to a family of RNS such as peroxynitrite, nitrogen dioxide, dinitrogen trioxide and *S*-nitrosoglutathione. It is known that NO functions as a signalling molecule in interaction with plant hormones under environmental stresses. NO plays a key role as a component in cells tolerance to oxidative stress and is also responsible for defence genes regulation encoding antioxidant enzymes.

Targets of NO need to be better defined and it will be important to ensure that they are physiologically relevant. Also of future interest will be more precise information about NO biochemistry and the natures of the mechanisms controlling the synthesis of NO. Although much has been studied about the relationship between environmental stresses and NO, our knowledge of the NO metabolism is still elementary. Identification of the RNS targets under stress conditions will be helpful to understand how these molecules participate in the mechanism of response to environmental stresses. The ability of NO to induce tolerance in plants to a broad spectrum of stressful agents seems to result largely from interactions with phytohormones. The investigations on the molecular basis of NO-mediated stress response and interactions with environmental cues will have a great influence on future application of these substances in plant growth and development. Clustering and gene network analysis can help to easily analyze genes and protein expression profiles. Combining transcriptomics, proteomics and bioinformatics approaches open a novel way of elucidating NO targets.

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Chapter 6
Brassinosteroids: Improving Crop Productivity and Abiotic Stress Tolerance

Renu Bhardwaj, Indu Sharma, Dhriti Kapoor, Poonam, Vandana Gautam, Ravdeep Kaur, Shagun Bali, and Anket Sharma

1 Introduction

Brassinosteroids (BRs) are polyhydroxylated plant steroidal hormone, which are structurally similar to animal steroid hormone such as progesterone and ecdysone. Brassinolide (BL) was a first characterized brassinosteroid which was discovered from the bee-collected rape pollen grain. Campesterol is found as precursor of BR, that is transformed into castasterone and consequently into brassinolide through early or late C-6 oxidation pathways (Yang et al. 2011). Seventy BR compounds have been isolated from plants till now. They regulate the diverse physiological processes like cell elongation, embryogenesis, vascular differentiation, senescence, fertility, developing seeds or fruits, ethylene biosynthesis, photosynthesis, proton pump activation, and adoptive responses to environmental stress (Krishna 2003; Vert and Chory 2006).

Brassinosteroids (BRs) regulate various growth and developmental processes of plants. They induce seed germination of almost all endospermic seeds, but they do not have an effect on germination of some of the non-endospermic seeds (Leubner-Metzger 2001). Li et al. (2002) reported that BL had enhanced the germination capacity of *Pinus tabulaeformis* and the length of hypocotyl. BRs were also found to stimulate the germination rate of clover broom rape (*Orobanche minor*) seeds (Takeuchi et al. 1995) and have tissue-specific effect on cell elongation. Epicotyls, hypocotyls, mesocotyls, and coleoptiles elongation can also be promoted by BRs, but they generally retard root elongation (Kim et al. 2007). Moreover, BRs promoted the elongation of etiolated squash hypocotyl segments and stimulated its fresh weight (Tominaga et al. 1994). Regarding this, Goda et al. (2002) observed

R. Bhardwaj (✉) • I. Sharma • D. Kapoor • Poonam • V. Gautam • R. Kaur • S. Bali • A. Sharma
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar 143005, Punjab, India
e-mail: dr.renubhardwaj@yahoo.in; renu_bhardwaj@rediffmail.com

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that exogenous application of BRs at nanomolar to micromolar concentrations stimulates a variety of physiological effects, which includes promotion of cell elongation and division. They also added that, promotion of cell expansion and regulation of photomorphogenic responses are among the most important roles of BRs that was recently analyzed by their molecular studies. BRs are also involved in the process of cell enlargement due to their effects on gene expression and on enzyme activity. According to Khripach et al. (2000), BRs importance in cell division was also confirmed by the finding that brassinolide can induce or retard cell division in *Petunia hybrid*-isolated leaf protoplasts which depends on the phase of cell development, auxins and cytokinin concentrations of the culture.

They also have major roles in various other physiological processes like induction of stem elongation, pollen tube growth, photosynthesis, xylem differentiation, leaf epinasty, proton pump activation, ethylene biosynthesis, gene expression, and adaptive responses to environmental stress (Krishna 2003; Yu et al. 2004; Vert and Chory 2006). It was recently reported that biosynthesis of BRs is promoted in the developing seeds or fruits of tomato, pea, and *Arabidopsis* (Shimada et al. 2003; Montoya et al. 2002; Nomura et al. 2007). BRs application can also enhance the ripening of tomato and grape fruits (Vardhini and Rao 2002; Symons et al. 2006). However, it was found previously that BRs have not been concerned with the regulation of early development of fruits, while Kamuro and Takatsuto (1999) have analyzed that exogenous application of BRs can induce fruit set. For this, cucumber—a monoecious annual cucurbit plant—was considered to be a good model for studying the fruit growth, when genotypes with different parthenocarpic capacity are existing. To confirm their role in fruit ripening, Asami et al. (2000) attempted to manipulate the BR levels in ovaries of Jinchun No. 4 (a non-parthenocarpic cultivar) and Jinchun No. 2 (a parthenocarpic cultivar) of cucumber (*Cucumis sativus* L.), through the exogenous applications of BRs and a BR biosynthesis inhibitor, brassinazole (Brz). BR levels were found to alter during ovary growth and cell division. Beside other stresses, exposure of plants to saline conditions retards plant growth and productivity (Abbas et al. 2010).

Effect of BRs in in vitro conditions were observed in *Arachis hypogaea* L. genotypes (M-13 and PBS24030) on their growth in the form of multiple shoots, chlorophyll content, Hill reaction activity (HRA) and also the activities of catalase (CAT), peroxidase (POX), polyphenol oxidase (PPX), and ascorbate peroxidase (APX) (Verma et al. 2012). In vitro effect was found best on shoot multiplication potential of both the cultivars at 1 mL L^{-1} with BA (3 mg L^{-1}). In PBS24030, flowering, rhizogenesis, total chlorophyll content, HRA, and antioxidant enzyme activities were enhanced in the medium containing BR. However, there was progressive decline in case of MDA content in the presence of BR. Shahid et al. (2011) reported that the EBL cause enhancement in seed germination, embryo axis length, and root and shoot length in the pea plants. Significant increase in the fresh and dry biomass, root and shoot length, photosynthesis rate (Pn), stomatal conductance (gs), total chlorophyll contents (Chl), proline contents, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), nitrate reductase activity (NRA), and nitrite reductase activity (NiRA) were also observed.

2 Physiological Roles of BRs

BRs have been found to be involved in many major physiological processes, including the following.

2.1 Cell Differentiation

Evidences suggested that in different plant species, BRs play active roles in vascular differentiation. At very low concentrations they were found to be effective in promoting the tracheid formation in Jerusalem artichoke explants and isolated mesophyll cells of zinnia (*Zinnia elegans* L., Fukuda 1997). These also regulated the expression of several genes which were involved in the development of xylem in zinnia mesophyll cells. In soybean epicotyls, they have been involved to play key role in xylem formation (Zurek et al. 1994).

2.2 Reactive Oxygen Species

BRs are involved in the regulation of reactive oxygen species (ROS) metabolism as they can provoke and regulate the expression of certain antioxidant genes and promote the activities of key antioxidant enzymes, that include peroxidase (POD), catalase (CAT), and SOD (Mazorra et al. 2011; Cao et al. 2005). Though BRs directly or indirectly modify the responses of plants toward oxidative stress remains unknown (Cao et al. 2005). However, for the induction and regulation of antioxidant systems in plants, both BRs and ROS act as vital secondary messengers under stress (Mazorra et al. 2011).

2.3 Enhancement of Crop Yield

Strategy of BR biosynthesis might offer a new and effective approach for promoting rice yield under a high-density planting (Sakamoto et al. 2006). They showed that without any discernible side effects, a rice phenotype with a more erect leaf type is related to an enhancement in the rate-limiting step in BL biosynthesis, which further increase productivity. Sakamoto et al. (2006) recognized two rice C-22 hydroxylases, among them one promotes shoot elongation and reproductive development (OsDWARF4L1) and the other helped in regulation of leaf inclination (OsDWARF4). Loss of function of OsDWARF4L1 gives rise to the formation of semi-dwarf plant with small seeds, while the loss of function of OsDWARF4 formed plants with

slightly shorter but erect leaves. Photosynthesis and yield was promoted by more erect orientation of leaves under high plant density (Sakamoto et al. 2006). Thus, manipulation of endogenous BL levels improved agronomically important trait without any adverse side effects.

2.4 Reproductive Biology and Senescence

It has been well accepted that BR-deficient and insensitive mutants possess reduced fertility and male sterility (Hewitt et al. 1985; Clouse and Sasse 1998; Clouse et al. 1993; Sakamoto et al. 2006). The uppermost levels of BRs have been found in pollen, from where they were first discovered. According to Szekeres et al. (1996), the BR-deficient mutant *cpd* was observed to be male sterile, due to the lack of ability to develop pollen tube following pollen germination. Li et al. (2010) reported that BRs actively take part in the regulation of the gravitropic reaction of *Arabidopsis* roots. External application of BRs promotes the activity of ROP2, a GTPase, and thus this protein mediated the BR-modulated polar auxin transport, that resulted in a better gravitropic response. Kim et al. (2007) recently presented additional evidence of BRs involvement in root gravitropic bending in *Arabidopsis*. They showed that in the root elongation as well as in gravitropic responses, BRs interacted with auxin differently. Besides, they also confirmed that BRs promoted an enhanced gravitropic response in *Arabidopsis* roots when the concentration of indole-3-acetic acid (IAA) was low, and at high concentration of IAA, the activity was reduced.

BRs applications have also been proved beneficial in plants exposed to chilling, drought, and salt stress (Clouse and Sasse 1998; Krishna 2003; Kagale et al. 2007). In bromegrass and tomato, 24-EBL has been found to increase the tolerance to both cold and heat stress (Wilen et al. 1995; Dhaubhadel et al. 1999; Singh and Shono 2005) and also alleviates the harsh effects of salt stress on growth, pigmentation, and NRA in rice (Anuradha and Rao 2003). According to Pinol and Simon (2009), in broad beans (*Vicia faba* L.) plants treated with a photosynthesis-inhibiting herbicide Terbutryn, the effects of 24-EBR were investigated on some key physiological attributes. It was confirmed that pre-sowing treatment of *Vicia faba* seeds with 24-EBR efficiently ameliorate the adverse effect of Terbutryn on chlorophyll fluorescence and net photosynthetic efficiency. 24-EBR in higher dose also mitigated the Terbutryn-induced inhibition in plant growth (Pinol and Simon 2009). Intermittent promotion of root elongation and formation of adventitious roots had been analyzed with very low (picomolar) concentrations (Clouse et al. 1993; Kagale et al. 2007; Arora et al. 2008). In various plant species, exogenous application of BRs by seed soaking, root treatment, and foliar spray had been broadly studied, indicating that BRs significantly improve the plant growth and development under various stress and non-stress conditions (Clouse and Sasse 1998; Yu et al. 2004; Cao et al. 2005; Houimli et al. 2008).

3 BRs Mediated Vegetative Growth of Plants

3.1 Promotion of Germination

Development of plants starts with the germination of seeds. It has been demonstrated by various studies that rate of germination of seeds increases by the application of BRs. Fathima et al. (2011) have studied the effect of BRs on the seed germination and seedling growth in *Gossypium hirsutum* L. var. Svpr 2 and *Vigna mungo* (L.) Hepper var. T9. Seeds of *Gossypium hirsutum* and *Vigna mungo* were soaked for 24 h in various concentrations of BRs and then planted in the pots and after regular intervals, various parameters were noted. The application of BRs on seeds of *Gossypium* and *Vigna* had showed significant increase in the seed germination. Application of exogenous brassinolide resulted in the elongation of hypocotyls in the sunflower seedlings (Kurepin et al. 2012). Sayed et al. (2009) had investigated that pre-soaking of seeds of *Cucurbita pepo* in lower concentration of brassinolide leads to the increased rate of germination and growth by increasing the metabolite activities.

3.2 Rhizogenesis

After the germination, root development starts in the plants. There are various factors which are responsible for the lowering of rhizogenesis, but it has been found that BRs play an important role in the development of roots. Studies by Mouchel et al. (2006) demonstrated that BRs use a transcriptional feedback loop to maintain root development. Studies were performed on *Arabidopsis* plant with brevis radix (brx) mutant with reduced root growth. The groundnut cultivars were grown on MS medium containing BRs and control for 4–5 weeks for shoot multiplication. The rhizogenesis was showed only in the plants grown with BRs (Verma et al. 2012). Rietz et al. (2010) have explored that BRs may cope the auxin to accelerate lateral root formation, which may be partially mediated by the patatin-related phospholipases A. It was found that BRs play a role in the development of lateral roots in *Arabidopsis* plants as the BR-deficient mutants has fewer lateral roots as compared to wild type and expression of DR5::GUS in root tips of BR-deficient mutants was less than wild-type (Bao et al. 2004; Fukaki and Tasaka 2009). BRs have also been investigated to regulate the root hair development. Kuppusamy et al. (2009) have showed that WEREWOLF and GLABRA2, which are two master epidermal patterning regulators were regulated by BRs and abnormal root hair development is shown by BR-related mutants. Swamy and Rao (2010a) have found the effect of BRs on roots of Coleus plants. One hundred and thirty-day-old stock cutting of Coleus plant was dipped in different concentrations of BRs for 5 min and immediately planted in the nursery cover. The root growth was examined on 30th and 60th day after plantation. The exogenous application of BRs resulted in the increased rhizogenesis in comparison to the control plant cuttings. Kwak et al. (2009) have

found that the application of lower concentrations of BRs has resulted in the increase in the number of adventitious roots. According to Kartal et al. (2009) application of homobrassinolide to barley seeds increases the primary root growth and also showed enhanced mitotic activity and mitotic abnormalities in comparison to the control material.

3.3 Senescence and Respiration

The maturation of fruits depends upon the production of ethylene. BRs have shown varied effects on senescence by effecting the production of ethylene. Zhu et al. (2010) have reported that in jujube fruit, BRs had helped in maintaining fruit quality by delaying fruit senescence by decreasing ethylene production. Application of exogenous BRs increased the grape fruit ripening and also showed improvement in the levels of endogenous BRs (Symons et al. 2006). Vardhini and Rao (2002) observed that application of BR enhanced the tomato fruit ripening by increasing the ethylene production.

Respiration is an essential activity to provide metabolic energy and carbon for growth and maintenance. There is very little knowledge about the role of brassinosteroids on the respiration of plants. Catterou et al. (2001) have investigated that in *Arabidopsis*, BR biosynthetic mutant bul 1/dwf 7 has increased the stomatal density. The brassinolide was found to promote the stomatal closure and inhibition of stomatal opening in *Vicia faba* (Haubrick et al. 2006).

3.4 Photosynthesis

Photosynthesis is the basis of life as it is helpful for the growth and sustenance of plants as well as for other living organisms. Brassinosteroids have found to increase the photosynthesis in various plants. Yu et al. (2004) had reported the role of brassinosteroids in promotion of photosynthesis in cucumber plants. Yuan et al. (2010) had studied the effect of brassinosteroids in tomato plants under water stress and found that exogenous application of EBR increased the relative water content and net photosynthetic rate. The effect of BRs on cucumber plant growth was studied by Xia et al. (2009a) which was associated with increased CO_2 assimilation and ϕPSII. In their experimentation, they have noted the effect of BRs and Brz on number of photosynthetic parameters including the amount and activity of Rubisco enzyme. The treatment of EBR had upregulated while the Brz had downregulated the expression of Rubisco and other photosynthetic genes. The activity of Rubisco had also increased with the treatment of EBR. Hayat et al. (2011) had found that foliar spray of two brassinosteroids HBL and EBL had enhanced the photosynthetic parameters. Foliar spray of BRs had enhanced the growth of Geranium plants by increasing the photosynthetic rate (Swamy and Rao 2010b). It has been observed that BRs had

detoxified the salinity and temperature stress and showed increase in photosynthesis in *Vigna radiata* plants (Hayat et al. 2010). The results of Jiang et al. (2013) have pointed out that application of BRs in *Cucumis sativus* had enhanced the recovery of photosynthetic apparatus from cold stress by various methods including activation of enzymes or by enhancing the antioxidant capacity. Haubrick et al. (2006) explored that stomatal aperture in *Vicia faba* plant is regulated by brassinolide and promote stomatal closure and inhibit stomatal opening.

3.5 Vegetative Growth in Plants

Brassinosteroids have been noticed to increase the vegetative growth in various studies. El-Bassiony et al. (2012) had performed experimentation to find the role of brassinosteroids on vegetative growth in snap bean plants. Different concentrations of brassinosteroids were sprayed and plant growth, yield, and pods quality of beans were examined. Application of brassinosteroids (25 and 50 ppm) to plants resulted in notable increase in total yield, pod quality, and vegetative growth, but there was not major difference between both the treatments. It had been described by Kang and Guo (2011) that BR treatments bring about the stimulation of elongation, cell division, and differentiation and resulted in the promotion of plant growth. Number of leaves in the cucumber plant showed increase with the treatment of BRs (Jian et al. 2012). Biopolymer cellulose contributes to cell wall formation during cell elongation and cell expansion. Findings of Xie et al. (2011) revealed that BRs regulate cellulose synthesis and thus helped in the cell elongation and expansion. The results of Fathima et al. (2011) demonstrated that exogenous application of BRs resulted in the improved growth and metabolite content. Pereira-Netto et al. (2009) observed that exogenous application of BRs enhanced the elongation and formation of shoots while treatment with the brassinozole led to inhibition of shoot elongation in the apple plants (*Malus prunifolia*). Nakamura et al. (2009) reported that in BR-related rice mutants there were approximately 10–30 % reduction in the cell length at the center of adaxial side of lamina joint. Figure 6.1 shows the different functions of brassinosteroids in plants.

4 BRs Mediated Reproductive Growth of Plants

4.1 Role in Flower and Fruit Development

Flowers are the important phase of the plants as they are the reproductive part of the plants. Development of flower is regulated by various signals of endogenous plant hormones including BRs. Manzano et al. (2011) have found that BRs have very minor role in the flower development in *C. pepo* genotypes. For this study,

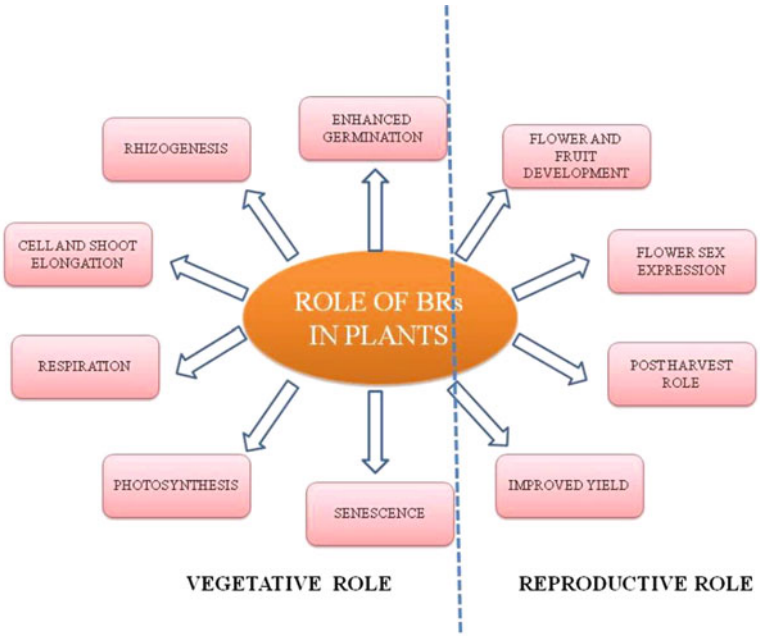

Fig. 6.1 Flow chart summarizing various roles of brassinosteroids in plants

C. pepo plants were grown in the pots and when a plant grown for four true leaves, brassinozole, and a brassinosteroid biosynthesis inhibitor was sprayed and flower development was studied. BR-deficient and BR-insensitive mutants show evidence of late flowering phenotype (Li et al. 2010). Studies by Fu et al. (2008) demonstrated that BRs play a vital role in early development of fruits in cucumber plants. EBR application on Jinchun No. 4, a cultivar without parthenocarpic capacity has induced parthenocarpic growth, while application of brassinozole inhibited fruit set. Applied BRs alters the expression of circadian rhythms of CCR2, CAB2, and CCA1 which control flowering time (Hanano et al. 2006).

4.2 Fruit Ripening

The time duration required for the ripening of fruit is very important from the commercial point of view. The BRs show varied effect on fruit ripening as in some plants it showed early ripening, while in some others ripening is delayed. Gabr et al. (2011) showed that BR application resulted in the advancing harvest dates of "Canino" apricot fruits. Samira et al. (2012) had found that exogenous application of 24-epibrassinosteroid resulted in the improved flower and fruit number and yield per plant in pepper. Zaharah et al. (2012) had observed that the exogenously applied epibrassinolide promoted fruit color development and softening of fruits during fruit development.

4.3 Flower Sex Expression

In various studies, it has been shown that BRs have positive effects in the expression of sex during flowering. Hartwig et al. (2011) had proposed that BRs play an important role in controlling sex determination in maize plants by the characterization of dwarf nana plant1 (na1). Male flowers were also found feminized by this gene. Papadopoulou and Grumet (2005) performed a series of experiments by treatment of cucumber, melon, and zucchini with brassinolide and reached to the result that cucumber was more sensitive, and showed reduced number of male flowers and at the same time promoted development of first female flower in the main shoot.

4.4 Post Harvest

Post harvesting effects include damage to the fruits and effects due to various diseases. Use of BRs reduces the post harvest diseases. Zhu et al. (2010) had found that in jujube fruit BRs suppressed the development of post harvest disease caused by blue mould rot. Activities of defense-related enzymes were also increased. Nakashita et al. (2003) had also reported that BRs can protect tobacco plant from diseases.

5 BRs as Potent Herbicides, Pesticides, and Insecticides

The resources of food and fiber supply for human feasting are unceasingly intimidated by herbivorous pests and insects. Moreover, there is a need to control parasitic insects and arthropod vectors of important diseases. Man-made pesticides play a key character in pest control. Though there is resemblance in the nervous system of insects and vertebrates, therefore these synthetic pesticides can exhibit substantial noxiousness toward higher animals and, therefore, their nonselective approach of deed may result in overwhelming ecological complications. In many pest insects, resistance to synthetic pesticides has been developed due to the extensive use of such compounds. So, the necessity of exploration for novel insecticides with a better effectiveness or a different mode of action is apparent. A concentrated exploration for substitutes less injurious to the environment has been started in laboratories around the world.

Brassinosteroids possess a structural similarity with ecdysteroids (insect molting and sex hormones). This similarity has given rise to several studies to determine an ecdysteroid-interfering action of brassinosteroids in insects. It was specified in an initial study that brassinosteroids contend to fix with the ecdysteroid receptors (Hetru et al. 1986). Lehmann et al. (1988) also reported an anti-ecdysteroid activity indicating that two brassinosteroids had a weak attraction for the incompletely purified ecdysteroid receptors from *Calliphora vicina*. The production of four new

brassinosteroids with 2β, 3β-diol functionality, A/B *cis*, and A/B *trans* ring junction was reported by Brosa (1994). It was also analyzed that these brassinosteroids could present activity as antiecdysteroids. Smagghe et al. (2002) studied the action of two brassinosteroids (24-epibrassinolide and 24-epicastasterone) in the cotton leaf worm *Spodoptera littoralis* using cultured imaginal wing discs from last-instar larvae. Fifty percent antagonism for binding with [(3)H] ponasterone A was observed at IC(50) of 1–3.6 μM. However, no initiation of evagination was established by culture of imaginal wing discs in different concentrations of brassinosteroids, even up to 100 μM. Dose of 10 μg of brassinosteroids in afresh molted last-instar larvae did not cause mortality above controls; higher mortalities were scored when brassinosteroids were injected late in the last instar. Decombel et al. (2005) showed that for 24-epibrassinolide, the lepidopteran cell line SeE-CLG4 allows the concurrent recognition of diverse cytotoxic properties that is particularly significant for likely hormone antagonists. In the transformed Bm5/ERE.gfp cells, this brassinosteroid had an antagonistic effect against 500 nM 20-OH-E deprived of changing the cell viability (Smagghe et al. 2002). Brassinosteroids (BRs) are recognized to defend the crops from the noxiousness of herbicides, fungicides, and insect repellents. In accordance with the results of experiments conducted by Xia et al. (2009b), BRs are capable natural bodies appropriate for inclusive use to diminish the hazards of human and environment disclosure to pesticides. An insecticide (chlorpyrifos) produced substantial declines of net photosynthetic rate and considerable yield of PSII in cucumber (*Cucumis sativus* L.) leaves. Absorption of this fly spray was fast-tracked and their enduring intensities were accordingly reduced in cucumber by the application of 24-epibrassinolide (EBR). It happened because due to the treatment of chlorpyrifos, EBR took a progressive influence on the stimulation of glutathione *S*-transferase (GST), peroxidase (POD), and glutathione reductase (GR).

Wachsman et al. (2000) reported that a natural brassinosteroid and a series of synthetic derivatives (analogues of the 24(*S*) ethylbrassinone) are good inhibitors of herpes simplex virus type 1 (HSV-1) and arenavirus replication in cell culture. Time-of-addition trials recommended that a late step in HSV-1 multiplication was affected, whereas arenaviruses remained vulnerable to the compounds throughout the replicative cycle.

6 BR as a Stress-Tolerant

Today in the era of climate change, plants particularly crops are exposed to wide range of environmental stresses. These environmental stresses could be classified into two broad categories: abiotic stresses (like osmotic stress, extreme temperatures [heat and cold], nutritional deprivation, drought and desiccation, water logging, photo-oxidative stress, heavy metal or xenobiotic stress, etc.) and biotic stresses (like pest and pathogens attacks). In response to these environmental stresses, plants acclimatize themselves by involving changes in biochemistry of their cells. Such changes include evolution of new biochemical pathways and

detoxification mechanisms, alterations in levels of antioxidative enzymes, antioxidative molecules and phytohormones, and synthesis of specific proteins (Bajguz and Hayat 2009) leading to decrease in growth and yield of the crop. BRs promote tolerance of plants to various environmental stresses (Krishna 2003). They also promote degradation of herbicides, fungicides, and pesticides by upregulating detoxifying genes playing crucial role in their metabolism. This reduces accumulation of pesticide residues in plants. As a result BRs can be used as environmental friendly method for crop protection and yield enhancement (Xia et al. 2010). BRs have been reported to enhance plant tolerance/resistance independently as well as through integration with other phytohormones (Divi et al. 2010). There are wide array of reports regarding positive or ameliorative effect of exogenously applied BRs against various abiotic and biotic stresses. There are very few reports for role of endogenous content of BRs in stress tolerance. Zeng et al. (2010) reported positive role of endogenous BR in tolerance to salt stress in *Arabidopsis*. Few recent studies of exogenous application of BRs and their physiological effect under given environmental stress have been summed up in Table 6.1.

Mechanisms by which exogenous and endogenous BRs provide enhanced tolerance against various stresses are still poorly understood and under investigation. Exogenously applied BRs induce accumulation of ROS in plants under stress, which in turn confers stress tolerance by upregulating genes involved in plant stress tolerance. ROS may also participate in BR-regulated physiological processes such as plant growth and development and photosynthesis (Xia et al. 2010). ROS also mediated BR-induced systemic tolerance (Xia et al. 2011). BR-induced short-term heat tolerance is found to be associated with abscisic acid (ABA) accumulation (Kurepin et al. 2008; Bajguz 2009). ABA partially mediates BR-induced chilling tolerance to *Chorispora bungeana* suspension-cultured cells by enhancing the antioxidant defense system, preventing the overproduction of ROS to alleviate oxidative injury induced by chilling (Liu et al. 2011). Zhang et al. (2011) have reported the role of nitric oxide (NO) in BR-induced oxidative stress tolerance by activating ABA biosynthesis in maize leaves. BR-induced production of NO is H_2O_2-dependent. NO mediates BR-induced abiotic stress tolerance by upregulating antioxidant genes which further enhance activities of antioxidant in cucumber plants (Cui et al. 2011). Pectin methylesterase (PME) activity enhanced during chilling stress which in turn increased stiffness of cell wall and further provide enhanced cold and chilling tolerance. Qu et al. (2011) reported BRs regulate PME activity. Thus BRs may provide enhanced chilling tolerance through enhanced PME activity. Exogenously applied BRs provide thermotolerance by synthesis of heat shock proteins and protection of transcriptional machinery from heat stress. Heat shock-mediated oxidative stress is alleviated by BRs levels but thermotolerance is independent of endogenous BR content (Mazorra et al. 2011). BRs play role in plant innate immunity by regulating plant pathogen responses. Brassinolide enhanced disease resistance against wide range of pathogen responses in tobacco and rice (Nakashita et al. 2003). Recently, contradictorily Vleesschauwer et al. (2012) have reported that BRs increased the disease susceptibility to root oomycete (*Pythium graminicola*) in rice (*Oryza sativa*). Their results suggested that the pathogen was found to seize the BR

Table 6.1 Application of BRs and their physiological effect under various environmental stresses

Type of stress		BRs applied	Plant studied	Effect	References
Biotic stresses	Bacterial infection (*Pseudomonas syringae*, *Xanthomonas oryzae*)	Brassinolide (BL)	*Nicotiana tabacum*, *Oryza sativa*	BL enhanced disease resistance	Nakashita et al. (2003)
	Fungal infection (*Oidium* sp., *Magnaporthe grisea*)	Brassinolide	*Nicotiana tabacum*, *Oryza sativa*	BL enhanced disease resistance	Nakashita et al. (2003)
	Oomycetes infection (*Pythium graminicola*)	Brassinolide	*Oryza sativa*	Both endogenous and exogenously applied BL suppressed root immunity to the rice pathogen	Vleesschauwer et al. (2012)
	Viral infection (tobacco mosaic virus)	Brassinolide	*Nicotiana tabacum*	BL enhanced disease resistance against viral attack	Nakashita et al. (2003)
	Nematode infection (*Meloidogyne graminicola*)	Epibrassinolide	*Oryza sativa*	BRs increased disease susceptibility at low concentration but at high concentration increased systemic immunity	Nahar et al. (2013)
Abiotic stress	High temperature	24-Epibrassinolide	*Brassica napus*, *Solanum lycopersicum*, *Brassica juncea*	EBR protected translational machinery, induced synthesis of heat shock proteins, improved photosynthetic efficiency, enhanced protein content, and antioxidant enzymes	Dhaubhadel et al. (2002), Singh and Shono (2005), and Kumar et al. (2012)
	Chilling	24-Epibrassinolide (EPI) and 2a,3a,17b-trihydroxy-5a-androstan-6-one (A)	*Zea mays* L., *Arabidopsis*	BRs enhanced chlorophyll content but does not regulate activity of photosystem I and II, regulated pectin methylesterases activity	Honnerova et al. (2010) and Qu et al. (2011)

Heavy metals (Cu, Cr, B, Pb, Cd, Ni)	28-Homobrassinolide, 24-epibrassinolide, brassinolide	*Raphanus sativus* L., *Vigna radiata*, *Chlorella vulgaris*, *Solanum lycopersicum*, *Sorghum vulgare*	EBL enhanced plant growth, yield, and nodulation, due to regulation of antioxidant system, photosynthetic machinery and proline content	Sharma et al. (2011), Choudhary et al. (2012a, b), Yusuf et al. (2012), Hasan et al. (2011), Bajguz (2011), and Hayat et al. (2012a)
Salinity	24-Epibrassinolide	*Fragaria*×*ananassa*, *Phaseolus vulgaris* L., *Lycopersicon esculentum*, *Triticum aestivum* L., *Capsicum annuum* L., *Pisum sativum* L.	EBR improved crop yield, plant growth by upregulating plant antioxidant system, carbon and nitrogen metabolism, water status, nutrient and mineral balance	Karlidag et al. (2011), Rady (2011), Slathia et al. (2012), Talaat and Shawky (2012), Samira et al. (2012), Hayat et al. (2012b), and Yuan et al. (2012)
Water	24-Epibrassinolide, 28-homobrassinolide	*Lycopersicon esculentum*, *Oryza sativa* L., *Capsicum annuum*, *Sorghum vulgare*	EBR influenced activity of antioxidant enzymes, endogenous ABA content, improved the leaf water economy and CO_2 assimilation, alleviated drought induced photoinhibition	Behnamnia et al. (2009), Farooq et al. (2009), Yuan et al. (2010), Vardhini et al. (2011), and Hu et al. (2013)
(a) Drought				
(b) Water logging	BR 120	*Triticum*	EBR increased wound flow the number of main stem green leaves, chlorophyll and protein content, improving photosynthetic rate	Xiaolin and Bingshan (2000)
Organic contaminants (PHE, pyrene, chlorpyrifos)	24-Epibrassinolide	*Solanum lycopersicum*, *Oryza sativa*	EBL improved growth, increased protein and proline content, upregulated antioxidant system, secondary metabolism, and the xenobiotic detoxification	Ahammed et al. (2012, 2013) and Sharma et al. (2012)
Nutrient deficiency (Fe)	24-Epibrassinolide (EBR)	*Cucumis sativus*	EBR plays negative role in alleviating Fe deficiency by suppressing ethylene production	Wang et al. (2012)

machinery and exploited BRs as virulence factors and improved disease susceptibility by antagonizing salicylic acid (SA) and gibberellic acid (GA)-mediated defense responses. Similar results are reported by Nahar et al. (2013) at low concentration of exogenous BR exposure, which enhanced disease susceptibility in *Oryza sativa* during infection with the root-knot nematode *Meloidogyne graminicola*. Plant steroid homeostasis is essential for innate plant immunity. In response to microbial-associated molecular patterns (MAMPs) BR can act antagonistically or synergistically. Synergistic activities BR in response to MAMPs require leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1 (Belkhadir et al. 2012). Albrecht et al. (2012) reported BRs inhibit MAMP-triggered immune signaling independent of the receptor kinase BAK1.

7 Exogenous Applications of BRs to Stress-Affected Plants

Numerous field and greenhouses have shown that exogenous BRs have the ability to ameliorate various environmental stresses such as salt stress, heat, cold, drought, heavy metal stress, or pathogen attacks (Xia et al. 2010). Exogenous application of BRs accelerated the seed germination, plant growth and development, yield and crop production and antioxidant enzymes. However, the extent of their effects in ameliorating stress may vary with the type of BR used, the concentration applied, mode and frequency of application, and the plant species (Ashraf et al. 2010). Table 6.2 summarizes few recent studies of exogenous application of BRs and their role in various environmental stresses.

8 BRs Mediated Genes in Crop Yield and Stress Tolerance

The redox-sensitive protein NPR1 (Non-expressor of Pathogenesis-Related Genes1), which is a master regulator of SA-mediated defense genes, is probably a vital component of EBR-mediated thermotolerance and salt tolerance, but not essential for EBR-mediated induction of *PR-1* (*Pathogenesis-Related1*) gene expression. BRs have anti-stress effects and also have interactions with other plant hormones. Microarray studies identified a large number of BR-regulated genes. Generally BR-regulated genes are related to the plant growth and development, such as hormone synthesis, cell wall modification, and cytoskeleton formation (Vert et al. 2005). BR has vital role in the regulation of gene expression as it binds to BRI1, a plasma membrane contained LRR-RLK. It induces association of BRI1 with its co-receptor BAK1 that promotes signaling output through reciprocal BRI1 transphosphorylation (Vert et al. 2005; Belkhadir et al. 2006). Binding of BR1 to BR inactivated BIN2, a glycogen synthase kinase-3, activates the phosphatase BSU1, whereas BIN2 negatively controls transcription factors BZR1 and BES1 by phosphorylating them and BSU1 positively regulates the signaling of BR by

Table 6.2 Exogenous application of BRs and their role in various environmental stresses

Brassinosteroid used and their mode of application	Plant studied	Type of stress	Physiological effects of BR in ameliorating given stress	References
24-Epibrassinolide (1 μM) Foliar spray	*Lycopersicon esculentum*	Drought stress	EBL application reduced content of H_2O_2, MDA and other aldehydes and increased shoot mass, the activity of antioxidants and antioxidative compounds	Behnamnia et al. (2009)
28-Homobrassinolide and 24-epibrassinolide (0.01 μm) Seed priming and foliar spray	*Oryza sativa* L.	Drought stress	BRs application improved the leaf water economy and CO_2 assimilation. Moreover, foliar spray had better effect than seed treatments under drought stress and of the two BRs, EBL proved more effective	Farooq et al. (2009)
24-Epibrassinolide and 2a,3a,17b-trihydroxy-5a-androstan-6-one (10^{-8}, 10^{-10}, 10^{-12}, and 10^{-14} M) Foliar spray	*Zea mays* L.	Long-term chilling stress	BRs diminished the degradation of chlorophylls. However, they did not improve the efficiency of primary photosynthetic processes and the activities of photosystem I or II	Honnerova et al. (2010)
24-Epibrassinolide (1 μM) Foliar spray	*Lycopersicon esculentum*	Drought stress	EBR ameliorated drought stress by elevating endogenous abscisic acid concentration and/or the activities of antioxidant enzymes	Yuan et al. (2010)
24-Epibrassinolide (0.5 and 1 μM) Foliar spray	*Fragaria*×*ananassa*	Salt stress	EBR improved cell membrane stability, nutrient uptake, plant growth, water status, and mineral nutrient balance in saline stressed plant	Karlidag et al. (2011)
4-Epibrassinolide (1 μM) Day 2 seedlings were treated with EBL	*Arabidopsis*	Chilling stress	BRs regulated activity of pectin methylesterases (PME)	Qu et al. (2011)
24-Epibrassinolide (5 μM) Foliar spray	*Phaseolus vulgaris* L.	Salinity and cadmium stress	EBL application increased growth of plant, green pod yield, pod protein, and the level of antioxidant system	Rady (2011)
28-Homobrassinolide (10^{-7} M) Shotgun method (pre-soaking seed with treatments)	*Raphanus sativus* L.	Chromium stress	Applications of HBL enhanced growth of plant, contents of chlorophyll, protein, proline, activities of all the antioxidant enzymes while decreased malondialdehyde content and guaiacol peroxidase	Sharma et al. (2011)

(continued)

Table 6.2 (continued)

Brassinosteroid used and their mode of application	Plant studied	Type of stress	Physiological effects of BR in ameliorating given stress	References
28-Homobrassinolide (10^{-8} M) Shotgun approach	*Vigna radiata*	Boron stress	Application of HBL improved growth, water relations and photosynthesis and further enhanced the various antioxidant enzymes and proline content	Yusuf et al. (2011)
28-Homobrassinolide/24-epibrassinolide (10^{-8} M) Foliar spray	*Lycopersicon esculentum*	Cadmium stress	Exogenous application of BRs improved the activity of photosynthetic machinery and that of antioxidant defense system	Hasan et al. (2011)
Brassinolide (10^{-8} M) Treatment	*Chlorella vulgaris*	Cadmium, lead, and copper stress	BL enhanced the content of indole-3-acetic acid, zeatin, and abscisic acid, chlorophyll content, protein content, monosaccharides, and phytochelatins content, while it lowered accumulation of heavy metals, reducing stress on growth	Bajguz (2011)
24-Epibrassinolide (10^{-10} and 10^{-8} M) and putrescine (1 mM) Foliar spray	*Lycopersicon esculentum*	NaCl stress	Co-applications of EBL and Put significantly enhanced the activities of antioxidant enzymes, improved protein content, and reduced membrane damages by declining MDA content	Slathia et al. (2012)
24-Epibrassinolide (0.1 μM) Foliar spray over 3 day seedlings	*Cucumis sativus* L. seeds	$Ca(NO_3)_2$ stress	EBL application enhanced nitrogen metabolism and increased total amino acids and total polyamines content which improved stabilization and biosynthesis of proteins	Yuan et al. (2012)
24-Epibrassinolide (0.1 mg L^{-1}) Foliar application	*Triticum aestivum* L.	Salt stress	EBL enhanced carbon and nitrogen metabolisms, glycinebetaine concentration, and polyamines accumulation	Talaat and Shawky (2012)
24-Epibrassinolide (10^{-6} M) Spray at vegetative, buds formation and early fruiting	*Capsicum annuum* L.	NaCl stress	EBL improved the flower number, fruit number, and yield per plant, but did not affect fruit mass and size. The effect was dependent on the plant development stage and frequency of EBL application	Samira et al. (2012)
24-Epibrassinolide (10^{-10}, 10^{-8}, or 10^{-6} M) Shotgun approach	*Vigna radiata* var. T-44 (Ni-tolerant) and PDM-139 (Ni-sensitive)	Ni stress	EBL caused upregulation of antioxidant enzymes as well as proline content which resulted in improved growth, nodulation, and yield in Ni-stressed plants	Yusuf et al. (2012)

24-Epibrassinolide (50 and 5 nM) Foliar and root treatment	*Lycopersicon esculentum*	Phenanthrene and pyrene	EBL increased detoxification of PHE and PYR by improvement in photosynthetic machinery and antioxidant enzymes	Ahammed et al. (2012)
Brassinolide (3 μM) Foliar spray	*Sorghum vulgare*	Saline soils	BL treatment enhanced detoxification of salt stress by lowering IAA oxidase, protease, and ribonuclease activities	Vardhini (2012)
28-Homobrassinolide/24-epibrassinolide (10^{-8} M) Foliar spray	*Solanum lycopersicum*	Cadmium stress	BRs enhanced growth, fruit yield, and quality of tomato	Hayat et al. (2012a)
28 Homobrassinolide (10^{-8} M) and salicylic acid (10^{-5} M) Foliar spray (after 30 and 45 days)	*Brassica juncea* L.	Salt stress	HBL excelled in alleviating salt stress symptoms sampling stages than SA. The combinations of the two hormones (HBL and SA) completely overcome stress toxicity at 45 days	Hayat et al. (2012b)
24-epibrassinolide (10^{-9} M) and spermidine (1 mM) Day two seedlings were treated with EBI and Spd	*Raphanus sativus* seedlings	Copper stress	Co-application of EBR and Spd modulated the expression of genes encoding PA enzymes and genes that impact the metabolism of indole-3-acetic acid (IAA) and abscisic acid (ABA) resulting in enhanced Cu stress tolerance	Choudhary et al. (2012a)
24-epibrassinolide (10^{-9} M) and spermidine (1 mM) Seedling were treated with EBL and Spd	*Raphanus sativus* L.	Chromium stress	Co-applications of EBL and Spd were more effective than their independent treatments in lowering the Cr-induced oxidative stress in radish, leading to improved growth of radish seedlings under Cr stress	Choudhary et al. (2012b)
24-Epibrassinolide (10^{-10}, 10^{-8}, 10^{-6} M) Shotgun method	*Brassica juncea* L.	High temperature stress	Exogenous application of EBL increased protein content and antioxidant enzymes	Kumar et al. (2012)
24-Epibrassinosteroid (0.01 mg L^{-1}) Foliar spray	*Capsicum annuum*	Drought stress	EBR alleviated the detrimental effects on photosynthesis by increasing the efficiency of light utilization and dissipation of excitation energy in the PSII antennae in leaves	Hu et al. (2013)
24-Epibrassinolide (0.1 μM) Foliar spray	*Solanum lycopersicum*	Phenanthrene and cadmium stress	EBL alleviated photosynthetic inhibition and oxidative stress by causing enhancement of the activity of antioxidant system, secondary metabolism, and the xenobiotic detoxification system	Ahammed et al. (2013)

dephosphorylating BZR1 and BES1. Activated form of BZR1 and BES1 accumulate in the nucleus and bind to CGTG (T/C) G motif in the promoters of BR biosynthesis genes *CPD* and *DWF4* (He et al. 2005) and also with the E box sequence (CANNTG) present in the *SAUR-ACI* promoter (Yin et al. 2005). Transcription factors like BIMs (Yin et al. 2005) and MYB30 performances as a helpful controller of the oversensitive cell death response and ELF6 and REF6 proteins intricate in maintaining flowering time. BES1 act together with those BIMs and MYB30 transcription factors and jumonji (Jmj) domain-covering proteins ELF6 and REF6 which results in conscription of diverse proteins by BES1 as one of the means through which BR have impact on miscellaneous biological progressions.

To an extensive variety of stresses, for example heat, cold, drought, and salinity, the tolerance is increased in plants by brassinosteroids. This increase is normally associated with greater appearance of stress indicator genes, such as heat shock protein (hsp) genes, RD29A, and ERD10 (Dhaubhadel et al. 1999; Kagale et al. 2007). It indicates that in brassinosteroid-treated plants improved expression of stress-receptive genes is accountable, partly, for the advanced stress tolerance. ARF2, an affiliate of the ARF family of transcriptional controllers, was phosphorylated by brassinosteroid-controlled BIN2 kinase which leads to damage of ARF2 DNA binding and suppression actions (Vert et al. 2008). Thus, ARF2 relates BR and auxin signaling pathways. Along with gene co-regulation, BR can correspondingly endorse auxin transportation (Li et al. 2010) and finest auxin deed is reliant on BR intensities (Hardtke et al. 2007).

In *Arabidopsis*, the nonexpressor of pathogenesis-related genes1 (NPR1) is probably a vital component of BR-mediated effects on thermotolerance and salt tolerance. BRs possess anti-stress effects independently as well as through interactions with other hormones. ABA reduces BR effects under heat stress and BRs share transcriptional targets with other hormones also. EBR promotes the basic thermotolerance of *Brassica napus*, *Lycopersicon esculentum* (Dhaubhadel et al. 2002) and *Arabidopsis* seedlings (Kagale et al. 2007). While the EBR effects on stress tolerance were most prominent when seedlings were grown in the long-term treatment of EBR (Krishna 2003). Treatment of EBR triggers significant improvement in the levels of hsps during heat stress in *B. napus* (Dhaubhadel et al. 2002); however, the effect of EBR on hsp levels in *Arabidopsis* was restrained (Kagale et al. 2007). No significant differences in the steady-state levels of hsp90 were analyzed between EBR-treated and untreated mutant seedlings which includes npr1-1 EBR-treated aba1-1 seedlings contained 3- and 2.5-fold higher levels of hsp90 at 3 and 4 h of HS, respectively, as compared to untreated aba1-1 seedlings.

EBR treatment also promoted the expression of the JA/ ET marker gene *PDF1.2* in WT, *aos*, *jar1-1*, and *eto1-1* backgrounds, but not as the same extent in *ein2*. The effect of EBR on *LOX2* expression was found different in Col and *jar1-1* backgrounds, while not in Col-6 and *aos* backgrounds. Improvement in the *HEL* gene expression by EBR was only minor. The ABA-responsive *LTP4* represented staged induction by EBR in WT and *abi1-1* background. EBR also upregulated the transcript levels of the ABA-marker gene *RD22* only slightly in *aba1-1* and *abi1-1* mutant seedlings, although significantly in WT, that indicates the interaction

between ABA and BR in affecting gene expression. For basal thermotolerance, a JAR1-reliant pathway is also compulsory (Clarke et al. 2009). By an assemblage of genotypes with basal thermotolerance whichever inferior or advanced than WT, it was established that EBR action might considerably upsurge the basic thermotolerance of these genotypes and that this upsurge was similar to the upsurge in WT.

9 Homeostasis

In recent times, it has been proved in a number of studies with *Arabidopsis thaliana* that for BR homeostasis, expressions of certain BR metabolic genes are restrained at mRNA levels. In answer to dropped aggregates of endogenous BRs in BR-lacking mutants over and above in wild-type *Arabidopsis* treated with a BR biosynthesis inhibitor brassinazole, the mRNAs of BR biosynthesis genes for instance DWARF4 (DWF4) and constitutive photomorphogenesis and dwarfism (CPD) rise (Noguchi et al. 2000; Asami et al. 2001; Choe et al. 2001). Distinctly, when BL is applied to wild-type plants, a BR inactivation gene, phyB stimulation marked suppressor 1 (BAS1), is augmented; that of mRNAs of DWF4 and CPD are reduced quickly (Mathur et al. 1998; Goda et al. 2002). Moreover, in BR-insensitive bri1 mutants in which BR discernment is imperfect, BL encouraged downregulation of CPD communication is cancelled which recommends that response terms of BR metabolic genes involve BR-insensitive 1 (BRI1) utility (Li et al. 2001; Bancos et al. 2002). Certainly, in bri1 mutants, the amassing of biologically dynamic BRs and mRNA advancement of BR biosynthesis genes deetiolated2 (DET2), DWF4, and CPD are detected (Noguchi et al. 1999, 2000; Choe et al. 2001; Bancos et al. 2002). For certain additional plant species, for example, barley (*Hordeum vulgare*), rice (*Oryza sativa*), pea (*Pisum sativum*), and tomato (*Lycopersicon esculentum*) parallel effects have been testified (Nomura et al. 1997; Yamamuro et al. 2000; Montoya et al. 2002; Chono et al. 2003).

For the regular progression and enlargement of higher plants, the homeostasis of brassinosteroids is vital. Brassinazole is a BR biosynthesis inhibitor. In BR-exhausted wild-type plants developed under brassinazole, two sterol biosynthesis genes (FK and DWF5) and five BR-precise biosynthesis genes (DET2, DWF4, CPD, BR6ox1, and ROT3) were upregulated. Instead, a sterol production gene (DWF7) and four BR-precise production genes (DWF4, CPD, BR6ox1, and ROT3) were downregulated and a BR inactivation gene (BAS1) was upregulated in brassinolide-nourished BR-excessive wild-type plants. However, their reaction to variation of BR intensities was greatly condensed (DWF4) or invalidated (the additional eight genes) in a bri1 mutant. A BRI1-mediated signaling pathway controls their feedback expressions. Furthermore, it is submitted by a weak response in the mutant that along with BRI1 intervention, DWF4 single-handedly is probably controlled in additional approach.

In *Arabidopsis* cell suspension, brassinosteroids might upsurge cell splitting up by cumulative CycD3 transcript intensities (Riou-Khamlichi et al. 1999; Hu et al. 2000). Variance manifestation arrangements of cyclin and CDK genes have also been examined throughout tomato fruit growth (Joubès et al. 1999, 2000).

10 Genetic Approaches for BRs to Enhance Crop Stress Tolerance in Plants

BRs have been testified to show a major character in stress protection in both biotic and abiotic stress in plants. Several responses to abiotic stress are controlled by brassinosteroids (BRs) and polyamines (PAs). Interaction of brassinosteroids (24-epibrassinolide) and polyamines (spermidine) enhanced copper stress tolerance in *Raphanus sativus* (Choudhary et al. 2012a, b). The expression of genes encoding PA enzymes and the genes, that influence the breakdown of IAA and ABA, was found to result in heightened Cu stress tolerance with the collective application of 24-epibrassinolide and spermidine.

11 Conclusion and Future Perspective

Various growth and developmental processes of plants are regulated by BRs. They play vital role in the regulation of diverse physiological processes like cell elongation, embryogenesis, vascular differentiation, senescence, fertility, ethylene biosynthesis, photosynthesis, and adoptive responses to environmental stress. By their molecular studies, it was analyzed that promotion of cell expansion and regulation of photomorphogenic responses are among the most important roles of BRs. Due to their effects on gene expression and enzyme activity they have also been involved in the process of cell enlargement. Involvement of BRs in the regulation of ROS metabolism can provoke and regulate the expression of certain antioxidant genes and promote the activities of key antioxidant enzymes, including POD, CAT, and SOD. Their similarity with ecdysteroids has given rise to several studies to determine an ecdysteroid-interfering action in insects. BRs possess anti-stress properties and also having interactions with other hormones. Recently, it has been proved that BR homeostasis expressions of certain BR metabolic genes are restrained at mRNA levels. The homeostasis of brassinosteroids is vital for the regular progression and enlargement of higher plants. Several responses to abiotic stress are regulated by the interaction of BRs with other plant growth regulators. New refined techniques are required for the synthesis of brassinosteroids, so that they can be commercially utilized in the agricultural lands for the enhanced crop productivity. Certain advanced phytoremediation techniques by using BRs should be developed to ameliorate various environmental stresses.

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Chapter 7
Ethylene: Role in Plants Under Environmental Stress

M.A. Matilla-Vázquez and A.J. Matilla

1 Introduction

When plants colonized the terrestrial ecosystems (some 475 million years ago), they had to acquire a number of organs necessary to keep erectile (i.e., root system, stem, and especially cell walls lignin enriched) (Kendrick and Crane 1997; Peter and Neale 2004; Martone et al. 2009). Likewise, terrestrial plants also had to develop a leaf system able to carry out both photobiosynthesis (i.e., carbohydrate and energy biosynthesis) and transpiration (i.e., gas exchange and generation of a force that allows the ascent and distribution of water and nutrients from the soil). However, the colonization process also caused serious problems of stress as a result of the transition from aquatic, motile ancestors into terrestrial, sessile organisms (Martone et al. 2009). Thus, the lack of mobility resulted in a complicated process of adaptation to the environment and the acquisition of defense mechanisms against diseases and predators (Ausubel 2005). In order to avoid a progressive disappearance, plants improved their physiological plasticity and developed a complicated set of signaling networks. These networks are tightly regulated by hormones that allow plants to survive by protecting them against biotic and abiotic stresses (Robert-Seilaniantz et al. 2011). Ethylene (Et), in combination with hormones such as jasmonic (JA) and salicylic (SA) acids, is one of the main players involved in the resistance and susceptibility to bacterial, fungal, and nematode pathogens (Adie et al. 2007; Kazan and Manners 2008; León-Reyes et al. 2009, 2010; Lin et al. 2009). The biosynthesis,

M.A. Matilla-Vázquez
Department of Biochemistry, University of Cambridge,
Tennis Court Road, Cambridge, CB2 1QW, UK

A.J. Matilla (✉)
Faculty of Pharmacy, Department of Plant Physiology, University of Santiago de Compostela (USC), 15782 Santiago de Compostela, Spain
e-mail: angeljesus.matilla@usc.es

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transport, and accumulation of the above-mentioned hormones trigger a cascade of signaling pathways involved in plant defense. Et and JA signaling pathways are activated in response to necrotrophic plant pathogens; whereas salicylic acid (SA) play a major role during the triggering of plant defenses toward biotrophic pathogens (reviewed in Glazebrook 2005; Thaler et al. 2012). In general, SA and JA/Et defensive signaling pathways have been demonstrated to be mutually antagonistic (van Loon et al. 2006; Adie et al. 2007; Pieterse et al. 2012). Recently, it was demonstrated that both SA- and JA-dependent disease resistance is inhibited by a simultaneously reduced red:far light ratio (De Wit et al. 2013). In addition, it seems fairly clear that: (1) Et production plays a role in plant responses to flooding, salinity, drought, and several contaminant agents (e.g., ozone); and (2) plant growth-promoting rhizobacteria (PGPR) can improve plant tolerance to drought, salinity, and metal toxicity (Haas and Defago 2005; Lugtenberg and Kamilova 2009; Barreto-Figueiredo et al. 2011; Hol et al. 2013), although the role of Et in this puzzle is not fully decoded. This chapter aims to give an overview on the role of Et in the defense mechanisms of land plants against different types of environmental stresses.

2 Updated Overview of the Plant Hormone Ethylene

Et is the simplest plant hormone. Zhong and Burns (2003) showed that 7 % of the 6,000 investigated Arabidopsis genes were regulated by Et. During the plants life cycle, Et regulates key processes such as root hair development, flowering, climateric fruit ripening, seed dormancy, and germination (for review, see Czarny et al. 2006; Delseny et al. 2008; Matilla and Matilla-Vázquez 2008; García et al. 2010). In general, with the exception of lateral root initiation and fruit ripening (see flooding below), elevated levels of Et are deleterious to plant health and growth. Likewise, Et is also involved in environmental stress signaling upon wounding and during the interaction with pathogen and non-pathogen microorganisms (Pieterse et al. 2007, 2012; Verk et al. 2009). Consequently, the biosynthesis and perception of Et must be tightly controlled within the plant. The biosynthesis of Et begins with the transformation of methionine (Met), a scarce amino acid in plants, to *S-adenosylmethionine* (SAM). This conversion is catalyzed by the SAM synthase (Peleman et al. 1989). SAM synthases are not specific to the Met cycle (Yang Cycle) since SAM also serves as substrate for several reactions, including cell transmethylations. Subsequently, the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS; *S*-adenosyl-L-Met methylthioadenosine-lyase) catalyzes the rate-limiting step in Et biosynthesis by converting SAM into ACC and 5′-methyl-thioadenosine (MTA), which regenerates Met in several steps (Bradford 2008) (Fig. 7.1, left). Besides plants, the Yang Cycle is also found in bacteria, archeae, and animals and it is well known that in higher plants it is tightly regulated (Rzewuski and Sauter 2008). The *ACS* gene was first cloned from *Cucurbita pepo* (Sato and Theologis 1989) and then significant efforts were conducted to study this key *ACS* multigene family. All *ACS* members are under strict regulatory control and the abundance of ACS proteins is

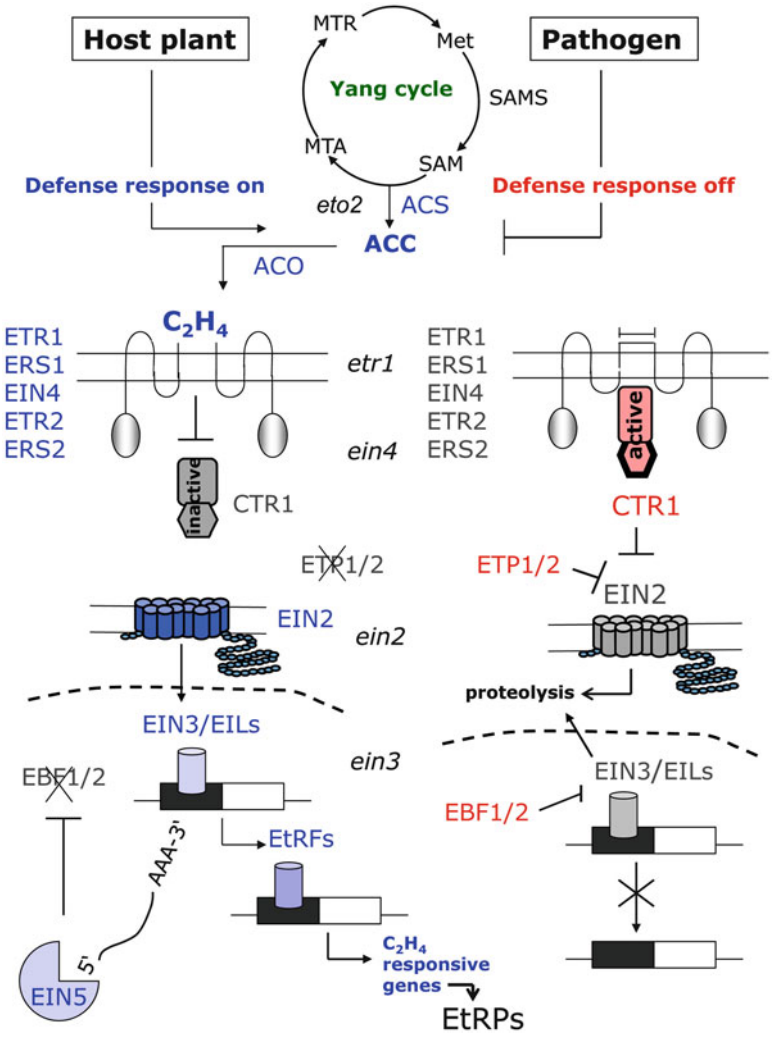

Fig. 7.1 Model explaining the involvement of the ethylene (Et) signaling in the plant defense mechanisms in the presence or absence of a pathogen. Adaptation of a scheme generously yielded by Dr. Ludwig-Müeller

closely correlated with the level of Et production in most plant tissues. Furthermore, various members of the *ACS* gene family were found to be differentially expressed in response to developmental and environmental triggers (Tsuchisaka and Theologis 2004a, b). The *ACS* family includes 6 members in rice (Rzewuski and Sauter 2008) and 12 members in Arabidopsis, of which only 9 appear to be implicated in Et biosynthesis (Yamagami et al. 2003; Vandenbussche et al. 2006; Vandenbussche and Van der Straeten 2007; Lin et al. 2009). Three types of ACS have been defined

based on their C-terminal regions, which are involved in enzyme stability. Five of the *ACS* genes and their expression patterns were described previously in deepwater rice internodes since at least two of them are sequentially induced during submergence (Rzewuski and Sauter 2008). Since *OsACS5* expression is induced within 60 min of submergence, this family member might be responsible for the early increase in ACS activity. By contrast, *OsACS1* expression is enhanced within 6 h of submergence. It has also been suggested that OsACS1 together with OsACS5 contributes to sustain Et production during long submergence (Zarembinski and Theologis 1997; van der Straeten et al. 2001). On the other hand, several MAPKs were found to regulate ACS activity (Broekaert et al. 2006; Schweighofer and Meskiene 2008). Thus, the phosphorylation of ACS2 and ACS6 by the MAPK MPK6 results in an increased production of Et (Christians et al. 2009). These phosphorylations also protect ACS2 and ACS6 from recognition and breakdown by the 26S proteasome pathway (Wang et al. 2004).

The last step of Et biosynthesis is catalyzed by ACC oxidase (ACO). In this metabolizing ACC reaction cyanoformic acid is also formed which is spontaneously degraded to cyanide (HCN) (Yip and Yang 1988). The HCN must be rapidly metabolized to keep its concentration below toxic levels. The molecular bases for HCN detoxification were recently studied in plants (Yi et al. 2012). The main HCN detoxification process described to date is catalyzed by β-cyanoalanine synthase (CAS), a pyridoxal phosphate-dependent enzyme that converts cysteine and HCN to HS and β-cyanoalanine. In Arabidopsis, the *CAS* gene family is composed of three members (Watanabe et al. 2008). The most abundant CAS protein (CYS-C1) is in the mitochondria, whereas CYS-D1 and DYS-D2 are found in the cytosol (Watanabe et al. 2008). Mitochondrial CAS is essential for formation of root hairs in Arabidopsis (García et al. 2010). HCN enhances the resistance of *N. tabacum* and Arabidopsis leaves to TMV and turnip vein clearing virus (TVCV), respectively (Wong et al. 2002). Likewise, it has also been proposed that HCN and Et are responsible for the resistance of young rice plants to blast fungus (*Magnaporthe grisea*) infection. In this fungus resistance mechanism, the induced *OsACS2* and *OsACO7* contributed specially (Iwai et al. 2006). On the other hand, plant pretreatment with KCN relieved stress induced by oxidative damage, and plainly induced the alternative oxidase (AOX) activity and Et production, proving a new fangled role of HCN against environmental stress (Xu et al. 2012).

In tomato and Arabidopsis *ACO* families are composed of four and six members, respectively (Babula et al. 2006; Lin et al. 2009). By contrast, in the rice genome six *ACO* members were found through computational analysis. Thus, in rice seedlings: (1) the highest expression of *OsACO1* was found in the very young growing internodes (i.e., *OsACO1* was induced after 4 h and at least up to 24 h of submergence; Mekhedov and Kende 1996); and (3) the expression of *OsACO2* and *OsACO3* were induced by auxin and Et, respectively, in a dose-dependent way (Chae et al. 2000). Taken together, Et biosynthesis is heavily regulated, including transcriptional and post-transcriptional control of the key enzymes (i.e., ACS and ACO). The presence of the enzyme ACC deaminase (ACCD), involved in the degradation of ACC to ammonia and α-ketobutyrate, is common in soil bacteria (Fig. 7.2), including biocontrol

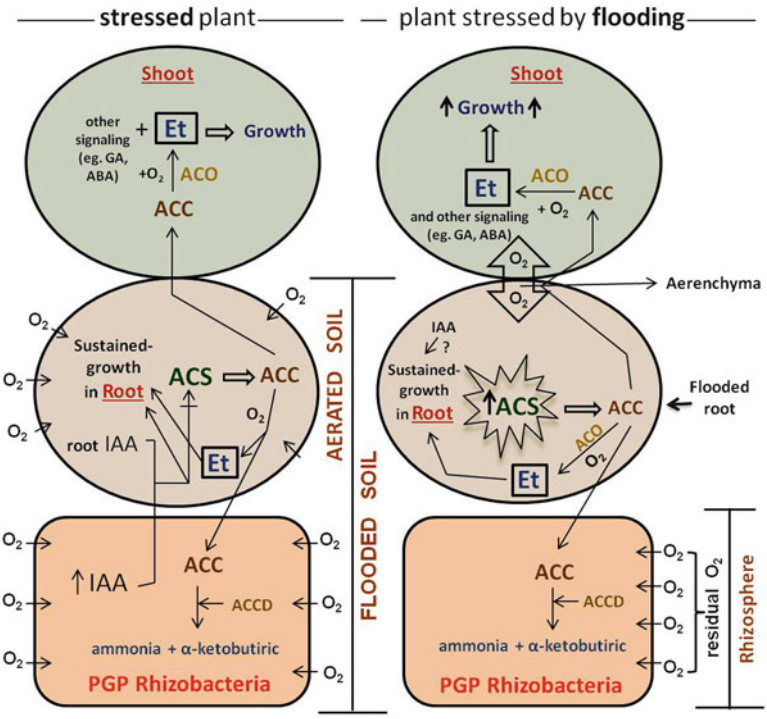

Fig. 7.2 Model explaining the role of plant growth promoting rhyzobacteria (PGPR) in generating plant growth under general stress conditions (*left*) and particularly under flooding (*right*). In the left model, PGPR synthesize and secrete IAA. Bacterial IAA, together with the IAA synthesized by the root, induce *ACS* transcription and consequently the production of ACC. A percentage of this Et precursor can be degraded by root-associated bacteria causing a notable decrease in the biosynthesis of Et. The remaining ACC is exported to the plant shoot where the ACC oxidase (ACO) catalyzes the synthesis of Et, triggering plant growth. In the right model, flooding is the environmental factor that induces ACS expression. The role of the ACC deaminase (ACCD) in both models is evident

bacterial strains (Glick et al. 2007; Chen et al. 2013; Roca et al. 2013). ACC is a frequent component of seeds, roots, and leaves exudates (Glick et al. 2007) and bacteria can act as a sink of ACC, lowering Et levels in the plant. As a consequence, plant growth can be promoted and some of the potentially deleterious consequences of high Et concentrations under environmental stresses (e.g., flooding, heavy metals, salinity, drought, and microorganisms attack) may be reduced (Glick et al. 2007; Gamalero and Glick 2012; Stearns et al. 2012). Interestingly, several plant-associated bacteria have a positive effect over the Et levels in the plants that they are colonizing. Thus, (1) some pathovars of the plant-pathogen *Pseudomonas syringae* have the ability to synthesize Et both in vitro and in vivo (Weingart and Volksch 1997; Sato et al. 1997); (2) the *Pseudomonas fluorescens* root colonization trigger an increase of ACO activity in vivo (Hase et al. 2003); (3) the expression amounts of *ACO1* and *ACO2* are up-regulated by the infection of *Botrytis cinerea*

(Adie et al. 2007); and (4) the transcriptional activation of *ACO* genes in tomato has been demonstrated in response to *P. syringae* infection (Weingart et al. 2001; Cohn and Martin 2005). Data on Et, JA, and SA production seems to conclude that a highly and tightly regulated Et biosynthesis may be used by pathogens Et producers to bypass defenses (Adie et al. 2007).

The Et signaling pathway is well established in Arabidopsis (de la Torre et al. 2006; Stepanova and Alonso 2009; Yoo et al. 2009). Thus, this gaseous hormone is sensed by receptors located in the endoplasmic reticulum. In the Arabidopsis there are five receptors (ETR1, ERS1, ETR2, ERS2, and EIN4), all of them with an active kinase domain (Stepanova and Alonso 2009; Yoo et al. 2009). The receptors operate as negative sensors of Et signaling and interact with Constitutive Triple Response 1 (CTR1), an Raf-like protein kinase (Fig. 7.1, left). In the absence of Et, CTR1 has a negative regulatory function, actively suppressing the Et signaling pathway. Upon Et-receptor binding, CTR1 is no longer capable of repressing Ethylene Insensitive 2 (EIN2) which is a transmembrane protein with homology to NRAMP metal ion transporters. EIN2 acts as a positive regulator of the Et responses. Et destabilizes the F-box proteins called ETP1 and ETP2, stabilizing EIN2 and promoting downstream effects (Qiao et al. 2009). EIN2 prevents the binding of the key Et Response Factors (EtRFs) EIN3 and its homolog EIN3-like 1 (EIL1) to EBF1 and EBF2 (EIN3 binding F-box proteins 1 and 2) which are part of an SCF E3 ligase complex (SCFEBF1/2) (An et al. 2010). Consequently, EBF1 and EBF2 are down-regulated by Et, suggesting that this gaseous hormone stabilizes EIN3/EIL1 by promoting EBF1 and EBF2 degradation by the proteasome complex. Thus, EIN3 (a short-lived transcription factor (TF) with five homologs in the Arabidopsis genome) and EIL1 are no longer degraded through the 26S proteasome pathway and induce transcription of EBF1 and EBF2 (Guo and Ecker 2003; Potuschak et al. 2003; Binder et al. 2007; Konishi and Yanagisawa 2008). When the Et levels decrease or Et is absent, EIN3 is ubiquitinated by SCFEBF1/EBF2 and degraded by the 26S proteasome. All this process is under control of EIN5, a $5' \rightarrow 3'$ exoribonuclease that acts downstream of CTR1 (Fig. 7.1, left). In the presence of Et, EIN5 promotes the *EBF1* and *EBF2*-mRNA decay, which allows the accumulation of EIN3 (Olmedo et al. 2006). In short, EIN3 is: (1) stabilized by Et; (2) phosphorylated by an MAPK cascade which can be activated by CTR1; (3) accumulated in nuclei after the increase in the Et levels with the subsequent binding to the promoter of EBF2; and finally, (4) together with EIL1, regulates the expression of target genes such as *EtRF1*, which encodes the transcription factor Et-Response Element Binding Protein (AP2/EREBP) involved in plant defense against necrotrophic pathogens (Glazebrook 2005; Verk et al. 2009; Zhao et al. 2012). EtRF1 and AtMYC2 are two notable regulators of Et–JA interactions in defense. However, AtMYC2 works in the opposite way to EtRF1 (for more information, see Adie et al. 2007). Interestingly, genes encoding group-VII EtRFs (Ethylene Response Factors) are up-regulated under anaerobic stress in several plant species (Nakano et al. 2006; Bailey-Serres et al. 2012).

Finally, it is especially important to note that during a stress process: (1) the Et action mode is modulated by the concentration of the hormone rather than by its

presence (Pierik et al. 2006); (2) Et, SA, and JA signaling pathways, individually or in crosstalk, play significant roles in the physiology of stress in land plants (Wasternack 2007; Thaler et al. 2012); (3) during resistance to necrotrophic pathogens, Et synergistically with JA plays a key role, as demonstrated by genetic approaches (Grant and Jones 2009; Pieterse et al. 2012); and (iv) ACC-JA conjugation may be fundamental for the Et-JA crosstalk regulation (Wasternack 2007; Fonseca et al. 2009).

3 Crosstalk Between Oxygen Deficient Stress and Ethylene Biosynthesis and Signaling

O_2 is the final electron acceptor in the mitochondrial respiratory chain. In soil, and more specifically in the rhizosphere, O_2 concentrations can be limiting (hypoxia) or absent (anoxia). The decrease of O_2 diffusion capacity in the soil (e.g., compact structure, water logging, and deep flooding) limits its availability for the root (Dat et al. 2004). Thus, the O_2 shortage in the soil generates a partial pressure around radical system incapable to oxygenate in the root the machinery of respiratory ATP biosynthesis. Additionally, the consumption of O_2 by aerobic rhizosphere microorganisms can further aggravate the root stress. Indirect and direct sensing of O_2 status may be responsible for the acclimatization responses that extend survival under O_2 deprivation (Bailey-Serres and Chang 2005). For this reason, plants can adapt to this energy crisis by promoting anaerobic metabolism and thus increase substrate-level ATP production (Magneschi and Perata 2009).

Rice (*O. sativa*) is a model plant for the study of metabolic control under O_2 limiting conditions since this semiaquatic organism is well adapted to a partially flooded environment. However, abrupt flooding can cause sharp submergence by imposing, among other factors, a complex stress due to a 10^3-fold reduction in the diffusion of O_2 and CO_2. The growth of deep water rice in wetlands is adapted to gradual flooding by means of acceleration in the elongation of submerged internodes to keep aerial tissues above the air–water environment. When sudden submerged, deepwater and most lowland varieties accelerate internode and/or leaf elongation to avoid the flooding. By contrast, lowland varieties tolerant to submergence save complete submergence through a constraint in shoot elongation and carbohydrate spending, thereby conserving energy reserves to restarting development upon desubmergence (Fig. 7.3). Consequently, an immediate response must be triggered by the plant in order not to block energy biosynthesis (Geingenberger 2003; Bailey-Serres et al. 2012). Thus, almost 50 genes responding to O_2-shortage, including EtRFs, were identified in several species such as Arabidopsis, rice, cotton, and poplar (Mustroph et al. 2010). Recent reports contain excellent updates on the molecular biology of O_2-shortage response (Mustroph et al. 2010; Bailey-Serres et al. 2012; Licausi 2011, 2012).

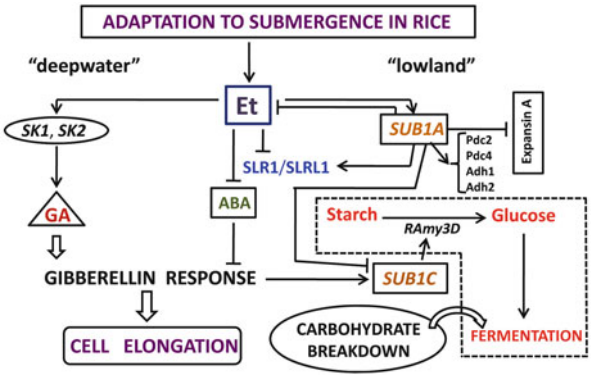

Fig. 7.3 Crosstalk between Et, ABA, and GA in submergence adaptation process of rice seedlings belonging to deepwater and lowland ecotypes

3.1 Role of Ethylene Response Factors Under Low-Oxygen Stress

A large quantity of microarray data for Arabidopsis and rice under low-O_2 stress (i.e., anoxia and hypoxia) are available, and these experiments have revealed much about plant responses to low O_2 (Licausi et al. 2010; Mustroph et al. 2010; Lee et al. 2011; Licausi 2012). For example, EtRFs are TFs unique to plants that bind specifically to TAAGAGCCGCC (GCC box) sequences found in the promoter regions of Et Response (EtR) genes (e.g., *Hookless1*). EtRFs are ubiquitous in the plant kingdom and their functional implications have been studied in a wide range of processes including response to biotic and abiotic stresses (for more information, see Pirrello et al. 2012). The EtRF family is a large gene family of TFs which is part of the *APETALA2* (*AP2*)/*EtRF* superfamily. AP2 is one of the largest families of TFs in plants, including three different sub-families which are characterized by the number of EtRF domains and by having either one or two AP2 DNA-binding domains. The EtRF, also known as the Et-Responsive Element-Binding Protein (EtREBP) family, has one AP2 domain, the RAV family has two domains (i.e., AP2 and B3), and the AP2 family has two AP2 domains (Nakano et al. 2006; Romanel et al. 2009). In Arabidopsis and rice, the EtRF family comprises about one hundred members which are categorized into ten clades. Clade VII has an MCGGAI/L highly conserved motif at its NH_2-terminal (Nakano et al. 2006). In all rice varieties studied, a sub-group VIIb exists where all members lack this NH_2-terminal motif. On the other hand, a major QTL responsible for tolerance to submergence, *Submergence1* (*SUB1*; located in chromosome 9), was identified in varieties of lowland *indica* rice (Fukao et al. 2006; Xu et al. 2012). This *SUB1* locus consists of a clade of three sub-group VIIb genes (*OsSUB1A*, *OsSUB1B*, and *OsSUB1C* genes), but the *SUB1A* is present only in *indica* and not *japonica* cultivars. *OsSUB1C* acts downstream of *OsSUB1A* (Fukao et al. 2006).

The expression of an Arabidopsis clade VII gene, *AtRAP2.2*, is induced by Et in shoots but not in roots (Hinz et al. 2010). RAP2.2 protein only affects to the induction of genes linked to sugar metabolism, fermentation, and Et biosynthesis (Hinz et al. 2010). Unlike rice, Arabidopsis possesses five genes within group VII, including *HYPOXIA-RESPONSIVE1* (*HRE1*) and *HRE2*. The plants overexpressing *HRE1* and *HRE2* showed an increased tolerance to anoxia, whereas the *hre1hre2* double mutant showed reduced tolerance (Licausi et al. 2010). A further study showed that in the presence of exogenous ACC transgenic seedlings with silenced *HRE1* displayed exaggerated apical hook curvatures. These results indicate a negative role of HRE1 in the Et responses (Yang et al. 2011). *HRE1* and *HRE2* shows a strong up-regulation under O_2 depletion, mediated by both Et-dependent and Et-independent signals (Licausi et al. 2010; Yang et al. 2011). Like *SUB1A*, *HRE1* transcript accumulation is induced by Et, which synergistically increases its rise during O_2 stress (Yang et al. 2011). Not long ago, another member of the *AP2/Etr2* family named *Octedecanoic-Responsive Arabidopsis59* (*ORA59*) was found to be as the more important integrator of the JA and Et signaling pathways. *ORA59* is induced and synergistically activated by JA and Et.

Et also induces the gene expression of alcohol dehydrogenase (*ADH1*) in Arabidopsis (Peng et al. 2001, 2005). Ethanolic fermentation through ADH1 activity contributes substantially to low-O_2 stress adaptation. For this reason, an *adh1* null mutant showed lower survival when exposed to low-O_2 pressure (Ellis et al. 1999). Likewise, the pyruvate decarboxylases (*PDC1* and *PDC2*) overexpression in Arabidopsis results in improved survival under low-O_2 conditions (Ismond et al. 2003). EtRFs are also involved in several developmental processes such as zygotic embryogenesis (Riechmann and Meyerowitz 1998) and abiotic and biotic stress responses (Fujimoto et al. 2000; Sakuma et al. 2002).

Finally, the degradation of clade VII-EtRF proteins is carried out by the N-end rule pathway (i.e., N-erp; Hinz et al. 2010; Gibbs et al. 2011; Bailey-Serres et al. 2012). More specifically, all five Arabidopsis VII-EtRFs proteins are N-end rule substrates. N-erp is a pathway to degrade proteins that relates the in vivo stability of a specific protein to the nature of its N-terminal. These N-terminal destabilizing residues are known as N-degrons (Varshavsky 2011). In eukaryotes, N-erp is a part of the ubiquitine (Ub) system (Graciet and Wellmer 2010).

3.2 Crosstalk Between Low-O_2 and Ethylene Under Submergence

Many investigations have demonstrated the involvement of Et in O_2-shortage responses (i.e., flooding and submergence). In contrast to flooding avoidance, which involves increased Et and enhanced stem elongation rates to permit the plant to have access to atmospheric O_2 (Kende et al. 1998), submergence tolerance is the result of an efficient reduction in the consumption of carbohydrates and an ethanolic fermentation-dependent metabolism, together with a reduced production of Et and

restricted cell elongation (Jackson and Ram 2003). Careful research in rice and a wetland dicot, marsh dock (*Rumex palustris*), pointed out that Et accumulation in submerged organs triggers a hormonal signaling pathway that cause the reduction of the antagonism between gibberellins (GA) and abscisic acid (ABA) which is usually responsible for the restriction of the internodal cell elongation. In submerged parts, the restriction of internodal elongation is achieved via a decreased responsiveness to GA arising from elevated levels of DELLA proteins that repress GA-induced growth (Fukao and Bailey-Serres 2008). *SNORKEL* (*SK*) *1* and *2* and *SUB1A* (EtRFs that confers prolonged tolerance to submergence in deepwater rice) genes are involved in the above signaling cascade (Hattori et al. 2009; Bailey-Serres and Voesenek 2010). The deepwater rice adaptation to flooding is the result of its ability to elongate the cell internodes. These internodes possess hollow structures which prevent plant drowning allowing gas exchange with the atmosphere. The internode elongation response in deepwater rice is regulated by Et (Hattori et al. 2009). Many physiological and molecular studies have shown that Et, GA, and ABA signaling are implicated in the elongation response. However, most of the gene(s) involved in this trait needs to be identified. Thus, the Hattori´s group found for the first time that the EtRFs-encoding genes *SK1* and *SK2* trigger deepwater response. Consequently, the deepwater rice requires *SK1* and *SK2* to extend the hollow stem to the water surface through the elongation of its stem internodes (Hattori et al. 2009). Therefore, under these deepwater conditions, Et accumulates and induces expression of *SK1* and *SK2* whose products triggers notable internode elongation via GA (Hattori et al. 2009).

As indicated above (section "Role of Ethylene Response Factors Under Low-Oxygen Stress"), several EtRF proteins from the major QTL SUB1 were demonstrated to have a main role in submergence tolerance in rice (Xu et al. 2006). Both flooding and submergence are controlled by *SUB1A*, *SUB1B*, and *SUB1C*. However, since the expression of *SUB1A-1* confers submergence tolerance to submergence intolerant rice plants, *SUB1A* is thought to be the key gene in this *SUB1* gene cluster (Xu et al. 2006). Some key features of *SUB1A-1* are described below. *SUB1A-1* overexpression in *japonica* rice, a flooding-sensitive cultivar, resulted in an enhanced *ADH1* expression and tolerance to flooding (Fukao et al. 2006; Xu et al. 2006). Several authors have proposed that the conferred submergence tolerance is the result of a complex signaling pathway that reduces carbohydrate consumption and growth elongation (Fukao et al. 2006; Xu et al. 2006; Perata and Voesenek 2007; Jung et al. 2010). *SUB1A-1* transcripts, as with *SK1* and *SK2*, are Et-induced. Additionally, (1) *SUB1A*-1 boosts the accumulation of *SLENDER RICE 1* (*SLR1*) and *SLENDER RICE-LIKE 1* (*SLRL1*), two negative regulators of GA responses; and (2) *SUB1A-1* protein ultimately limits Et biosynthesis (Fukao et al. 2006; Fukao and Bailey-Serres 2008). Other effects induced by SUB1A-1 were described by Bailey-Serres et al. (2012). All together, *SUB1A-1* seems to be included in an appropriate point in the signaling pathway belonging to submergence response. Thus, SUB1A-1 maintains cell viability and prevents plant growth during submergence stress. Furthermore, during a subsequent recovery period (i.e., reoxygenation), SUB1A-1 is also involved in homeostasis restoration. The reduced elongation response is only beneficial when the submergence is deep and/or relatively short

lasting. However, when the submergence is prolonged but relatively shallow floods, several plant species have been shown to elongate their stems in a hormonal-dependent manner. Thus, the accumulated Et inhibits ABA biosynthesis and increases its degradation resulting in reduced levels of ABA (Benschop et al. 2005, 2006; Saika et al. 2007). The decline of ABA levels results in the release of the repression of GA biosynthesis promoting the increase of the concentration of bioactive GA in the submerged tissues. Additionally, in response to Et and submergence, the sensitivity to GA is also enhanced, through yet unknown mechanisms. *SK1* and *SK2* genes, belonging to the same APETALA2/EtRF subfamily as the *SUB1A-1* gene, play a role in rice elongation when submerged (Hattori et al. 2009). Although it is not known whether the *SK* genes interfere with GA biosynthesis or action, it has been demonstrated that they act upstream of GA. A rapid underwater elongation requires carbon and energy, and, therefore, depends on the accessibility to nonstructural carbohydrates. Chen et al. (2010) shown that the translocation of newly fixed carbon to the elongation tissues and the mobilization of starch can both be induced under submergence conditions (Chen et al. 2010). Model explaining the relationship between Et, ABA, and GA in submergence adaptation process of rice is indicated in Fig. 7.3.

SUB1A perhaps can represses cell elongation though an involving expansin-A, increase in ethanolic fermentation via control of ADH gene expression, and a decrease in carbohydrate consumption, among other metabolic factors (Bailey-Serres and Voesenek 2010). Strikingly, SUB1A represses SUB1C which acts in an antagonistic way by promoting GA-induced carbohydrate breakdown and cell elongation. Both SUB1A and SUB1C are induced by Et. However, since SUB1A responds to Et at concentrations two orders the magnitude lower than SUB1C, is expected to be induced earlier. Therefore, in the presence of SUB1A, a delay in the induction of the expression of SUB1C during submergence is observed (for more information see Rzewuski and Sauter 2008). Although it is clear that several hormones, cell wall loosening proteins and carbohydrates are required for the elongation response, nowadays is poorly understood which part of the signal transduction pathway may cause the differences within and among naturally occurring species. In contrast to wild species, more research has been done in cultivated rice varieties to explain the variation in underwater elongation.

Recently, Chen et al. (2010) suggested that, under submergence conditions, the variation in the elongation rate of the petioles of the wetland plant *Rumex palustris* is controlled by an Et-regulated pathway that alters the dynamics of endogenous ABA levels in the petioles. This variation in the endogenous ABA concentration affects the responsiveness to GA and consequently the underwater petiole elongation rate. In this wetland species, the stimulation or inhibition of the underwater elongation is controlled by the *AP2/EtRF* genes (Voesenek and Bailey-Serres 2009). The slow elongating varieties maintain relatively high levels of ABA, which then results in a limited GA responsiveness and thus reduced growth rate. The effect of ABA on GA in the model species *R. palustris* suggests a novel role of ABA regulating GA. Notoriously, if we compare this study with previous research investigating the role of Et and ABA under submergence conditions in the fast and slow elongating species *R. palustris* and *R. acetosa*, respectively, the results strongly indicate

that differences between and within species in petiole elongation induced by flooding are controlled by the same switch point(s) and pathway(s), i.e., by regulating the levels of ABA and the subsequent GA responses (Benschop et al. 2005; Chen et al. 2010). It may be hypothesized that the inter- and intra-species genotypic variation in wetland plant species is the result of the strong selective force exerted by flooding stress.

3.3 Ethylene and Flooding

To survive flooding, many plant species have evolved by developing new adaptive traits (Bailey-Serres and Voesenek 2010; Bailey-Serres et al. 2012). The privatization of O_2 to the roots is the main consequence of flooding. Flooding together with salinity, dryness, and temperature is an important generator of abiotic stress and significantly affects distribution of plants in terrestrial environments. Shortage (i.e., hypoxia; $[O_2] < 50$ mmol m^{-3}) or absence (i.e., anoxia) of O_2 in waterlogged environments generates different responses in root systems (Matilla and Rodríguez-Gacio 2013). Under flooding, gases diffuse 10^4-fold slower. Thus, within the first 60 min of flooding, a decline from 20.8 to 7.9 kPa in the partial pressure of O_2 was observed, which continues to decrease to 1 kPa after 24 h. Under low O_2 conditions, soil microorganisms are the main consumers of the available O_2 and several toxic compounds may accumulate in the rhizosphere. The O_2 consumption by soil microorganisms generates a strong stress around the roots. Some plants (e.g., rice) may remain temporarily in soils with low O_2 levels and show a positive response to Et and enhanced tissue sensitivity to GAs (Knaap et al. 1996). Therefore, survival of rice upon a great increase of the water level depends on the fast elongation of the stem, which is Et-regulated. *OsACS1* alone, or in combination with *OsACS5*, maintains Et production during submergence (van der Straeten et al. 2001; Rzewuski and Sauter 2008). It was hypothesized the increase in the *OsACS* expression, together with the increase in the activity of OsACS due to the escape of OsACS1 from OsEOL-mediated degradation, result in a rising of Et production within the first hours of submergence (Yoshida et al. 2006). The appearance of aerenchyma vessels (i.e., soft tissues), which allow O_2 exchange from the aerial parts to the root tissues and adventitious roots, was an evolutionary key for the flooding adaptation (Vartapetian and Jackson 1997; Watkin et al. 1998; Bacanammwo and Purcell 1999; Gunawardena et al. 2001; Aschi-Smiti et al. 2004). Aerenchyma formation occurs through two different processes: schizogeny and lysigeny. Schizogenous aerenchyma is characteristic of *Rumex* spp. and involves reorganization of the cell wall (CW) and cell separation. However, in plant such as Arabidopsis or rice, programmed cell death (PCD) is responsible for the formation of lysigenous aerenchyma. Many of the adaptive growth responses occurring in roots under hypoxic conditions, including aerenchyma formation, occur in response to Et which is stored by physical trapping in flooding soil solution and submerged parts of plants at concentrations of 10^3 mm^3 dm^{-3} (Voesenek et al. 2006). Therefore the application of Et

induces the aerenchyma formation in hypoxic maize roots, while the presence of Et inhibitors repress its appearance (Dat et al. 2004).

The expression level of genes responsible for Et biosynthesis is up-regulated under flooding conditions (van der Straeten et al. 1997; Peng et al. 2005). Thus, in root tissues ACO activity is inhibited (Voesenek et al. 1993) and ACS activated (Van Der Straeten et al. 2001; Rieu et al. 2005), generating high levels of ACC (Geisler-Lee et al. 2010). Notably, since ACC is a mobile molecule does not necessarily require to be produced at sites where Et acts. Under flooding, ACC synthesized in plant roots is transported via the xylem to enable the biosynthesis of Et in the distant tissues (Finlayson et al. 1991). Therefore, the ACC must be transported to the next aerobic zones (i.e., shoots) for its conversion into Et. In tomato plants, English et al. (1995) showed that ACO activity regulates the Et production in response to flooding. On the other hand, during flooding of *Rumex palustris*, Et biosynthesis seems to be limited at the level of ACO activity rather than by ACS (Voesenek et al. 1993). However a portion of ACC biosynthesized by the roots is translocated to the rhizosphere and become available to bacteria possessing ACCD (see above, section "Updated Overview of the Plant Hormone Ethylene") (Fig. 7.2). If bacteria with ACCD are not abundant in the rhizosphere, the ACC is mostly translocated to the oxygenated upper parts of the plant for subsequent transformation to Et (Grichko and Glick 2001). Interestingly, aerenchyma formation does not always require Et. In some species such as Arabidopsis, constitutive lysigenous aerenchyma is formed in response to Et and H_2O_2 signaling (Mühlenbock et al. 2007). In support of the latter, the involvement of ROS, Ca^{2+} signaling, and CW metabolism in aerenchyma formation was recently demonstrated under waterlogged conditions (Rajhi et al. 2011).

Finally, to summarize rice adaptation to flooding: (1) the triggered Et biosynthesis and accumulation leads to an increase in bioactive GA and appearance of PCD; (2) PCD of epidermal cells facilitates emergence of adventitious roots at the nodes of the submerged stems, while GA induces the internodal growth; (3) Et prevents ABA biosynthesis and consequently the GA action on growth and PCD.

3.4 Crosstalk Between Low-O_2 and Ethylene in Seeds

The production of seeds is crucial and represents the main strategy that allows most plants species to maintain their genetic diversity, survive, and spread. Before germination is triggered, viable seeds can overcome long periods of severe desiccation and dormancy (Iglesias-Fernández et al. 2011; Graeber et al. 2012). Indeed, one of the key milestones during plant evolution has been the acquisition of desiccation tolerance (Linkies et al. 2010). Under desiccation conditions, the seed undergoes strong metabolic and hormonal readjustments, such as an increase in dehydrin and ABA levels (Rodríguez-Gacio et al. 2009; Leprince and Buitink 2010). During seed development and early imbibition, the internal high metabolic activity and the outer seed layers (i.e., seeds coats) prevent O_2 diffusion. Likewise, this hypoxic environment inside the seed causes an ATP deficiency (Borisjuk and Rolletschek 2009).

Hence, the seed needs to develop strategies to reduce or prevent O_2 restriction besides an ability to adjust its endogenous levels of O_2 as well as O_2 demands. For this, the seed requires mechanisms for O_2-sensing and O_2-dependent regulatory systems (Bailey-Serres and Chang 2005; Borisjuk and Rolletschek 2009). Although the O_2 sensors have not been definitely identified, in Arabidopsis seedlings, two independent research groups have recently demonstrated that one branch of the Ub-dependent N-end rule pathway functions as a mechanism for sensing O_2 (Gibbs et al. 2011; Licausi 2011; Licausi et al. 2011). Additionally, an increasing amount of data supports the leading role for the non-symbiotic hemoglobins/NO (nsHbs/NO) cycle in O_2-sensing (Sairam et al. 2009; Siddiqui et al. 2010; Matilla and Rodríguez-Gacio 2013). However, it has not yet been successfully demonstrated whether Et biosynthesis and signaling are involved in triggering processes of hypoxia in seeds. The down-regulation of the nsHbs1 biosynthesis in *Hordeum vulgare* (barley) enhanced the production of Et in *Zea mays* (maize) suspension cells during hypoxia (Manach-Little et al. 2005). On the other hand, studies in *Gossypium hirsutum* showed that *GhnsHb1* expression is up-regulated by Et, SA, and JA, suggesting that GhnsHb1 may be involved in defensive mechanisms (Qu et al. 2006).

4 Ethylene and Plant Defense Against Microorganisms

Land plants are anchored to the soil and therefore the root system is in close contact with the neighboring soil environment (Darrah and Roose 2007). The release of nutrients in the form of root exudates to the rhizosphere (Loyola-Vargas et al. 2007; Newman and Römheld 2007; Uren 2007; Badri and Vivanco 2009) results in a highly active and dense population of microorganisms. In fact, bacterial population densities in the rhizosphere can reach 1–2 orders of magnitude higher than in the bulk soil (Molina et al. 2000; Morgan et al. 2005). The root exudation occurs through root hairs and both the apex and young parts of roots (Newman and Römheld 2007; Uren 2007) and influences microbial root colonization (Lugtenberg and Bloemberg 2004; Gamalero et al. 2005; Watt et al. 2006). At the same time, rhizosphere colonizing microorganisms can directly alter the metabolism and development of the root system (Ahemad and Khan 2011; Berendsen et al. 2012).

The presence of rhizosphere microorganisms can affect the root exudate properties due to an active degradation of its components (Jones et al. 2003). Furthermore, rhizosphere microorganisms can also increase the exudation levels and alter the root exudates composition, facilitate the availability of some soil nutrients and promote the plant growth (Phillips et al. 2004; Rosas et al. 2006; van Loon 2007; Lugtenberg and Kamilova 2009; Matilla et al. 2010). Additionally, plant-associated microorganisms can synthesize plant hormones such as cytokinins, GA, and auxins (Preston 2004; Vessey 2003; Ahemad and Khan 2011; Roca et al. 2013) besides releasing Et (Freebairn and Buddenhagen 1964; Weingart and Volksch 1997; Sato et al. 1997). Microorganisms use two different Et biosynthetic pathways, both different from

that of higher plants (see above). Thus, most of these microorganisms produce small traces of the hormone via the 2-keto-4-methylthiobutyric acid (KMBA) pathway, in which the NADH:Fe(III)EDTA oxidoreductase generates hydroxyl radicals from molecular O_2 (Fukuda et al. 1989; Nagahama et al. 1992). However, several microorganisms can synthesize Et using 2-oxoglutarate as precursor via an Et-forming enzyme (Weingart and Volksch 1997).

During evolution, plants have acquired a complex system of defense mechanisms that protect them against plant-pathogenic fungi, oomycetes, and bacteria, besides viruses and nematodes (Bari and Jones 2009). Successful plant pathogens can interfere or block the plant immune system whereas beneficial plant–microorganisms associations can promote plant growth and help to overcome different environmental stresses. However, beneficial microorganisms are firstly recognized as potential pathogens and the plants can react to their presence by activating an immune response (Pieterse et al. 2012). Thus, the recognition of pathogen- or microbe-associated molecular patterns (PAMP/MAPS) by the plant can also trigger the so-called effector-triggered immunity (De Vleesschauwer and Höfte 2009). Found mostly in plant-associated bacteria, PAMP/MAPS are bacterial determinants such as flagella, lipopolysaccharides, siderophores, and antibiotics, amongst others (reviewed by Bakker et al. 2007; De Vleesschauwer and Höfte 2009; Vlot et al. 2009). Recently, it was shown that microbial elicitors and JA differentially modulates the plant's innate immune response (Flury et al. 2013). Plant pathogen infection may result in the induction of systemic acquired resistance (SAR), a broad spectrum, and long-lasting disease resistance. SAR is generally involved in the protection against (hemi-)biotrophic pathogens (Glazebrook 2005) and its induction requires the accumulation of SA. Moreover, SAR-induced plants show increased expression of pathogenesis-related (PR) genes (Durrant and Dong 2004; Vlot et al. 2009; Fu and Dong 2013). On the other hand, the plant root colonization by certain non-pathogenic PGPRs can suppress disease by triggering systemic induced resistance (ISR). ISR is phenotypically similar to SAR but it is dependent of the Et and JA signaling pathways (van Loon and Bakker 2005; De Vleesschauwer and Höfte 2009) (Fig. 7.4). In general, ISR is associated with defense against necrotrophic pathogens and herbivorous (Glazebrook 2005; Pieterse et al. 2012) and is not associated with an enhanced expression of PR genes (van Loon and Bakker 2005; De Vleesschauwer and Höfte 2009). Interestingly, the ISR induced by the rhizobacteria *Pseudomonas fluorescens* WCS417r is not associated with the endogenous increase of the JA and Et, suggesting that enhanced hormonal sensitivity causes this improved defense (Pieterse et al. 2000; De Vleesschauwer and Höfte 2009 and references therein). PGPR-mediated ISR has been shown to be efficient against a broad range of plant pathogens on both monocotyledonous and dicotyledonous species (reviewed by Bakker et al. 2007; De Vleesschauwer and Höfte 2009) and it is well known that for its induction an effective colonization of the rhizosphere is required (Raaijmakers et al. 1995). Both SAR and ISR signaling pathways have been shown to be dependent on the transcriptional activator NPR1 (Non-expresser of Pathogenesis-Related; Pieterse et al. 1998, 2007; Niu et al. 2011; Zhang et al. 2012) (Fig. 7.4).

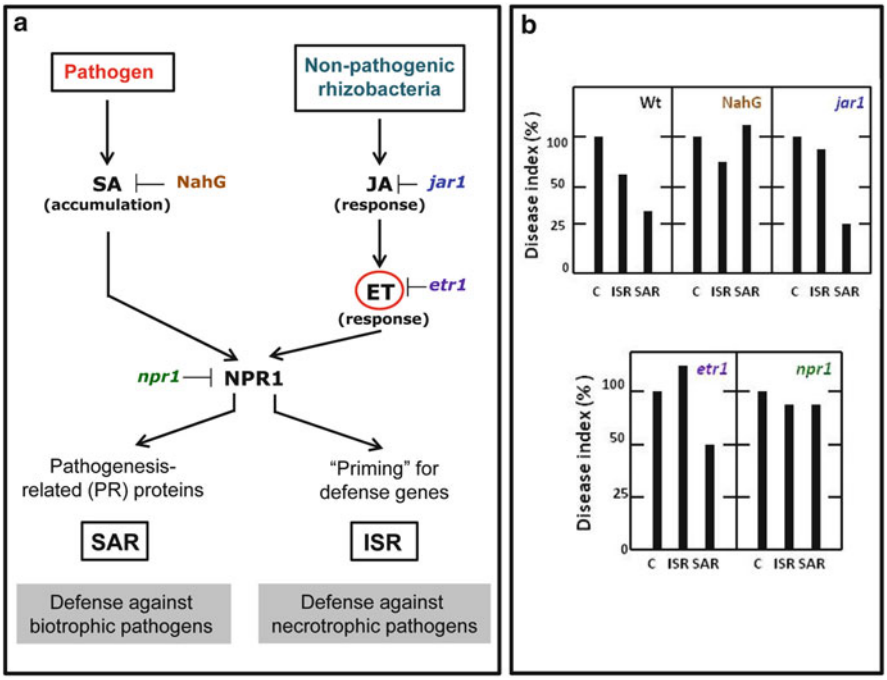

Fig. 7.4 Elicitation of induced systemic resistance (ISR) and systemic acquired resistance (SAR) transduction pathways in *Arabidopsis thaliana*. (**a**) Simplified model for triggering of SAR and ISR. *etr1* (ET receptor mutant 1 plants); *jar1* (JA response 1 mutant); NahG (SA non-accumulating transgenic plants); *npr1* (non-expressor of PR genes 1 mutant plants). (**b**) Quantification of ISR and SAR in Arabidopsis plants infected with *P. syringae* pv. tomato DC3000. ISR was induced by inoculating plant roots with the rhizobacterium *P. fluorescens* WCS417r. SAR was triggered by infiltrating plant leaves with an avirulent variant of *P. syringae* pv. *tomato*. Disease index represents the percentage of leaves showing symptoms relative to the control plants. Wt: wild type; C: non-treated plants. Adapted from Pieterse et al. (1998) with permission of Dr. Pieterse

4.1 Involvement of Ethylene in Pathogenic Infections

In ISR-triggered plants no defense mechanism is activated before the recognition of a pathogen. However, the plant tissues are sensitized to react faster and strongly in response to the pathogen, a phenomenon known as "priming" (Verhagen et al. 2004; Conrath 2009). For example, experiments with endophytic biocontrol strain *Enterobacter radicincitans* DSM 16656 demonstrated that this bacterium is capable of inducing priming via SA or JA/Et signaling pathways to protect plants against potential pathogen attack (Brock et al. 2012) (Fig. 7.4). Importantly, primed plants show a wide spectrum of resistance with low impact on the plant fitness (i.e., plant growth and seed production) (Van Hulten et al. 2006). A number of studies show that priming: (1) often depends on the induced disease resistance key regulator Non-expresser of Pathogenesis-Related genes (NPR1) (León-Reyes et al. 2009);

and (2) is an evolutionary advantage over constitutive activation of defense response (Van Hulten et al. 2006; Conrath 2009).

A hypothesis on the involvement of the Et signaling in the plant defense mechanisms in the presence or absence of a pathogen is shown in Fig. 7.1. It has long been known that Et can act positively and negatively on plant immunity (van Loon et al. 2006). Thus, pathogen attack activates Et production in many plants (Broekaert et al. 2006; van Loon et al. 2006 and references therein) and rhizobacteria-mediated ISR requires responsiveness to Et and JA (van Wees et al. 2008; Pieterse et al. 2007). Unfortunately, the role of Et during the plant–pathogen interaction has remained secondary and deserves more attention. Thus, after the infection, plants often respond with a rapid rate of Et biosynthesis (Iwai et al. 2006; van Loon et al. 2006 and references therein). Pathogenic infection triggers a rapid and low Et biosynthesis from pre-existing ACC in affected tissues. This first Et wave may be a protective response by the plant (van Loon et al. 2006). Subsequently, the activation of the transcription of the ACS genes to generate a net biosynthesis of Et immediate precursor and then a highly elevated ACO activity provokes a second wave of hormone (Iwai et al. 2006; van Loon et al. 2006 and references therein). If the pathogenic attack is ongoing, autocatalytic biosynthesis of Et takes place. This remarkable process is highly damaging for the infected plant. Therefore, it is logical to suppose that (1) the inhibition of the biosynthesis of Et decreases the severity of infection; and (2) transgenic plants with high expression of ACCD are strongly protected against some pathogenic attacks (Czarny et al. 2006; Glick et al. 2007).

The ISR model system Arabidopsis-*Pseudomonas fluorescens* WCS417r is one of the best characterized (Pieterse et al. 2007; De Vleesschauwer and Höfte 2009 and references therein). In this model, the Arabidopsis mutants *etr1* (ET-response) and *jar1* (JA-response) were unable to trigger resistance against the pathogen bacteria *P. syringae* after colonization with *P. fluorescens* WCS417r (Pieterse et al. 1998). Investigation with other mutants in Et signaling concluded that the establishment of ISR requires an intact Et signaling pathway (Ton et al. 2002a). Particularly interesting results emerged from the study of the *eir1* mutant, insensitive to Et in the roots but not in the shoots. Arabidopsis *eir1* plants were unable to show ISR after root colonization by the rhizobacteria WCS417r. However, *eir1* mutants exhibited ISR when the strain WCS417r was infiltrated into the leaves, suggesting the importance of responsiveness to Et at the site of application (Knoester et al. 1999). Interestingly, in Arabidopsis, *etr1* plants failed to exhibit ISR after treatment with ACC or JA. However, *jar1* plants were able to response to JA but not to ACC suggesting that JA pathway acts upstream of Et pathway in the signaling cascade (Pieterse et al. 1998).

It is interesting to point that the locus *ISR1*, encoding a key component of the Et signal transduction pathway, is required for both ISR and basal resistance in Arabidopsis (Ton et al. 1999, 2001, 2002b). Likewise, the endogenous Et levels are crucial for the development and fine-tuning of appropriate defense responses (Zhao et al. 2012, and references therein). The importance of Et content in plant defense responses may have led to the development of Et-producing pathogens. These evolved pathogens might interfere with the Et plant status altering or preventing the defense response to their benefit.

As described previously, Et alone or in combination with other hormones is involved in determining the most appropriate defensive response. However, the function of Et in plant defense is complex and highly regulated. This is reflected in the enumeration of Et-associated mutants and their susceptibility to phytopathogens (van Loon et al. 2006). For example, although *ACS* expression is poorly understood during pathogenesis, recent results indicate that the rice OsEDR1 (Enhanced Disease Resistance 1; ortholog of Arabidopsis EDR1) is a positive regulator of Et biosynthesis. Thus, the expression of the ACS gene family was suppressed in OsEDR1-defective mutants resulting in rice plants more resistant against the biotrophic pathogen *Xanthomonas oryzae* pv. *oryzae* (Shen et al. 2011). The TFs EIL1 and EIN3 regulate the expression of the Et transcriptional activator ERF1. Likewise, ERF1 regulates EtR and Et defense-related genes (e.g., Pathogenesis-Related gene 3 (PR-3) and Plant Defensin 1.2) playing a role in the defense against necrotrophic pathogens (Berrocal-Lobo and Molina 2004; Adie et al. 2007). In Arabidopsis, Et appears to act antagonistically in SA signaling. Thus, it was demonstrated that EIL1 and EIN3 repress SA biosynthesis by binding to the *isochorismate synthase 1* promoter, a well-known SA biosynthetic gene (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012). Conversely, Et potentiated the response of Arabidopsis plants to SA, resulting in a increased expression of *PR-1*, an SA-responsive gene (De Vos et al. 2006). Moreover, in tobacco (*Nicotiana tabacum*) Et was shown to be key player for the establishment of SA-dependent SAR against TMV (León-Reyes et al. 2009 and references therein).

Considerable research in recent years has demonstrated that Et regulates the expression of defensive genes such as *PR-2* (β-1, 3-glucanases), *PR-3* (chitinases), and *PR-12* (plant defensin factors) (van Loon et al. 2006). However, Et works as a component of a tangled network of signaling compounds including SA, JA, and ABA. Likewise, in different plant species the presence of the GCC box (see section "Role of Ethylene Response Factors Under Low-Oxygen Stress") was demonstrated to be essential, and sometimes sufficient, for the regulation of the expression *PR* genes by EtRFs (Adie et al. 2007). The EtRFs–GCC binding can also take place in promoters of *EtR* genes not involved in pathogenesis (e.g., *Hookless1*), evidencing a wider role for GCC box in the transcriptional regulation by Et. On the other hand, EtRF family members can activate or repress concrete defense pathways, often with opposite effects, resulting in susceptibility or resistance to the attacking pathogens (Berrocal-Lobo and Molina 2004; McGrath et al. 2005; Ham et al. 2006). Other examples of the involvement of Et in plant defense are listed below. In Arabidopsis, Et has also been involved in both local and systemic defensive responses against the necrotrophic fungus *Alternaria brassicicola*. Et, but not SA or JA, was capable of inducing the expression of the Arabidopsis secreted lipase GLIP1, which shows antifungal activity against *A. brassisicola* (Oh et al. 2005). More recently, the elicitation of systemic resistance was shown to not significantly alter the structure community of rhizosphere bacteria (Doornbos et al. 2011). Referring to aggressive pathogens, the necrotrophic fungus *Botrytis cinerea* is one of the most stressful and destructive (Williamson et al. 2007). Et, synergistically with JA, plays a key role during resistance to necrotrophic pathogens (van Loon et al. 2006; Grant and Jones 2009). In a recent study, Zhang et al. (2012) found that the mutation of the Arabidopsis

mediator complex subunit 16 (MED16) blocks the expression of several Et and JA response genes compromising, consequently, the plant defenses against necrotrophic pathogens such as *B. cinerea* and *A. brassicicola*. Furthermore, studies with Arabidopsis have shown that the *ein2* and the *ein3eil1* double mutant, both Et-insensitive, are more susceptible to *B. cinerea* (Alonso et al. 2003). Several EtRFs (e.g., ORA59, RAP2.2, and EtRF1) have been also recognized as remarkable regulators in the *Botrytis* resistance (Nakano et al. 2006; Wehner et al. 2011; Zhao et al. 2012). Moreover, ectopic expression of EtRF1 and ORA59 enhanced resistance of *Arabidopsis* to *B. cinerea*, *Fusarium oxysporum*, and *Plectosphaerella cucumerina* (Berrocal-Lobo and Molina 2004; Pré et al. 2008). Taken together with the RAP2.2 function in low-O_2 tolerance (see section "Role of Ethylene Response Factors Under Low-Oxygen Stress"), the Zhao group's data suggested that RAP2.2 (1) may act as a global regulatory protein in the Et signaling pathway and could play a dual role in the low-O_2 tolerance and *Botrytis* resistance; and (2) might serve as a global TF involved in the regulation of the Et signaling pathway and as node in the crosstalk signaling between biotic and abiotic stress responses (Zhao et al. 2012). Recently, it has been shown that EtRF6 is a notable regulator of biotic stress defense. Thus, EtRF6 controls the ROS-responsive genes expression after activation by MPK3/MPK6 (Wang et al. 2013). Likewise, ERF6 plays a dual role under stress as it activates both stress tolerance and growth inhibition, and both roles take play independently from each other (Dubois et al. 2013).

4.2 Non-pathogenic Infections and Induced Ethylene Production

As described above, different biotic and abiotic stresses can cause an imbalance in the Et production of land plants and the increased level of gaseous phytohormone can inhibit the overall plant growth or the length of specific organs including roots (Bleecker and Kende 2000; Mayak et al. 2004; De la Torre et al. 2006; Matilla and Matilla-Vázquez 2008). Et and JA have been shown to be required for the establishment of a broad-spectrum ISR response, stressing the crucial modulating role of Et in plant defense (van Wees et al. 2008). Thus, Et and JA are indispensable for the development of ISR in leaves after root colonization by beneficial microorganisms such as *Piriformospora indica* (Verma et al. 1998) and *P. fluorescens* (van der Ent et al. 2009). The fungus *P. indica* colonizes plant roots and promotes Arabidopsis growth and seed production. Interestingly, the growth of Arabidopsis Et-related mutants *etr1*, *ein2*, and *ein3eil1* was not promoted by the *P. indica*, although the roots were more colonized by the fungus (Camehl et al. 2010). Conversely, the overexpression of EtRF1 reduced *P. indica* colonization and constitutively activated plant defense. Camehl et al. (2010) suggested that the Et homeostasis is required to balance fungal colonization and defense responses. Recent studies have also demonstrated that *P. indica* induces ACC biosynthesis (Khatabi et al. 2012). The ability to inhibit the Et biosynthesis without the necessity of applying exogenous inhibitors has allowed the study of the accurate role of Et in multiple stress and developmental-related phenomena. Thus, the heterologous expression of the *Pseudomonas ACCD*

gene in tomato plants showed to greatly decrease the production of Et (Klee et al. 1991). No apparent vegetative phenotypic abnormalities were detected in these tomato transgenic plants. However, there were notable alterations in the reproductive phase (i.e., several weeks delayed fruit ripening). After these early results, the ACCD was considered as a marker for the Et role in many stress and developmental processes. Interestingly, degradation of ACC in tomato inhibits Et biosynthesis but does not prevent the ability of fruits to sense Et and no ripening defects were observed in transgenic fruits exposed to Et (Klee et al. 1991). On the other hand, during the symbiotic association between rhizobia and legumes, the exogenous application of Et inhibits the formation and functioning of radical nodules. As an example, a *Medicago truncatula* Et-insensitive mutant showed increased nodulation by its symbiont *Sinorhizobium meliloti* (Penmetsa and Cook 1997). Additionally, the results of Stearns et al. (2012) support the possibility of a direct connexion between Et and auxin response, and evidenced the stress-reducing benefits of ACCD-expressing PGPRs (Fig. 7.2). Thus, some ACCD-encoding rhizobial strains can decrease Et production in the plant and therefore enhance the formation of nodules. This increased nodulation was enhanced when ACCD-containing PGPRs and rhizobial strains were co-inoculated (Baby et al. 2011). Soil bacteria expressing ACCD reduce the level of Et and confer resistance and growth of plant under various stresses (Glick et al. 1998, 2007) including flooding and pathogen attack (Wang et al. 2000; Farwell et al. 2007; see section "Updated Overview of the Plant Hormone Ethylene"). It has been hypothesized that under conditions of stress, the root excretes the majority of ACC to the rhizosphere where it is degraded by the ACCD of appropriate bacteria (e.g., *Pseudomonas* sp.; Zahir et al. 2009). Therefore, rhizobacteria with ACCD activity have the ability to reduce Et production in roots and promote plant growth (e.g., root elongation) under several stress conditions (Siddikee et al. 2011; Chen et al. 2013) (Fig. 7.2). For example, in vitro experiments showed that ACCD-producing PGPRs enhanced the salt tolerance of important crops such as canola (Cheng et al. 2007), tomato (Mayak et al. 2004), and wheat (Zahir et al. 2009). Much work is still required to transfer these results to field conditions in order to gain insight on how microorganisms induce ACC biosynthesis in plant roots. However, some progress has already been made in this regard (Ma et al. 2004; Gamalero et al. 2008; Gamalero and Glick 2012).

5 The Relationship Between Ethylene and Other Environmental Stress-Inducing Factors

5.1 Ozone

Ozone (O_3) is a highly unstable and reactive allotrope of O_2. O_3 is a common constituent of troposphere, with powerful oxidizing properties and the most phytotoxic air pollutant affecting plants, causing damage to the photosynthetic apparatus

(Ashmore 2005; Wittig et al. 2009). Surface O_3 concentrations (i.e., >60 nL L^{-1}) have been shown to negatively affect the yields of crops (Fiscus et al. 2005). Et production is (1) the quickest and most commonly observed response to O_3 (Kangasjärvi et al. 2005), including in many important crop plants (Wilkinson and Davies 2009); (2) highly correlated with O_3 injury (Tamaoki et al. 2003); and (3) clearly associated with the induction of Hypersensitive Response (HR) and PCD (Kangasjärvi et al. 2005; Overmyer et al. 2003, 2005). On the other hand, in some species it was demonstrated the prominent role of JA in the O_3–Et signaling pathway (Tamaoki et al. 2003; Grantz et al. 2010).

Rice, a moderately O_3-sensitive crop species, has significant reductions in its yields (~15–20 %) due to elevated O_3 levels (Shi et al. 2009). Moreover, O_3 also induces a quick stomatal closure response (Wittig et al. 2007; Wilkinson and Davies 2009). ABA is considered the main regulator of stomatal functioning in plants and induces stomatal closure via a network of chemical messengers (Acharya and Assmann 2008) and Et has been shown to antagonize the stomatal response to ABA (Tanaka et al. 2006). Thus, plants pretreated with 1-methylcyclopropene (1-MCP), an Et perception antagonist, were able to close the stomata normally in response to ABA (Wilkinson and Davies 2009). On the other hand, when O_3 penetrates the plant leaf through the stomata, it is quickly transformed to ROS (e.g., O_2^- anion and H_2O_2) in the apoplast (Baier et al. 2005). Subsequently, in Arabidopsis, the H_2O_2 production in guard cells as a consequence of oxidative stress of O_3 causes stomatal closure in an Et-dependent manner (Matilla-Vázquez and Matilla 2012; and references therein). In this process, Et also induces the stomatal closure stimulating the production of H_2O_2 by the NADPH-oxidase AtRbohF (Matilla-Vázquez and Matilla 2012). For more detailed information about the O_3 harmful effects on stomata movements, see Wilkinson and Davies (2010).

As indicated above (section "Cross-Talk Between Oxygen Deficient Stress and Ethylene Biosynthesis and Signaling"), when the root system is subject to stresses like flooding, the ACC is transported from there to the oxygenated parts (e.g., shoots) and transformed in Et by ACO. However, to our knowledge, studies on spatial alterations of ACC content and Et production in response to O_3 still remain to be performed. Several mutants and accessions of Arabidopsis described as O_3-sensitive have now been demonstrated that overproduce Et (Kangasjärvi et al. 2005), and Arabidopsis mutants insensitive to Et are O_3-tolerant. Recently, (1) an essential JA–Et interaction was found to be mediated by JA-Zim domain (JAZ). These JAZ proteins repress the transcription of JA-responsive genes and interact with TFs involved in mediating responses to Et (Wager and Browse 2012); and (2) O_3 surface levels induce plant physiology responses in *Gossypium barbadense* with no increase in the production of Et (Grantz et al. 2010; Grantz and Vu 2012). However, when the plants were exposed to high O_3 levels, Et biosynthesis was induced and further enhanced in MeJA-treated plants (Grantz and Vu 2012). In *G. barbadense*, the application of MeJA as an anti-ozonant has been proposed.

5.2 Freezing

Although Et regulates several specific aspects of plant responses against biotic and abiotic stress (sections "Cross-Talk Between Oxygen Deficient Stress and Ethylene Biosynthesis and Signaling" and "Ethylene and Plant Defense Against Microorganisms"), their definite role in freezing stress remains unclear (Zhang and Huang 2010 and references therein). In general, high levels of Et production are associated with chilling sensitivity (see Morgan and Drew (1997) for review of earlier literature). Nevertheless, the TFs known as C-repeat Binding Factor (CBF), belonging to the AP2/ERF superfamily, are involved in the well-understood cold signaling pathway (CBF/DREB) transcriptional regulatory cascade. Recent results in Arabidopsis demonstrated the negative effect of Et biosynthesis and signaling over the plant freezing tolerance by repressing type-A *Arabidopsis Response Regulators* (ARR) genes and the cold-inducible CBFs (Shi et al. 2012). Namely, ETR1 and EIN4, in contrast to EIN2 and EIN3/EIL1, have positive roles during the modulation of the plant adaptations to freezing. Diverse and contradictory implications of Et biosynthesis in chilling sensitivity were previously shown in maize, mung bean, tomato, cucumber, and tobacco plants (more information in Shi et al. 2012).

6 Conclusions and Future Perspective

At present, there is no doubt about the critical role of Et in plant defense strategies against biotic and abiotic stresses. Et participates in a highly complex and tightly regulated signaling network that also includes crosstalk with JA, SA, GA, and ABA signaling pathways. In order to obtain goods and services orientated to the development of modern agriculture, the knowledge of all these plant signaling networks has undergone a strong progress during the last decade. As a result, the number of biocontrol and biotechnological strategies designed to improve plant responses to stressful environmental cues, such as low O_2, freezing, and pathogens, is growing exponentially. It seems beyond doubt that the level of endogenous Et is critical for the establishment and adjustment of appropriate plant responses, and that these processes require tight spatial and temporal regulation of Et biosynthesis. A major research priority to improve the understanding of the Et signaling at molecular level was the identification of transcriptional networks that regulate the synthesis of developmental modulators. Thereby, functional analysis of the large ERF family is helping to characterize how Et coordinates plant adaptive responses to stress. Ultimately, unscrambling how plants alter their microbiome and the mechanisms by which plant-associated microorganisms control plant health will provide an excellent opportunity to enhance crop productivity and quality. However, the molecular mechanisms by which rhizosphere microorganisms are recognized to subsequently activate Et-mediated responses are still poorly understood.

Due to ET action is included in a plant hormone network, it is indispensable to unravel the ET crosstalk with SA-, JA-, and ABA-depeudent signaling pathways.

The result of this extensive study is to understand the plant response to a particular type of stress. This biotechnology challenge will require the characterization and contribution of the molecular components involved in this tangled network. To fill this complicated puzzle, molecular platforms as microarrays, protein–protein interactions, knock-out gene collection, or RNA-seq facilities must be utilized to this aim without ruling out new -omics technologies.

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Chapter 8
Scenario of Climate Changes in the Context of Agriculture

Rida Rehman, Anber Hamdani, Aisha Naseem, Muhammad Ashraf, and Alvina Gul Kazi

1 Introduction

Every creature requires certain conditions to sustain life. In the solar system, only earth supports life. Earth is heated by sun emitting different radiations and these radiations aid in global warming. Global warming is the rise of earth's atmospheric temperature. This rising of temperature is aided by a number of factors and presently is the most threatened issue which is triggering climatic changes across the globe. The data shows that global warming is the intermediated phase between two ice ages and the distance between two ice ages is approximately 100,000 years (Wallington et al. 2004). The fact that earth revolves around the sun aids in the environmental changes over a long period of time. With the change in earth's orbit, the temperature falls to many degrees. At present we are in the middle of two ice ages and the temperature changes to few tenths of the degree Celsius by every thousand years. There are a lot of factors that indicate to fast approaching ice age including significant retreat of mountain glaciers in many locations all over the world, the continuously decreasing ice that covers the Northern hemisphere, sea level rise and decreased extent and thinning of Arctic ice. Climate change as a result of global warming is considered as the most serious threat to our environment ever encountered in human history (Environmental Protection Agency 2011).

There is nearly 1.5 °F increase in the temperature of earth since 1880 and has been rising since late 1970s. Over the past century, the unusual rise in the average temperature of earth's surface predominantly as a result of release of certain

R. Rehman • A. Naseem • M. Ashraf • A.G. Kazi (✉)
Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan
e-mail: alvina_gul@yahoo.com

A. Hamdani
Crops Science Institute, National Agricultural Research Center, Islamabad, Pakistan

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greenhouse gases (GHGs) to our atmosphere is global warming. Between the years of 1906 and 2005, the rise in surface temperature of earth was recorded to be 0.6–0.9 °C. In the last 50 years, this rate has nearly been doubled. It has been claimed that temperature will further increase unless protective measures are observed to mitigate the emission of GHGs. The "Intergovernmental Panel on Climate Change" in 2009 speculated that the increasing temperature, floods, drought, desertification, and weather vagaries will severely affect the agriculture and a rise of 4.2 °C in mean temperature of earth is anticipated at the end of the present century, i.e., the twenty first century (Khajuria and Ravindranath 2012). Various international reports suggest that a large disparity will exist among the developed countries as well as the developing nations in the context of agriculture vulnerability to the change in climate (Rosenzweig and Parry 1994).

The origin of climatology dates back to the eighteenth and the nineteenth century. A Swiss Scientist, proposed that our atmosphere is like a greenhouse, protecting both the earth's surface and its inhabitants from temperature extremities. Later John Tyndall (British Scientist) did experiments and confirmed the greenhouse effect. Few scientists at that time feared differing progression of climate change, i.e., global cooling and reappearance of an ice age that could threaten humanity. Louis Agassiz is considered as the very first scholar to put up the history of climate change. Svante Arrhenius (1896), a Swedish chemist, followed Tyndall demonstrating the effects of CO_2 on atmospheric temperature and welcomed the idea and named it as global warming. With the passage of time by 1930, when people analyzed the previous half century, they started to realize that regions of the USA and Northern Atlantic have significant increase in temperature. Most scientists were of the view that it was just a phase of some natural atmospheric cycle of mild affectivity with unknown causes. Among all these, an amateur G.S. Callendar insisted that it was not ordinary phase or any temperature cycle rather it was greenhouse warming on its way everyone thought that it would be better if this continues to happen. He supported Arrhenius findings and refined the understanding of the role played by CO_2 in climate change. By the 1950s scientists were provoked to look into the questions raised by Callender. The thing that gave it way and resulted in a sharp increase of government funds was the weather and the seas during the cold war suffered by the military agencies. At that time it was assumed that carbon dioxide could be trapped up in the atmosphere and be the reason of warming.

In 1950s, modern climatic science was born. Roger Revelle and his colleagues at the Scripps Institution of Oceanography began their work on temperature across the different layers of ocean and made pivotal contribution to the field. Revelle also sponsored the research of Charles David Keeling who measured the atmosphere's level of carbon dioxide which was continuously increasing, known as "Keeling Curve." In 1960, the measurements were observed and it was deduced that with every year the level of this gas is increasing in the environment. In the start, scientists were successful in finding the single matter key to change in climate but after research they realized that climate comprises of an intricate system that responds to a number of influences involving eruption by volcanoes, changes in the solar system, even the human activities. It was a surprise to know that the timings for ice ages had been set by astronomical cycles. Apparently balance between the climates was

so delicate that almost any small change in the movement, quality, and behavior might set off a great shift. Chaos may result due to sudden shift as a result of this perturbation. Apart from greenhouse effect concerns, it was also being pointed out that human activity is also resulting in putting the particles of dust and smog into the environment. There they act as a blockade, inhibiting the sunlight to pass through and cool the earth. It was predicted that a cooling trend has begun in Northern Hemisphere in late 1940s, as predicted by the Northern Hemisphere weather statistics. The inhabitants accompanied by mass media were confused whether to wait for a flood over the entire globe or another ice age (Intergovernmental Panel on Climate Change 2001b, c). Panels to carry out studies were set in the USA as well as in many other places which claimed that a severe threat may be posed by one or other kind of climate change in the future. All the scientists agreed that their knowledge regarding climate change is insufficient hence more research is to be done. In order to overcome this problem, schemes aiming the gathering of data were devised in which even the satellites orbiting the earth were mobilized along with the mobilization of oceanographic ships' international fleet.

2 Discovery of Global Warming: Climate Change

The United Nations Framework Convention on Climate Change (UNFCCC) defined climate change as "a change of climate which is attributed directly or indirectly due to anthropogenic interventions that alter the composition of the global atmosphere and which are in addition to natural climate variability observed over comparable time periods." In the history of previous 4.5 billion years, our earth has gone through considerable changes. Earth's temperature keeps fluctuating between very hot, very cold, and stable. For eons, the stability remained rendering earth favorable for cultivation and growth of flora and fauna, and subsequently to the ever-growing population of mankind.

The balance that exists between mankind and environment is pretty delicate. The race for development coupled with anthropogenic activities (GHG emissions and land use) has disturbed the environment by threatening the delicate matter of equilibrium between objects. The balance of natural ecosystem (forest, rivers, basins, sea level) and socioeconomic system (agriculture, fisheries, and irrigation) is affected by the climate change. The increased industrialization and human activities over the past 100 years has disturbed the natural balance of the climate, increasing the concentration of GHGs in the atmosphere of earth resulting in global warming.

Scientists have been studying the climate for centuries and basic physics of climate changes has been known for more than a century but in recent decades the science of global warming has been firmly established. History shows that in early nineteenth century, the scientific discovery of changes in the climate rooted for the first time. Spencer R Weart (2003, 2007) demonstrated this history in his famous book "The Discovery of Global Warming." Some of the important events pertaining to the discovery of climate change are illustrated as under.

2.1 1800–1850

The history of climate change is as old as the discovery of carbon dioxide by Joseph Black in 1753. The nineteenth century is the era of industrial revolution. In early nineteenth century the concentration of CO_2 in the atmosphere as measured in ancient ice age was found to be 290 ppm. Jean-Baptiste Fourier (1827) proposed that earth would be much cooler if it lacks atmosphere, an atmospheric effect exists which keeps the earth warmer.

2.2 1850–1900

In 1861, John Tyndall (Irish physicist) conducted research and concluded that the gases present in the atmosphere including CO_2 and water vapors trap infrared radiations and perturbation in the concentration of these gases can lead to climate change. With the prediction made by Svante Arrhenius, a Swedish chemist, in 1896, the predictions about changes in the climate as a result of human activities were also started. Immediately, he took into account the revolution taking place in industrial sector and deduced that the concentration of CO_2, which is being released in the environment, is also increasing. Other than this, he was of the view that the amount of CO_2 in the atmosphere will increase continuously side by side along with the increase in consumption of fossil fuel, particularly coal. Even at that time, his assessment led him to predict the CO_2 role in increasing earth's temperature. He noted that the average temperature of earth is nearly 15 °C. This was due to the ability of water vapor and carbon dioxide to absorb infrared radiations, in other words, the natural greenhouse effect. He was of the view that as a result of CO_2 doubling in earth, earth's temperature would further rise by 5 °C. Chamberlin (1897) demonstrated a model for global carbon exchange.

This topic was long forgotten after the findings of Arrhenius and Chamberlin. In that era, it was thought that human influences were insignificant hence do not aid in global warming as compared to the natural activities like the solar activity and ocean circulation. It was also believed that the oceans will act as carbon sinks and they will automatically clear out the pollution. At that time, water vapors were more feared to be potential threat towards global warming.

2.3 1900–1950

In the 1930s, global warming trend of the whole nineteenth century was observed and reported. It was also reported that the changes in the orbits are the reasons of all ice ages. In 1938, Callendar argued that carbon dioxide, which is a GHG, is the seed of global warming. Several advancements in the field of infrared spectroscopy led to the measurements of radiations with longer wavelength. In that period the

hypothesis, greater CO_2 concentration in the environment results in greater absorption of infrared radiations, was proved. It was also discovered that the ability of water vapor and carbon dioxide to absorb radiations is different.

2.4 1950–Onwards

Gilbert Plassa bridged the above results in 1955 and concluded that increased CO_2 concentration in the atmosphere will affect the radiation balance, which is otherwise lost in the space. In 1956, Philips demonstrated a more pragmatic computer model for the global atmosphere. It was still thought that oceans would absorb most of the carbon dioxide. In 1950, evidence was obtained which claimed that CO_2 have a time span of approximately 10 years for which it can stay in the environment but the fate of CO_2 molecule was still not understood. Further investigations revealed that ocean cannot act as a sink for all the CO_2 in the atmosphere. Revelle in 1957 figured out that anthropogenic CO_2 is not readily absorbed by the oceans. Telescope studies in 1958 showed that the temperature of Venus has increased above the temperature at which water boils just because of greenhouse effect.

Charles Keeling took advantage of the available modern technologies and formed concentration curves for the CO_2 present in the atmosphere of Antarctica as well as Mauna Loa. Keeling accurately measured the amount of CO_2 (315 ppm) and detected annual rise of the curves made from 1940s to 1970s presented a downward trend. Simultaneously, research carried out on the ocean sediment showed that nearly 32 cold–warm cycles have been occurred over the time span of past 2.5 million years rather than only 4. This led to the fear of a new ice age. The data was ignored by media and a number of scientists who were in favor of global cooling. Calculations made in 1963 advocated that feedback with vapors of water in the atmosphere could result in climate extremely sensitive to CO_2 alterations. The boulder meeting carried out in 1965 was held on the reasons and causes of upcoming global warming. Lorenz and others pointed out the muddled nature of climatic system and speculated the probability of abrupt shifts. International Global Atmospheric Research Program was established in 1967 with a manifesto to have information in hand for an improved short-range prediction of weather as well as climate. Manabe and Wetherald (1967) made a conclusive calculation that if the concentration of CO_2 will be doubled, the temperature in turn would decrease several degrees. Nimbus III satellite in 1969 (Hanel et al. 1970; National Environmental Satellite Center 1970) reported elaborated measurements on the atmospheric temperature of the globe. Concern regarding environmental effect caused by airplanes emerged which formed the basis of investigation on trace gases concentration in stratosphere and discovered harmful effects to the ozone layer.

By 1980s, the curve of global annual mean temperature began to rise. People started questioning the theory of new ice age upcoming. It was late 1980s when this curve began to increase in a very steep manner. This was the time when global warming theory began to win terrain rapidly. Environmental NGOs came into action

and started to work out methods in order to prevent further global warming. This topic also gained the attention of the press and soon it was a news flash all around the globe. Finally in 1988 acknowledgement was passed that there is an increase in climate more than ever observed. The greenhouse effect theory was named and IPCC was established by UN Environmental Program and the World Metrological Organization with an objective to anticipate, according to the climate models as well as available knowledge in the literature, the impact of greenhouse effect. The panel formed consisted of 60 different countries of the world and more than 2,500 scientists and other technical individuals were part of it. These scientists belonged to different research fields including climatology, ecology, economics, medicine, and oceanography. IPCC is considered as a historical project with the largest peer-viewed scientists' cooperation.

In 1990, due to a number of ambiguities in the model outcomes as well as data set, greenhouse theory was also in question by different scientists. The basis of theory was the global mean temperature recorded annually and it was objected too. They were of the view that there is an ambiguity in the measurements and the data obtained from oceanic study was missing. The global warming data did not explain the cooling trends. Upon observing satellites, totally different temperature records were obtained from the initial zones. The data caught fire that the model generated on global warming had overestimated the trend in temperature increase over the past 100 years. This idea made IPCC to review the initial data they had on global warming. Still they did not reconsider that whether an actual trend exists. From the data obtained, now we are familiar that 1998 was the warmest year on record so far all around the globe which is followed by the years 1997, 2001, 2002, and 2003. Since 1990, the 10 warmest years have occurred. IPCC is keeping an updated record as being challenged by a number of scientists resulting in new research and frequent responses by IPCC. The topic of global warming is still in debate and data is constantly being checked and renewed. Amendments are also made in models to keep them updated and in accordance with new theories.

So far, nothing has been done to control the devastated condition of climate. This is the result of major uncertainties that still hold the theory. It is a global problem, so all countries ought to join hand in hand to solve it. For this purpose in 1998, the Kyoto Protocol was negotiated in Kyoto, Japan with the principle that countries that play a role in it will try to minimize their anthropogenic GHG emissions (CO_2, CH_4, N_2O, HFCs, PFCs, and SF_6) by at the minimum 5 % below the levels recorded in 1990 during the commitment period, i.e., 2008–2012. 186 countries became part of this commitment and signed Kyoto protocol in Bonn, 2001. The USA and Australia retreated from the pact. The greenhouse effect terminology started to change from 1998 onwards. As a consequence of influence generated by media, the people started using terms like global warming and climate change.

With the advent of revolution in industrial sector, it led to a quest in the utilization of nature's blessings including hydrocarbonaceous fuels leading to increase in the emission of gases such as carbon dioxide and other nauseous gases never reported before in the earth's evolutionary history. This along with particulate matter increases the atmospheric trapping of radiated heat from the sun. Changes are being

detected by scientists, who indicate that with every passing day the climate is becoming, on average, hotter and more variable. This variability is the gift of increasing carbon dioxide in the atmosphere. The scientific studies suggest that since last 650,000 years, planet never had more carbon dioxide trapped in it as it does today.

3 Factors Contributing to Global Warming

Among the most prominent reasons of global warming are the GHGs contributing in the greenhouse effect, radiative forcing of climate change, and ozone depletion. The three most prominent reasons of global temperature changes are: (1) high and continuously increased concentrations of carbon dioxide in the atmosphere which is due to the combustion of fossil fuels primarily. The carbon dioxide concentration has been increased from approximately 280 to 394.25 ppm at the end of 2012 since 1750 (Dlugokencky and Tans 2013). This increase in concentration leads to substantial alteration in earth's system, (2) changes in the global nitrogen cycle leading to changes in biogeochemistry, and (3) ongoing changes in the land as one half of the world is being transformed by humans. These three components contribute in the devastating changes in environment and loss of biodiversity (Vitousek 1994). The expected consequences to this problem include flooding in the coastal areas, increase in extreme weather, spreading disease and mass extinctions.

3.1 Greenhouse Effect and Greenhouse Gases

Over the past few centuries, greenhouse effect has drawn a great attention towards itself. The accumulation of CO_2 and GHGs causes this greenhouse effect. The prominent GHGs are (1) carbon dioxide (CO_2), (2) methane (CH_4), (3) nitrous oxide, and (4) chlorofluorocarbons (CFCs) contributing 76 %, 13 %, 6 %, and 5 % to global warming, respectively. Among GHGs, CO_2, CH_4, and N_2O are more closely associated with agriculture activities, contributing 26 %, 60 %, and 14 %, respectively, to total GHGs (Azam and Farooq 2005). Although CO_2 is considered as main driving force behind global warming, water vapors (WV) which accounts for 95 % of greenhouse effect are not considered as GHG in most global warming studies. A significant portion of atmospheric WV originates from agricultural crops.

The study of greenhouse effect goes back to the Jean Baptist era, in 1827. According to the studies, the emission of longer wavelength radiations of sun cool down the earth atmosphere and the shorter ones warm it up. These GHGs block the emission of longer wavelength radiations and thus heats up the earth. The retention of radiations is facilitated by GHGs. According to John Tyndall, ice age is caused by variations in the atmospheric levels of these gases. Large amount of CO_2 is released due to burning of fossil fuels. The heat in the atmosphere is trapped by water molecules, methane and carbon dioxide. The only method of cooling earth is

Table 8.1 Emission and per capita consumption of CO_2 (2011)

Country	CO_2 emission	Per capita emissions tc per person per annum
China	28	4.7
USA	16	2.0
Europe	11	1.8
India	7	0.5
Globe	_	1.4

the emission of infrared radiations. At infrared frequency earth acts as a black body that absorbs all while releasing none.

The greenhouse effect is also important in a way that it keeps the earth's atmosphere warm and heated. In the absence of this, the earth would cool down to the temperature that would not support life at all. In the light of above factors, global warming is the phenomenon of enhanced greenhouse effect that is expected as a result of increase in the atmospheric concentrations of GHGs associated with activities done by humans.

3.2 Carbon Dioxide (CO_2)

The most abundant GHG is the carbon dioxide with the most hazardous outcomes. Estimates of CO_2 emissions depicted that the total global emission is unequal to the sum of the gas released from all the countries. In 2011, the global CO_2 emissions were ruled by emissions China followed by the USA, Europe, and India as shown in Table 8.1. Combustion of fossil fuels primarily contributes to the increased CO_2 concentration in the atmosphere. Before the industrial revolution, gases released from burning of fossil fuels started and became the dominant source of human-induced emissions around 1920 till now, i.e., 2013 (Munhoven et al. 2009; Randerson 2013). These emissions occur in active carbon cycles that are responsible for the circulation of carbon between atmosphere, ocean, and terrestrial biosphere (Archer et al. 2009). During 2002–2011, 89 % of total emissions were caused by fossil fuel combustion and cement production and 11 % by land use change. The total emissions were partitioned as atmosphere (46 %), ocean (27 %), and land (28 %) (Le Quéré et al. 2013; Shakun et al. 2012).

Deforestation plays a major or key role in increasing the concentration of CO_2. The atmospheric burden of CO_2 increases at the rate of "$3.3 + 0.2$ GtC yr^{-1}" (where GtC stands for Giga tones of Carbon). A study conducted in 2001 showed that at that time CO_2 concentration was 370 ppm and is increasing at a rate of 1.5 ppm per year and this level was 30 % above the preindustrial time. Records show that the current concentration of carbon dioxide is greater than the past 420,000 years. Data published by Dr. James Hansen who is the director of NASA's Goddard Institute for space studies and others show that CO_2 emissions are not the observed atmospheric warming. There are certain other gases more harmful than CO_2 gas.

CO_2 gas emission also produces aerosols and these aerosols have a cooling effect on the global warming. This magnitude equalizes the warming effect produced by aerosols. This claims that there is no net effect produced by the effect of CO_2 on global warming. This issue cannot be justified. Aerosols are short-lived while CO_2 continues to heat the environment for hundreds of years (Cox et al. 2013; Bardgett et al. 2013).

Energy released by fossils is the large reservoir of CO_2. The deep ocean acts as a large sink for the kinetically slow disposing carbon dioxide. The oceans of the world act as large source as well as large sink for carbon dioxide reservoirs and CO_2 is being continuously exchanged between these two. Due to this continuous exchange, it is difficult to predict atmospheric lifetime for CO_2. The atmospheric lifetime for CO_2 is typically stated as 100 years. The trapped CO_2 changes the rain patterns as well. Carbon dioxide is supposed to be disposed of in proper thermohaline currents that have a very large equilibrium capacity. The Mediterranean under current entering the Atlantic is one such current. It has the capacity to absorb all the CO_2 till 2100 produced in Europe only (Marchetti 1976; Sitch et al. 2013).

3.3 Methane (CH_4)

After CO_2, the most harmful GHG is methane. It is the most well mixed gas after carbon dioxide whereas it is removed via chemical reactions with hydroxyl (OH) radicals. The sources of methane are natural as well as anthropogenic. Since the start of industrial revolution, the concentration of methane has increased by 148 % (Riebeek 2010). Natural gas facilities, mines filled with coal, petroleum industry, coal combustion, enteric fermentation, rice paddies, the burning biomass, landfills, animal waste, and domestic sewage are major anthropogenic sources with estimated emission of 40, 30, 15, 15, 85, 60, 40, 40, 25, and 25 $Tg(CH_4)\,yr^{-1}$. Methane produces an effect 21 times greater than CO_2. Since preindustrial times, the concentration of methane has been doubled. Human sources produce 1.5 times methane as all natural sources. The primary natural source of methane is microbial decay. Half of the human-induced warming is due to methane. One hundred million tons of methane is produced every year. Livestock digestive system produces 85 % of this methane while 15 % is released by massive lagoons (Environmental Protection Agency 2013).

3.4 Nitrous Oxide (N_2O)

The large increase in the amount of nitrogen fixation has led to the production of increased amount of nitrous oxide. This stable gas, nitrous oxide, produces greenhouse effect as well. The production of nitrous oxide is 30 % greater than the disposable concentration (Keller et al. 1986). One of the positive effects of large amount of nitrogen fixation in the atmosphere is that it provides more life benefits

to all the producers and some of the consumers as well. Symbionts, producers, and consumers all are affected by large nitrogen concentrations. The increasing concentrations of nitrogen in the atmosphere also affect the global nitrogen cycle (Vitousek and Walker 1993). The effect produced by this disturbance is the greatest compared to any other global component. This fixation is also important in the prediction about global warming. Land use change also affects the diversity of the living species along with all other factors.

3.5 Other Factors

There are certain other chemicals that affect the change in the global environment. They also add their bit of affect in warming of the atmosphere. Some of them are discussed below.

3.5.1 Worldwide Distribution of Synthetic Organic Compounds

The most persistent compounds such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) have been used all over the world and have globally disturbed the biota. The CFCs have affected the ozone layer in the stratosphere, depleted it, and increased the entry of ultraviolet radiations into the atmosphere over the Antarctic region resulting in decreasing the marine life (Rowland 1989).

3.5.2 Alteration in Biogeochemistry of Global Element Cycle

Human activity has also affected the sulfur cycle along with carbon and nitrogen. The concentration of sulfur dioxide produced from burning of fossil fuel exceeds the concentration of all other natural gaseous emission combined (Charlson et al. 1992). The sulfur dioxide emissions lead to sulfuric acid rain and increased sulfur aerosol concentration (Keller et al. 1986). These aerosols act as effective nuclei for cloud condensation (Fan and Harden 2012).

3.5.3 Biological Interruptions by Non-native Species

The immense increase in the GHGs and other factors that affect the atmosphere also affect the living species. The species that are transferred from one area that forms their habitat to other, directly or indirectly affects the climate of the atmosphere. For example, certain annelids like earthworms reside in soil and provide fertility to it. If earthworm is transferred to a barren land and its life is supported there, it can fix nitrogen and provide fertility to soil which results in provision of support to the plants for their growth. This would take a couple of years for trees to grow

but they will affect largely the environment. Certain nitrogen-fixing bacterial species may also lead to changes. All these small changes group up to form a reason of global changes.

3.5.4 Volcanic Eruptions

A volcano is an opening in the earth surface that allows or aids the eruption of hot magma, volcanic ash, and gases from the earth's crust into the environment. The climatologists may not agree on what percentage of earth's warming is natural and how much is aided by humans but all scientists agree on the fact that volcanism acts as a wildcard in the climate change and produces a significant cooling effect for at least some years following a major eruption (Houghton et al. 1996; NASA 2011).

The activity of volcanoes is a continuously on-going process with more or less a dozen volcanoes active at any given time. Most of these eruptions are small with minimal effects, shortly living and contained within lower atmosphere near volcano. Occurrences of major volcanic activities are extremely rare. These volcanic activities and eruptions are able to release gases and eject ashes like sulfur dioxide (SO_2) as high in the atmosphere as 80,000 ft or more. The ashes from the eruptions fall out within 6 months to a year. The sulfur dioxide is immediately converted into aerosols of sulfate and can stay for 2 or more years in the high atmosphere, which is pretty stable. These aerosols may block some of the incoming solar radiations and result in the lowering of earth's temperature overall. Over the period of 2–3 years, an average lowering in temperature is from 0.2 to 0.5 °C (de Silva 2010).

Little volcanic activity was observed during the period of 1920–1940. This coincided with immediate increase in the solar radiations and warming in the oceans that continued for a number of decades. This resulted in the warmer temperature all around the globe. Sun and oceans are believed to be the main culprits in the warming of globe but lack of volcanic ash may have also aided in the warming. In this perspective, 1960s became highly active as a number of significant eruptions took place in those years. These eruptions kept aerosol levels higher in the atmosphere. This followed lesser degree of radiations from the sun resulting in a quitter sun and relatively cooler cycles in Atlantic and Pacific Oceans. Those were the coldest 10 years in the last 5 decades. Temperatures began to rise with increased solar activity and warm temperature at Pacific and lower globe temperatures gave rise to a number of eruptions in different parts of world. This is clearly evident that volcanic eruptions are somehow linked to the percentage of aerosols in the stratosphere, which is represented as optical thickness of aerosols to the satellite-derived lower troposphere temperatures.

3.5.5 Oceans as Heat Reservoir

Seventy-five percent of all earth is water whereas the rest 25 % is the land. Water has a great potential to absorb large amount of heat. With more than 1,000 times as

compared to the strength of atmosphere to tolerate heat, the oceans, all over the world, are the biggest reservoir to absorb heat produced as a result of global warming. Oceans are thermodynamically stable and their overall temperature is not easily affected because of their large heat capacity. Long-time and continuous effect only will be able to bring any change in it. The temperatures of air and land can be easily changed by short-term activity when compared with the oceans. Their temperatures can easily be changed even without the warming effect produced due to the increasing temperature of earth. This characteristic of air and land makes it difficult to obtain valuable data as a great deal of "noise" is generated due to continuous and fast changes. But since the ocean water is much less sensitive to short-term effects, there is a low level of noise in oceans. If the warming up of oceans is detected, this will enable us to know that long-term effect is occurring and it can be used as a reliable indicator to detect and study this warming trend.

Since 1955, ocean temperature is being measured which is necessary for making such evaluations. With the improvement in technology, the databases are becoming more and more extensive as well as reliable. All the previously recorded information reveals that the temperature of oceans is continuously increasing over a period of time and the greatest increase in temperature is happened in recent years.

3.6 Arguing the Possible Reasons

The heating that is occurring inside the earth is largely due to the radioactive decay of stable isotopes inside the earth. These isotopes are scattered at an uneven percentage in the earth's interior and they also observe movement along with the convection occurring underground. Therefore, it can be deduced that the convection, that moves the isotopes underground, may bring them together and lead to increase in the radioactive decay. The probability of this decay cannot be accurately calculated until and unless the size and distribution of isolated isotope concentration is not known. However, when further investigation of this matter was carried out, this became clear that this phenomenon was not actually happening. The reasons of global warming are widespread and are occurring on short notice by certain geological as well as human standards. This contradicts with the idea of large amount of interlinked activity that brings the isolated concentrations of isotopes together within the interior of earth. This activity would require a time frame from 100 to 1,000 decades. If the assumption is made that this activity has occurred in the past and the effects are now visible, even then it is not something that has happened suddenly or forms itself with uniformity over time. The measurements that were carried for calculating the infrared radiations of the surface of earth have not shown large areas of unusual rise in temperature. There are some of the isolated hot spots where such activity has been observed such as Yellow Stone National Park in the US state of Wyoming (that extends to Montana and Idaho). However these spots are limited in both; spatial dimensions and in changes occurring in temperatures. The heating produced on such levels do not equate the global temperature changes. They might

contribute to a small fraction of increase in temperature. Studies show that these spots are active for many millenniums and are also included in global heat budget so they might not be contributing to this warming. The evidence provided by the level of geological activity observed on the map is insufficient and could not possibly account for the observed increased heat (Taylor et al. 2012).

Furthermore, if it may be considered that the heating is due to earth's internal activity, inverse gradient to distribution of temperature within oceans would be observed. The ocean floor, i.e., the ocean bed forms the thinnest crust on planet. Most of the heat that would escape from the surface into the atmosphere would occur from there. The geological activity such as volcanic or seismic events would have made this happen. The direct conduction via rocks may also help in this phenomenon. The evidence contradicts this scenario. It says that temperature drop occurring at the oceans is dramatic and uniform all over the world. As depth increases, the temperature further cools. The ocean is the coldest at the depth of ocean floor. If this is accepted that short-term heating within the earth is occurring, then we would observe the opposite of it. Via research, we consider that upper portions of oceans are heating up and yet there is no evidence that this heating has affected the lower portions of oceans. This proves that the oceans are getting heated up from top to bottom and not the vice versa. The earth's activity, hence proved, is not aiding much for heating up the earth's temperature.

If speed and magnitude of observed global warming is considered and recorded data is analyzed, then it will be evident that warming cannot be an attribute of increase in interior warming of earth. This also supports that only a small percentage of heating is aided by earth's internal activity. The only possible explanation about the internal heating can be in a way that sizable increase in global temperature can be aided by naturally occurring warming up of the globe. This would be occurring if the sources inside the earth produce small increase in temperature over a time span of many years. Plus, this heating is trapped or retained in the atmosphere by some trapping force. This would also require the greenhouse activity otherwise all heat will radiate into the space and a thermal equilibrium would be attained.

Now considering manmade effects, we can realize by observing statistical data that human activity has increased dramatically. Over a last few centuries, population and industrial sector has progressed exponentially. This gave rise to increased heat produced by humans. It is justified to consider the fact that any manmade heat produced would stay in the atmosphere and aid in the global warming before radiating into the space. Nearly 10 years of energy or heat release in the environment was observed. According to it, heat would retain in the environment. This is possible only in the case if atmosphere has increased its efficiency of greenhouse effect. More importantly, the recorded data only shows the heating of oceans and does not speak for the heating in the atmosphere. It does not have any information about the heat produced by land masses and heat radiated into the space. All energy-generating sources are the main reservoirs of energy addition to the environment. The prominent energy-generating sources are solar, wind, and hydroelectric power units.

These arguments rule out the possibilities of internal heating either contributed by humans or naturally occurring as the sole reason for global warming. This only

leaves one possibility that it would be a gift of external heating. If the solar activity is questioned and claimed as a possible source of warming, then we can pin out two ways by which it can be achieved: (1) either by increase in solar radiation absorption rate or (2) by increase in the value of solar constant. If the first possibility is considered, then this would support the hypothesis of greenhouse effect.

Second is the measurement of solar irradiance and in this regard it is observed that it is decreased over a period of time. According to the observations made, over the past 20 years solar forcing has declined whereas the temperature of the surface has risen continuously. The contradictions revealed the mechanism that resulted in any kind of solar variability effects on the climate. Such mechanisms have ceased in the twentieth century and it was due to certain other factors. It is also being claimed that the changes in the solar irradiance is responsible for nearly 50 % of the observed global warming. Another theory that contributes to global warming is the theory of solar/cosmic ray. This theory states that clouds are formed by cosmic rays and they decrease the earth's temperature. The activity of solar radiation has increased its magnitude and delivers a better protection to the charged particles that form cosmic rays. This ends up in failing of cloud formation which eventually results in less cooling effect. As a result of this, warming trend around the globe is observed (Rogelj et al. 2012).

Along with all these arguments, there are many other claims. There are many possible hypotheses but a few possess the stronger evidence. The temperature changes are dependent upon large number of factors hence it is nearly impossible to evaluate all of them and come up with a satisfying theory.

4 Effect of Changes in Climate Variations

Climate change is explicit. The perturbations in climatic system modify all sustainable development dimensions and hence the potential development pathways for a given nation or a region. It is even considered as the "mother" of all problems to show its irreversible impacts (Kumar and Yalew 2012). The set of mechanisms in which climate change affects economic and environmental outcomes are too vast and complex to investigate comprehensively. Its impacts, vulnerability, and adaptation issues have drawn many scholars from the political, academic, and research sphere. Climate change specifies general and overall changes in an atmosphere of a region or area because of different disturbances in that particular area. These disturbances may occur due to natural reasons, human activities in that area like urbanization, deforestation, air pollution, etc. One of the main reasons of the major climatic changes occurring all over the world is global warming plus the factors influencing and involving it. These factors are described in detail in the portion related to global warming above. In this chapter only the type of climatic changes and the reasons of their occurrence will be discussed in detail. The effects are already being observed in various regions of the world with more droughts, floods, storms, and heat/cold waves (Frank et al. 2013; Goldman et al. 2012; Hansen et al. 2013; Harris et al. 2013).

4.1 Shrinking of Arctic Ice

The global warming has not imparted uniform effects on the entire globe. The Arctic region is going through rapid and severe climatic changes (Hassal 2005). Arctic summer ice is constantly decreasing because of continuous changes in climate and global warming. Arctic sea ice extent for January 2013 is 13.78 million sq km, which is 1.06 million sq km below with respect to 1979 to 2000 average for the month (Vizcara 2013). This thing is an attractive plus immediate concern for the weather and climate analysts and scientists all over the world because this decrease in ice cover triggers ocean waves and storm surges striking the coastlines harder and longer breaking the permafrost which will cause the release of CO_2 and CH_4 to the atmosphere that has been frozen for millennia (Bosnjakovic 2012). This means that further melt down of Arctic region ice cover will result in more emissions of CO_2 contributing further towards climate change and global warming. This will result in loss of whole Arctic ice within next 2–3 decades. This is also thought that the pace of this effect is increasing. The scientists working in this regard are proposing in view of their careful calculations and evaluations that this constant and continuous melt down is going to impose and have serious consequences on our future well-being. This is because Arctic summer ice or Arctic region in general helps in regulating climate by reflecting the excess and harmful sunlight off, hence cooling the earth's climate. Plus if excessive meltdown occurred then due to less ice in Arctic region, this will cause more moisture entrance in the atmosphere from oceans. As a result, more powerful and much frequent storms will occur. This rate of increase in storm intensity and frequency would likely to affect most populated areas of the world, which in turn would lead to excessive damages like life and resource loss, etc.

The Arctic region is the first and foremost point when it comes to impact upon climate change. We should consider it seriously but unfortunately we keep on forgetting that impact of climate change will not stop at Arctic region but will eventually spread further on to the entire world because it is a global phenomenon that, if preventive measurements will not be taken immediately, will only increase in magnitude and power in its impact upon world over the passing minute.

4.2 Extreme Rainfalls in the Tropical Region

The serious issue of global warming prevailing in the world because of which change in climate is occurring continuously resulting into constant rise in temperatures is the major cause of excessive rainfalls in the tropical region. Although the well-documented literature is available on rise of global temperature, no clear estimates on long-term trends in global precipitation are available (IPCC 2001b) because of lack of dependable oceanic rainfall estimates. Recent studies have shown that there is a significant increase in precipitation since 1950s in tropical regions. It is now being confirmed recently that every 1 °C rise in temperature will result in 10 % heavier rainfalls in the tropical region, according to the latest.

Research study performed at MIT. This eventual increase in rainfalls can have utmost and major impact on flooding in populated regions of tropical region. Scientists involved in this particular research are convinced and of the view that excessive rainfalls occurring in tropics are mainly because of global warming. Lau and Wu (2007) confirmed the earlier studies, which showed that extreme rain events in tropics will be more sensitive to the warming climate. However, the guess behind this increased and heavy rainfalls in tropics are basically well known. When some GHGs like carbon dioxide (CO_2) are emitted in the atmosphere, the more the GHGs in the atmosphere, the higher the temperature will be and then because of this, the amount of water vapors increases in the atmosphere. If more amount of water vapors is present in the atmosphere, heavier will be the rainfall due to increase in overall humidity of the region, which helps in facilitating the increase in the frequency and intensity of the resulting storms.

Scientists from MIT have confirmed by satellite observations and study of occurrence of excessive rainfall between latitudes of 30° north and 30° south that global warming is the major issue ruling this climatic change in tropics. Researchers also discovered one clear pattern that showed strong evidence of excessive rainfalls because of El Niño in tropics.

4.3 Forests: Feelers of the Heat of Climate Change

The climatic changes have many negative impacts on forests regarding many different angles. Increase in temperature is not only causing forest fires, heat stress, and drought conditions but also becoming a serious and hazardous cause of widespread insect population (Kumar and Yalew 2012). Scientists and researchers from various parts of the world are already studying forest mortality. As a matter of fact that not all species of trees are affected or impacted upon in the same way as some of the species are more resistant than other ones, regarding factors like age, size, nutritional requirement, etc. of the tree.

It is being feared by many researchers from all over the world that in the prevailing condition of global warming, many forests will cease to exist in the coming 3–4 decades and eventually turn into grasslands at the least or even barren lands like deserts. This factor could further increase the bad impact upon climate change because forests in world act as major and large carbon sinkers. If deforestation is not prevented or forcefully stopped on global level, then because of the decomposition and break down of dead trees, more CO_2 will be released into atmosphere, hence more increase will occur regarding global warming. The leftover debris from cut or dead trees is also an important contributing factor in increased risk of forest fires which will eventually result in even more CO_2 release in the atmosphere. In the USA, Canada, Europe, and Australia, outbreak of pests and diseases, hurricanes, heat waves, and increased risk of forest fires are affecting forest lands (Kirilenko and Sedjo 2007).

So in order to avoid the serious threat of further increase in the prevailing levels of global warming, deforestation should be stopped worldwide forcefully in order

to protect and preserve nature. Also if in any part of world, some forest area has been demolished, then the trend of reforestation should be encouraged on the national level (Sugde et al. 2008). This should also be done practically on the whole world basis because nothing artificial can replace the role of forests. Forests play an important and vital role in not only absorbing CO_2 back from atmosphere but are also the major contributors in regulating weather and climate of the general area plus water purification. Forests are also an important factor regarding water, nitrogen, and nutrient cycles plus also provide fodder and homage for millions of different species of animals and plants.

4.4 Warm Climate: Short-Term Extinction of Species

Fossils and geological records, going back around 540 million years ago, are being reexamined recently by the British scientists and researchers from the universities of York, Glasgow, and Leeds. This reexamination was undertaken so that to discover and confirm connection between biodiversity and global warming in the world. This reexaminational study of fossils clearly showed that warmer time spans in the past at first were accompanied by increase in the rate of extinction of some species. Also it was seen through fossil record study that after a period of long time span, environmental and climatic changes promote devolving of new species causing an increase in overall world's biodiversity.

Through careful calculations, analysis, and examination, it was concluded that in normal conditions biodiversity in the world increases as the world warms up. Exclusive climatic changes and rapidly rising trends of continuous increase in temperature hinder the increase of global biodiversity because of rapid climatic changes. However, researchers and scientists from all over the world disagree that current and rapidly increasing levels of global warming and impact of climatic changes are good for existing species, in view of the present scenario at least. This is so because large variations in earth's biodiversity need billions of million years. Right now we can only predict one thing with surety and with reasonable evidences, provided through proper and careful studies and scientific calculations, is the short-term losses in the present biodiversity of earth.

4.5 Impact on Human Health

Climate change can affect human health by four means: (a) some diseases such as kidney stones are aided by the rise in the mean temperature of the earth (Brikowski et al. 2007), (b) heat stress and cardiovascular disease prevalence is increased due to extreme weather, (c) the reproduction, spatial and seasonal distribution of some disease causing vector (such as mosquito) and bacteria (e.g., salmonella in food poisoning) are affected by the temperature, precipitation, and wind variability,

and (d) drought, flooding, and tropical cyclones affecting indirectly. In a nutshell, climate change leads to morbidity and mortality rate of human beings (Kumar and Yalew 2012).

The prevailing rate of the level of variations in climatic changes and the factors influencing them have an important effect of enhancive nature upon emerging of new infectious diseases, reemerging of the previous strains of infectious entities and the modified form of infections caused by them (Patz et al. 1996). This is happening in addition to the multiple human determinants, including biological and ecological factors. A rise in temperature by 2 °C will bring various serious and (in some cases) yet incurable infectious diseases.

In the last 2–3 decades, the incidence of insect-borne diseases through various species of mosquito has increased and come forward to global concern in the form of epidemics. Some examples include malaria, dengue, and viral encephalitis. These diseases are among those, which are sensitive to climate and the changes influencing it (IPCC 2007a). It can be said with evidence provided by scientific and geographical studies that climatic changes directly affect disease transmission and its epidemic form by shifting geographic range, increased reproduction levels, and the level of biting rates of the specific infectious entity containing vector and pathogen incubation period is also affected as it becomes shortened. It has also been observed by scientists from world over that some climate-related changes can be a cause of increase in the temperature of sea surface and in the sea level. This can be a leading cause of the higher rates of incidences of the water-borne infections and bacterial and industrial toxin-related diseases like water-borne cholera, shellfish poisoning, etc.

The increase in urbanization and human migration from one part of the world to another in pursuit of better lifestyle may influence and damage health infrastructure of that particular region. As a result of this massive human movement, from different parts of the world (particularly towards the cooler regions of the world) cause an increase in deforestation to stand multistory buildings in order to provide working places and housing to them. This will cause a major climatic change in that region as forests (natural buffers of nature) will be demolished in order to accommodate human population explosion over there. This irreversible increase in climatic variability can indirectly contribute to disease propagation and transmission as temperature of that region will increase dramatically due to many humans living in already congested areas.

There is also a main issue regarding climatic stress upon agricultural assets and products that can result into general malnutrition conditions, which can prevail through whole of the world. Also some potential mutations or alterations in immune system of humans, which may be caused by increased flux of the ultraviolet radiation due to continuous erosion of ozone layer can altogether increase humans' susceptibility towards infectious diseases.

The analysis of special and highly influential role played by climatic changes in the emergence of the infectious diseases requires global cooperation among medical physicians, geobiophysicists, climatologists, biologists, and social scientists. The increasing disease surveillance which includes the observation, check, and

maintenance along with use of data systems based on geographical studies can afford much anticipatory measures by the medical society from the world over. If the understanding will be made of the linkages between climatological and ecological changes as determinants of disease emerging contributing factors, redistribution will eventually and ultimately help in order to optimize preventive strategies to slow down the increase in threat of emergence of new and resistant infectious diseases (Diffenburg 2013).

4.6 CO_2 Emissions from Soil Because of Global Warming

There can be one major accelerative influence of global warming upon rate of decomposition of soil organic matter which increases release of carbon dioxide (CO_2) into the atmosphere which will in turn further increase and tend to enhance the trend of global warming (Jenkinson et al. 1991). CO_2 is responsible for approximately 55 % of increase in radiative forcing which arise from anthropogenic gas emissions into the atmosphere. Plus it has also been calculated by conduction of various experiments that round about twice as much amount of carbon is present in top 1 m of soil crust as present in the atmosphere.

For this purpose, Rothamsted model was employed for use in order to do turnover of organic matter of the soil to calculate the exact amount of carbon dioxide expected to release from world's store of soil organic matter, if the rise in temperatures occurred as it has been predicted but keeping constant the annual return of plant debris to soil. The temperature of the earth if rises by 0.03 °C per year (this increase is considered most likely by Intergovernmental Panel on Climate Change), we can estimate upon that additional or excess release of carbon dioxide from soil organic matter will be approximately 61 10^{15} gC in the coming 60 years. The estimate is that of approximately 19 % of carbon dioxide that will eventually get released by consumption and combustion of the fossil fuels in the coming 60 years if the present use of this irreversible natural resource continues excessively in an unchecked manner.

4.7 Unique Impact of Increase in Urban Population

It has been found out that the ever-increasing growth rate in the urban population can lead to more than 25 % rise in the CO_2 release in the atmosphere in some of the developing countries. Increase in the economic plus social growth and ever bending interests towards the achievement of much better lifestyle associated with people moving into the cites or already situated dwellers over is directly related and proportional with increase in CO_2 emissions. This is largely because of the higher formation and consumption of resources preferred by an urban population.

5 Climate Change and Agriculture

Agriculture is an important sector of many of the world economies. It provides us with food, fiber, shelter and feed for the livestock. In addition to it, it contributes billions of dollars to the economies of many countries especially the agricultural-dependent economies and is the most dependent area as climate change is the main determining factor for agricultural productivity (Adams et al. 1998) and has threatened world agriculture productivity both economically and physically (Shakoor et al. 2011). Various elements shape and drive the agricultural sector. It is influenced by market fluctuations, national and international policies, practices in management, trading terms, technology availability, biophysical factors, etc. (Kurukulasuriya and Rosenthal 2003). Being dependent on resources present naturally, agricultural produce is at the mercy of uncertainties driven by climatic variability. Hence, vulnerability of agriculture sector can be classified in two broad categories of effects induced by climate: (1) effects having direct impact due to variability in temperature, precipitation, and content of carbon dioxide and (2) changes in soil quality and the occurrence, dispersal of infestation by pests and diseases having indirect impact. Therefore, susceptibility of agriculture sector can be assessed with the understanding of three major factors that are: environmental, biophysical, and socioeconomic factors. Some impacts are anticipated to be adverse while others being favorable. The distribution of impacts will change depending upon the ability to respond to these effects along with what sort of resources is used across various nations.

Changes in agricultural sector due to impacts of climatic variability are anticipated to manifest directly from changes in water resources and land. Climatic variability is expected to result in water and other resource shortages, affecting soil properties, variation in intensity, and frequency of droughts, flooding, sea-level rise, and storm surges, desertification and disease and pest outbreaks on agricultural lands and livestock. The areas susceptible to climate change will experience losses in crop yields (Rosenzweig et al. 2002). Dell et al. (2008) reported that 1 °C rise in temperature will reduce economic growth in poor countries by 1.1 %. He used the per annum variability in temperature and precipitation from 1950 to 2003 on 136 panel of world countries and reported devastating effects of climate change on economic growth especially of the poor countries and significant loss in industrial output (food, brewery, and textile) too. With respect to crop production, it is anticipated that change in climate will come along with impact as well as opportunities (FAO 2008) and would significantly affect the living patterns, the ability to access food and socioeconomic conditions of the majority of the people living in different regions of the world especially the arid, semiarid, and coastal areas (Chijioke et al. 2011; IPCC 2007b; Schmidhuber and Tubiello 2007). In contrast, it will bring beneficial effects in temperate regions and high latitude regions (Mendelsohn and Nordhaus 1999).

According to various international reports, it is projected that developing countries will be most affected for three reasons that are: (a) the changing climate will have its most negative effects in tropical and subtropical regions (Rosenzweig et al. 1993; IPCC 2001b), (b) most of the expected population growth will occur in

developing world in 2030 (United Nations Population Division, DoEaSA 2009), and (c) more than half of the entire labor force in the developing world is engaged with agriculture (FAO 2005). This will exacerbate the situation in rural community. Earlier estimates depict 4–24 % agricultural losses in developed countries while 14–16 % in developing countries (IPCC 1996). The report of FAO published in 2008 indicated that the number of hungry and malnourished people have increased from 90 to 225 million from the years 1970 to 2008 and has anticipated further 100 million by 2015.

Although agriculture soils contribute about 15 % of global GHGs emissions (Gitz and Ciais 2003), these emissions include both exchange of GHGs in the arable fields, and indirect emissions from the use of agricultural inputs. The agro-ecosystems contribute emissions of nitrous oxide (nitrification, denitrification, and use of N-fertilizers), carbon fluxes between soil–plant interaction system and atmosphere, and methane exchanges to the atmosphere (Lehuger et al. 2007). But agriculture as emitter of GHGs is not an important issue rather how to protect the agriculture from changing climatic scenario is of paramount importance to ensure food security for the coming generations.

From various reports, it is evident that within and across the regions the vulnerability to changes occurring in climate will vary. In the absence of pragmatic policies to long-term climate changes, region-specific impacts will become more evident. The regions where strategies to address the issue of climatic variability are poorly structured will cause the high cost of maladaptation. Therefore, in the coming decades such policies should be devised which minimize the devastating effects of climate change and aid in increasing the agricultural productivity to meet the requirements of continuously increasing population and ensuring access to food for future generations.

5.1 Climate Change and Productivity of Plants

Our planet Earth has undergone an ecological shift. Plant productivity globally is thought to be at the verge of decline, mainly due to stress due to droughts caused by global warming. This global turnaround was discovered during an observation conducted by NASA satellite. The data was obtained by Maosheng Zhao and Steven Running from the University of Montana in Missoula. This data was analyzed against a data containing only a 6 % increase, which was obtained 2 decades ago. It is being observed that the recent decade's decline is slight which is just 1 %. However, this global shift can have an impact on food security, biofuels, and nitrogen, carbon and nutrient cycle.

In accordance with previous knowledge based upon old researches, it was held that productivity of plants was rising steadily. It has been confirmed through proper observations that rate of global plant productivity has increased by 6 % between the years 1982 and 1999. This was what occurring for two whole decades and it was reasonably thought that variations in temperature, solar emissions, and availability

of water which are altogether influenced by climate changes affected by global warming are hence favorable for the growth of the plants . But these results were challenged and then nullified because new research through modern technology and analysis showed that the effect of global warming upon climatic changes which in turn influence productivity of land plant growth and vegetation need not to be positive (Nemani et al. 2003). It is also observed that the effect of the regional drought overruled the positive influential effect of longer growing season which in turn has driven down the global plant productivity between the years 2000 and 2009.

Scientists have predicted a very serious type of warning that in future, the much warmer and ever rising temperatures will be dangerous for plant growth. This has been discovered through a carefully conducted analytical study of plant productivity data with the help of an instrumental machine known as MODIS (Moderate Resolution Imaging Spectroradiometer) placed upon one of the NASA's satellites that is known as "Terra." The observations made by using MODIS about plant productivity were in combination with increase in the rate of ever growing and seasonal climatic variations, which include change in temperature spectra, range and intensity of solar radiations, and water availability. An algorithm was made based upon the data obtained by observation done upon and study of factors influencing and affecting plant growth and variations in climate which describes and explains about restricted growth of plants at different geographical locations in the world. It has been observed through careful analytical study by using this particular algorithm that plant growth is generally restricted and limited at high latitudes by steady rise in temperature and in desertificated areas by less water availability. The countries of north latitude indicate net positive impacts of climate change but projections for most developing countries are negative (Reynolds 2010). But one thing, which should be, kept in mind is that such varying and restricting plant growth regional limitations can also vary in their degree of impact and effect on growth of vegetation throughout the growth season around the world.

The above conducted analysis depicted that since the year 2000, the ecosystems flourishing in northern hemisphere with high latitude have continued to get benefited by the warmer variations in temperature and periods of longer growth season (Zhao and Running 2010). It has been seen that this particular effect was counterbalanced by the droughts associated with global warming which in turn has restricted and limited the plant growth in the southern hemisphere which, as it has been feared, resulted in the net global loss of land plant's productivity.

The steady and fast decline in terrestrial plant productivity in the last 3–4 decades depicts that in future, a complex type of interplay between ever rising temperature variations, amount and frequency of rainfalls, the patterns of cloud formation, concentration of CO_2 in the atmosphere, nutrients' availability and the programs and phenomena of land management, in combination will determine the patterns and trends in vegetative productivity around the world. Various scientists and researchers are now considering upon maintaining the record of the trends of variations of such atmospheric and environmental factors. This is the major concern of them because for one thing, the global plantation act as the carbon dioxide "sink" and the ever shifting of plant productivity levels towards constant decline is linked with the

shifting levels of the influence of GHGs into the atmosphere. The other reason due to which the scientists are much bothered is the fact that the extent of such environmental and atmospheric fluctuations also exerts huge and negative stress upon the plant productivity and growth throughout the world which can seriously challenge food production and threaten meeting its requirements in the present and continuously growing situation of population explosion.

In the future, the potential damage that the global warming will cause further and multiple decline in global plant production plus availability of natural resources is not promising well enough and also the ability of the world's biosphere to support and maintain multiple demands of the population of the world for meeting agricultural needs and production, fiber production needs, and the increase in the rate of demand and need of biofuel and its production in the world (Zhao and Running 2010). It has been demonstrated on the basis of various observations and careful study that even if the declining trends in the rates of ever reducing plant productivity, etc. as depicted by the study of rates of the past 3–4 decades does not continue to proceed, still the managing forests and croplands for getting and meeting the requirements and the multiple benefits for the world's population including food production, biofuel harvesting, and carbon resource's storage will become excessively and increasingly full of challenge for the world in the near future, in the present scenario of the negative and immensely possible impacts left by these decline inducing changes in (sometimes irreversible) resources like forests, carbon reservoirs, biofuels, etc.

An observation of the earth was made in the year 2003 in the form of a snapshot, which traced the rates of plant productivity in the regions of the world having increased plant productivity and decreased plant productivity. But recently it has been observed by pattern study done by NASA scientists that the plant productivity tracked between the years 2000 and 2009; the global net decrease in plant productivity was because of the regional droughts under the influence of global warming.

5.2 Impact on Crop Production

Extensive literature has been developed on the impacts of climate change on agriculture sector, mainly focusing on the sensitivity of this sector. The available literature depicts that the extent of vulnerability of this sector to climatic perturbations is dependent on various local environmental and management factors which includes local conditions that are biologically active such as soil physical properties, type of crop, awareness about the climate change, the support from government, and the ability of stakeholders to undertake necessary measures for remedies to address the impact of changes in climate. The increased uncertainty of climatic effects poses an additional problem that farmers have to take into account. For example, the poor condition of soil, financial limitations, and lack of access to market can limit agricultural output to begin with, regardless of climatic effects.

Agriculture is an economic activity, dependent on many biophysical factors. Change in climatic variables could have significant impact on plant growth and development. The climatic variability may pose direct implications on biophysical factors including plant and growth of animals and the physical infrastructure related to processing of food and its distribution (Schmidhuber and Tubiello 2007). Most of the models designed for crop response to climate change take into account temperature, moisture, and carbon dioxide. But many other processes not integrated into these models could have significant effects including incidence of pests and diseases, exposure to heat waves, elevated ozone, loss of irrigation water, and an increase in inert-annual climatic variability related with a phenomenon like El-nino (Reynolds 2010).

Different crop simulation models have been designed to estimate the implications of changing climatic scenario on the crop production. The quantitative projections on impacts of climate change have been primarily based on the studies from experiments as well as cross sections. The experimental methodology includes agro-economic models, which is similar to controlled experiment where climatic variables are adjusted and their effects on crops are estimated. Similarly, agro-ecological zone analyses are carried out where estimates are made about specific agro-ecological zone. In the end, the results are merged into models of economic and general circulation to anticipate the impact's range as well as scale.

Using these simulation models, scientists undertook studies to estimate and quantify the impacts. Newman (1980) concluded that the corn belt of the USA would shift to northeast with every 1 °C increase in temperature. Rosenzweig (1985) reported that under changing climatic scenario winter wheat production in Canada would increase.

The continent of Africa is anticipated to be 2–6 °C warmer on an average according to the third report of IPCC on assessments. Sivakumar (1992) reported the shift in rainfall pattern from 1965 to 1988 in Niger and depicted reduced growing season by 5–20 days in Pearl millet (staple crop) across various locations in the country. He observed reduction in the net absolute amount of rainfall and change in its timing. Fischer and Van Velthuizen (1996) concluded that the agricultural productivity of Kenya will robust under changed climate if amount of precipitation is increased along with elevated levels of CO_2 and warmer air temperature. In Zimbabwe maize production is anticipated to decrease by 11–17 % (Makadho 1996) because of reduction in the grain filling period. Yates and Strzepek (1998) speculated that high dependency of Egypt on natural resources will make it more vulnerable to impacts of climate change. Benson and Clay (1998) using information from countries like Namibia, Zimbabwe, South Africa, Mozambique, Malawi, Lesotho, and Botswana demonstrated that industrial economies of these countries will be more vulnerable than the developing countries of Africa.

Numerous studies indicate that the agricultural sector of Asian countries will be more vulnerable especially the tropical zones (South and Southeast Asia). Impacts of climate unevenness are more devastating in southern part of Asia and may result in 50 % reduction in wheat productivity (MoE 2009). Rosenzweig and Parry (1994) reported reduction in grain yields by 25–40 % with a 4 °C rise in the temperature in

India. Seshu and Cady (1984) anticipated a decrease in rice yield with minimum temperature increasing from 18 to 19 and 0.5 °C rise in temperature in winters could decrease duration of crop by 7 days and 0.45 tons/ha yield and 2 °C increase would reduce yield in many parts of India (Aggarawal and Sinha 1993). Murdiyarso (2000) reported 7.4 % decrease in rice production of Asia. Mirza et al. (2003) reported changes in inundation of land will significantly implicate on rice production and cropping pattern in Bangladesh. Pakistan is ranked 28th among the countries which will face massive vagaries of climate change (Shakoor et al. 2011). Since 22 out of 28 countries are in Africa, crop production is affected by the following physical effects of climate change (Hulme 1996; Chijioke et al. 2011):

- Variability in temperature
- Effect of CO_2 concentration
- Change in precipitation amount and pattern
- Incidence of pests and diseases
- Extremity of weather events
- Rise in the level of sea

5.2.1 Variability in Temperature

The IPCC report (2007a) by combining the results of GCMs (general circulation models) predicted that the temperature rise in the coming 7 decades would be more than 5 °C. Variability in mean, maximum, and minimum temperatures is anticipated for most regions of the world due to climate change and northern countries would expect a higher temperature rise (Milanova 2012). Variability in temperature will affect the soil moisture content and the duration of growing season in different parts of the world. It is projected that countries lying in low latitude would be generally at a risk of reduced crop yields even at 1–2 °C of warming (Parry et al. 2007) especially in those areas where temperatures are close to or at optimum level for a crop growth to start with because levels of transpiration and evaporation with low levels of soil moisture content benefit in a predominant manner (IPCC 2007c). As a result some cultivated areas and some tropical grassland may become arid at increasing pace (IPCC 2001a). In temperate regions, the higher temperature will predominantly bring benefits to the agriculture by expanding the cropping areas and increased growth period and posing positive effects on crop yields (Kurukulasuriya and Rosenthal 2003; Schmidhuber and Tubiello 2007). The decreased fertility of soil of higher latitude will affect some of the fruits of an extended growing season (Rosenzweig and Hillel 1995). A moderate warming in temperate and some humid regions may increase productivity of pasturelands whereas in arid and semiarid regions pasture yields will decline (IPCC 2007a).

Plant growth rate is dependent on temperature, increases from a base value and decreases beyond an ambient limit. The plant biomass yield is a product of rate of biomass deposition and the growth period. The biomass accumulation is governed by the rate of photosynthesis of canopy. The period of growth is directly

proportional to the temperature. Higher temperature leads to increased respiration rate, lesser time period for seed formation and hence lower biomass production. The increase in temperature will result in shorter period for grain filling, lighter grains, and lower grain quality. Increased temperatures will lengthen the duration of vegetative growth and reduce the risk related to spring and winter frosts (Milanova 2012). Plants photosynthesize at optimum temperature, which is generally higher in C4 plants than C3. Temperature affects dark respiration, increasing exponentially with rising temperature and hence net photosynthesis rate becomes sensitive to temperature response (Rosenberg et al. 1983). Vu et al. (1997) proposed that the response of doubling the carbon dioxide concentration at 35 and 32 °C for rice and soybean increased the photosynthetic rate but decreased with further increase in temperature. The results from the various scientific studies indicate that temperature increase may offset the luxuries of increasing CO_2 concentration on crop yield. The warm air temperature accelerates the rate of grain growth, reduces the period of grain filling and grain weight. The reduction in grain weight of cereals is considered as the effect of temperature on rate and length of grain growth period (Fuhrer 2003). The range of many pests may also expand and the ability of pest population to withstand the cold climate and attack on the spring crop will increase (Schmidhuber and Tubiello 2007).

5.2.2 Effect of Carbon Dioxide Concentration

Agro-ecosystems may be influenced strongly by the expected increase in carbon dioxide content and related climate unevenness and change. Plants respond to their surroundings, CO_2, and temperature. Increasing CO_2 has positive effects on the plant growth because water use efficiency is increased and photosynthetic rates will be higher as CO_2 gradient will increase between leaf and atmosphere (Streck 2005). The current amount of CO_2 379 ppm in the atmosphere (Chijioke et al. 2011) is inadequate to saturate the ribulose 1,5-biphosphate that drives photosynthesis in C3 plants (Taiz and Zeiger 1991). The concentration of CO_2 is expected to rise by 57 % by 2050 (Hulme 1996) but projected to rise about 550 ppm under IPCC scenario by 2100 and business scenario greater than 800 ppm (Schmidhuber and Tubiello 2007). But if the atmospheric CO_2 increase will be accompanied by the rise in air temperature, it may offset the advantages of an increasing CO_2 concentration.

Stomata do not have a direct response to the CO_2 concentration. CO_2 concentration is regulated in the stomatal cavity (Ci) by plants and there is a constant ratio with atmospheric concentration (Ci/Ca) at a given vapor pressure deficit (Mott 1990). This ratio under stationary condition is 2/3 for C3 and 1/3 for C4 plants (Wong et al. 1979). Therefore it can be concluded that the partial closure of stomata at elevated CO_2 concentration will be the result of Ci/Ca regulation. The possibility of acclamation stomatal movement to exposure, to escalated CO_2 has been pointed out. Rice crop has shown marked acclimation and soybean appears to be less affected (Campbell et al. 1988). Wheat did not show any down-regulation of photosynthesis

to elevated CO_2 concentration in the field (Nie 1995) in contrast to when raised in pots (Sage 1994).

In commercial greenhouses, CO_2 enrichment has been practiced since long time. The history of this practice has been reviewed by Wittner (1967) and reported increase in yield and ameliorated quality has been achieved in lettuce, tomato, cucumber, and some flower crops. A small number of studies have reported decreased yield at escalated CO_2 concentrations (Rosenberg et al. 1983). Lawlor and Mitchell (1991) reported that if C3 and C4 crops were provided with sufficient water, nutrients, and pest control, the yields of these crops grown at 700 μmol CO_2 mol^{-1} would increase by 40 % and 9 %, respectively. Only at very extreme conditions, there is deleterious effect of CO_2 concentration.

Where CO_2 metabolism is considered in plants, three major categories exist: C3, C4, and CAM. Each of these categories responds differently to the higher concentrations of CO_2. Generally the photosynthetic pathway of C3 is considered as less efficient when compared with C4 pathway. In C3 plants, the increase in optimum CO_2 concentration suppresses photorespiration. The increased concentration of CO_2 increases carboxylation and decreased rubisco activity and hence reducing the loss of CO_2 through photorespiration. Therefore, a net photosynthetic increase occurs (Taiz and Zeiger 1991). The concentrating mechanism of C4 and CAM plants tends to allow the leaves of these plants to maintain increased photosynthetic rates when internal concentration of CO_2 levels are lowered Therefore, photosynthetic rates of C4 and CAM plants are considered as less prone to heightened CO_2 concentration. The yields of crops are expected to increase nearly 10–30 % in C3 plants (wheat, rice, and soybean) and 0–10 % in C4 plants (maize, millet, sorghum, and some grasses) (IPCC 1996; Streck 2005; Schmidhuber and Tubiello 2007). An atmosphere with elevated CO_2 would result in higher photosynthetic rates but the quality with higher yields may not increase. Some cereals and forage crops showed lower protein content at higher CO_2 (IPCC 2001a). About the effect of increasing CO_2, a great many uncertainties exist and the response of different species to this increase may be different.

5.2.3 Change in Precipitation Amount and Pattern

The availability of water is a critical factor in determining the impacts of climate change in different regions of the world. Numerous studies demonstrate that it is critical to determine whether the precipitation and duration of growing season will be affected by climate change either in a positive or in a negative way (Hulme 1996; Sivakumar 1992). Rise in the level, timing, and variability of precipitation may be beneficial for semiarid areas by enhancing moisture to the soil, but could exacerbate problems in areas with plenty of water, while a positive effect may be posed by reduced rainfall. It will vary from region to region. It is projected that due to climate change, the temperate region may become wetter and drier areas of tropical region may become drier (FAO 2008). The variability in rainfall will affect rate at which soil erodes and moisture content of the soil, which are important factors in plant

growth and development. A temperature increase along with reduced rate of precipitation would result in the loss of cultivated lands due to low moisture, high aridity, salinity, and groundwater depletion (Bals et al. 2008). To overcome the water shortages, more capital and technological requirements will be needed for irrigation, which will cause high input costs.

5.2.4 Incidence of Pests and Diseases

Climate change due to anthropogenic interventions has the ability to influence significantly the biology of all living organisms. Limited literature is available on the variability in the incidence and severity of pests affecting agricultural products and diseases. This factor has not been incorporated into the estimates of climate change impacts. The rise in global mean annual temperature associated with climate change will likely favor winter survival of insects pests that may modify the predictions regarding dynamics of insect population (Denlinger and Lee 2010). Many pests and fungi survive under comparatively warm temperatures, humid climates, and increased carbon dioxide level (Chidawanyika et al. 2012). This would cause new problem for farmers especially for the farmers of developing and the poor world.

Short-term fluctuations in temperature may be stressful for small insects as their body temperatures exist in equilibrium with optimum temperatures. Therefore, it is necessary for these insects to be able to cope with such changes. Physiologically, insects have the ability to adjust themselves with respect to thermal tolerance over short duration, a phenomenon named as "hardening" (Bowler 2005; Lagerspetz 2006). But over a long time period temperature tolerance may be altered to acclimatization in the field in response to changes in the environment (Huey and Berrigan 1996). Environmental factors directly influence the survival, development, reproduction, and dispersal of insect pests. The invasion potential of some pests may increase in response to the changing climate. For example, the maize stem borer, which was accidently introduced in Africa at first was able to survive and establish itself in many African countries, rendering more destruction as compared to species that were indigenous to that place (Sithole 1990).

5.2.5 Extremity of Weather Events

Extreme events are not new to agriculture, but it is anticipated that their frequency and intensity will increase and the areas subjected to these events will expand (Schmidhuber and Tubiello 2007). Huge agricultural losses can occur from extreme weather events like droughts, floods, storms, sudden heat, cold waves, etc. Extreme events can harm crop and reduce yield especially before the harvest period, which will pose serious consequences to food production and food security. Climate variability has been directly linked to the decline in economic activity (Brown 2009). A higher frequency of droughts will put increased pressure on water supplies varying from transpiration of plants to their allocation (Rosenzweig and Hillel 1995).

According to Lobell et al. (2011), yields will reduce by 1.7 % per degree if a day is spent over 30 °C under drought conditions. Whereas increased rainfall intensity in various regions can result in higher rates of eroded soil, leaching of chemicals involved in agricultural sector and runoff that contains agricultural waste to water bodies.

5.2.6 Rise in Sea Level

Sea level is expected to rise as a result of global warming, endangering the coastal and low lying areas triggering coastal inundation, salinization of soils, and intense rainfalls. Climate change will bring inundation in low lying agricultural lands associated with increased runoff from tropical storms while sea level rise will increase level of soil salinity and water logging. Salinity affects the plant growth by increasing the ionic concentration, which causes osmotic stress and the accumulation of these ions in plant tissues impair plant metabolism. Water logging leads to the displacement of air from the soil pores leading to hypoxia. This would cause reduction in the crop production leading to loss of farmer's income and food supply system of the affected region.

The sea level has been increased by 1.7 mm/annum on an average from 1870 to 2000, for a total rise in the sea level of 221 mm (0.7 ft or 8.7 in.). Since 1993, the satellites of NASA reveal that the level of sea is rising more steadily, about 3 mm/year, for a total rise in the level of sea by 48 mm (0.16 ft or 1.89 in.) between the years of 1993 and 2009. Projections on rise in sea level indicate that it will continue to rise for centuries after temperature stabilizes (Reynolds 2010). Satellite measurements depict that the Greenland and West Antarctic ice sheets are losing nearly 125 billion tons of ice yearly, enough to raise sea levels by 0.35 mm/yr (0.01 in./yr). If the melting hastens, the rise in the sea level could be significantly higher (Riebeek 2010).

5.3 Impact on Livestock

Livestock can also be affected by climate change. It can be affected by two ways: (a) reduction in the quality and quantity of forage from pastures and (b) the direct effect of higher temperatures on the livestock. Warmer temperatures are anticipated to have a suppressing effect on the appetite of animals hence leading to lower weight gain (Adams et al. 1998). Extensive evidence exists that properly managed livestock systems have more potential to adapt to climatic variability as compared to crop systems because they are better able to adapt to extreme events.

Livestock may be threatened by heat waves directly. All animals are affected by heat stress either directly or indirectly. Vulnerability to diseases may also be increased with it along with the reduction in fertility and milk production. On the other hand, drought may affect pasture and feed supplies. The amount of forage

quality is reduced by drought rendering it unavailable to grazing livestock. Some areas may observe more intense and longer droughts that may in turn result from increase temperatures in summer and deceased precipitation. Changes in crop production due to drought may cause problem for animals relying on grazing. The grasslands of mid to high latitude are anticipated to show higher productivity under changing climatic scenario (IPCC 1996). The arid and semi-arid pastures are projected to have reduced livestock fertility and increased mortality rates (IPCC 2007b).

The incidence of parasites and diseases affecting the livestock may increase with change in climate. The survival of many parasites and pathogens may become easy with the early onset of spring and comparatively warmer winters. Moisture-dependent pathogens could thrive in areas where rainfall is increased. The productivity of pastures may increase with higher CO_2 concentration but there might be a decline in its quality. The increased gas concentration can in turn increase the plant productivity that is utilized as fodder for livestock. However, it has been indicated via studies that decrease in quality of forage found in pasture lands may occur as a result of higher CO_2. In short, more fodder will be consumed by cattle to obtain the same nutritional benefits.

5.4 Impact on Fisheries

Along with sectors, the industry of fisheries is also anticipated to affect from global climate change. Fisheries are already going through many stresses, including overfishing and pollution of water. Climate change may heighten these stresses; particularly temperature changes could pose significant impacts (Environment Protection Agency, USA).

Due to climate change, there might be a change in the ranges of many fishes and shellfishes. A lot of marine species have ambient temperature ranges at which their survival is possible. For instance, the North Atlantic cod requires below 54 °F temperature of water. Even temperatures at the bottom of the seas above 47 °F can retard their ability to reproduce. In this century, it is likely that both threshold temperatures would exceed (USGCRP 2009). Many species of the seas are able to find areas of streams with lower temperatures and lakes or move northward along the coast or in the ocean. However, escorting to a new area may put these species into competition over food and other resources with many other living organisms.

The prevalence of some diseases, which affect the aquatic species, may increase with the increase in the temperature of water. For example, in southern New England, lobster catches have dramatically declined. This decline is due to a temperature-sensitive bacterial shell disease, which was the cause of this die-off event. Migration and reproduction timings may be affected by changes in seasons and temperatures (CCSP 2008).

In addition to warming, due to atmospheric CO_2 increase, the acidity of oceans is also increasing all around the world. This increase in acidity could have a harmful

effect on shellfish by weakening their shells formed from calcium. The structures of ecosystems that are relatively sensitive may also be threatened by acidification upon which some of the fish and shellfish rely.

Agriculture and fisheries are considered as most vulnerable sectors to the climate changes which, on the other hand, will affect other sectors and future world market and trade. The fourth IPCC assessment report defined vulnerability as "the degree to which a system is susceptible to or unable to cope with the adverse effects of climate change including variability and extremities." The vulnerability of agricultural produce to climate changes depends on the physiological feedback of affected plant and the capability of affected socioeconomic system to tackle with the changes in yields along with the changes in the drought frequency and floods. Adaptive and mitigating strategies are needed to prepare the communities, regions, and countries for the penalties of climate change.

The combined effect of climatic variables (temperature, precipitation, carbon dioxide, extreme events) on crop yields, livestock, and fisheries is estimated to vary from one crop to another, species to species as well as from region to region. From the available literature and research on impacts of climate change it has become evident that there will be regional winners and losers from climate change, given that net reduction potential in the yield will be greater in warmer, low latitude, arid and semiarid areas, and the developing world. It implies that climate change may affect the comparative advantage of agriculture production region, which is expected to shift to the areas in which specific crops are raised, within the borders and across the borders. It would affect the agricultural revenues of various regions and countries and alter the patterns of agricultural commodities trading among countries. The economic consequences of reduction in yield will depend on the adaptations made by the farmers, governments, consumers, and other related institutions.

6 Global Climate Change and Security of Food

Climate change and agriculture are inter-related processes. It is a leading agendum today and a growing concern on global scale in context of its impacts on crop production and food security. It is considered as the biggest challenge to agriculture and security of food because global warming is projected to have significant implications on conditions affecting agriculture. Crop and livestock production both will be influenced by climate change and the way it affects may vary from crop to crop, region to region, and from season to season (Dell et al. 2008). Agriculture is dependent on climatic variables that include: maximum and minimum temperatures, incident solar radiation, precipitation, wind speed, and relative humidity. Other variables include the concentration of CO_2, sea-ice extent, mean sea level pressure, sea level and storm surge frequencies. The increasing climatic variability brought about by these variables is a major environmental challenge to the world today with significant implications to ecosystem, food security, and economic stability and will affect both farm income and food security. It affects production of food directly through

changes in conditions of agro-ecology and indirectly by affecting distribution of incomes along with the growth.

The FAO (1996) defined food security as "it exists when all people at all times have physical and economic access to sufficient, safe, and nutritious food to meet their daily dietary requirements to ensure an active and healthy life." It depends on availability and access to food and utilization of food (FAO 2000). Climate change will affect the security of food through its impact on all components of local, national, and global food systems. The main pillars of food security are: food availability, its access and utilization (FAO 2000), which are hierarchical.

Food availability is essential but not enough for access and access is essential but not sufficient for utilization (Webb et al. 2006). These three facets of food security need to be ensured to overcome the risk of food paucity at local, national, and global levels, which may be affected by climatic variability. The first pillar refers to the existence of quality food in sufficient quantities, supplied by either domestic production or import. Changes in agricultural supply result from the changes in yield and crop acreage. The domestic consumption requirements give the estimate of deficit or excess of food availability in a certain region. It is most often used as a measure of food security.

Food accessibility refers to the ability of individuals, communities, and countries to purchase sufficient quantity and quality of food. The access to food is determined by physical and financial resources and social and political factors. Food access consists of affordability, allocation, and preferences. The physical availability of food does not necessarily mean an individual has access to food. It depends on many factors such as poverty, infrastructure, prices, and preference of household. Food costs and capacity to procure food are directly proportional to the changes in commodity supply and resultant price changes.

Utilization of food refers to an individual's capacity to consume or benefit from food (FAO 2011). Climate change will also affect the ability of individuals to use food effectively by altering the conditions for food safety. A household who has physical and economic access to food could be food insecure if it is unable to get a balanced diet.

In future global and regional weather conditions will become more variable with increase in the severity and frequency of extreme events, which will bring greater fluctuations in the crop yields and local and international food supplies that will affect the stability of food supplies and thus food security. No doubt, climate change will impart significant negative impacts on the crop yield, and hence a huge challenge to the livelihood and food accessibility to most of the people. Crop production and food accessibility are key determinants whether a region is food-secured. Climate change will affect all the components of food security. Any change that will affect crop production would have significant implications to food availability, accessibility, and utilization. Climate change is considered as one of the root cause of high food prices. The major negative impact of high prices will be burdensome for small farmers especially of the developing world. The household may be forced to reduce the quantity/quality of food or consume less preferred food to meet other socioeconomic demands of the family. In short, climate change will bring low

production and low productivity that will cost high food and feed prices and some people may be unable to access food, leading to malnutrition, poverty, diseases, and starvation (Albaladejo 2013).

All assessments depict that the first decades of the twenty-first century are expected to see less impacts of climate change, but also decreased overall incomes and a higher dependence on agriculture.

7 Adaptations, Mitigation, and Climate Change

The IPCC defines mitigation as "implementation of policies to reduce GHG emissions and enhance sinks" (IPCC 2007a). It is concerned with how to limit the GHG emissions due to anthropogenic interventions. Mitigation strategies are today's world need so as to limit the vagaries of climatic variability. It can only be effective if such measures are organized globally and strong coordination and dissemination linkages are established between various research institutes, universities, governments, etc.

To stabilize the warming climate near 2 °C would require reduction of global emissions about 1.5 % per annum, i.e., about 50–70 % reductions in GHG emissions (World Bank 2010; Johnsson et al. 2012). The climate modeling reports depict that reduction in emission of 50–85 % in carbon dioxide are needed to stabilize atmospheric concentrations of GHGs at 440–490 ppm, corresponding to a global temperature increase in equilibrium of 2–4 °C (IPCC 2007b). By 2050 emission should be 50 % below the 1990 levels and zero by 2100 (World Bank 2010). Currently the per capita emission of CO_2 is 7 tonnes and by 2050 the per capita emission should be 2 tonnes provided the world population is about nine billion by 2050 (Bosnjakovic 2012).

Currently the developed countries are contributing more transmission of GHGs in the atmosphere than the developing nations. In rich countries, even if emissions fall to zero, still poor countries will need to limit emission about 2–2.5 tonnes, because eight billion of the world population resides in poor countries especially Asia which is the most populated region in the world and India and China are considered as key players. Therefore, it is necessary that poor countries should be at the center of any global deal. The USA, Canada, and Australia emit about 20 tonnes per capita of CO_2, Europe and Japan around 10 tonnes, China about 5 tonnes, India about 2 tonnes, and most of the sub-Saharan African countries emit round about less than 1 tonne. At current emission scenario and adopting the principle of equity, the USA, Australia, and Canada would need to cut down the reductions by 90 % up to 2050, European countries to 80 %, and China to a level of 60 % to achieve the target of an average of 2 tonnes globally (Bosnjakovic 2012; Rogelj et al. 2013).

In agriculture sector, GHGs mitigation can be achieved by four basic mechanisms (Khajuria and Ravindranath 2012): (a) reduction of methane and nitrous oxide emissions from agricultural production, (b) producing various forms of biomass for use as energy source as substitute of fossil energy sources, (c) minimizing

desertification by supporting forests via reforestation, afforestation, and adopting agro-forestry, and (d) storage of carbon by increasing the organic content of soils (Caldeira and Myhrvold 2012).

It would be difficult to cut back away the emissions from agriculture sector as compared to other sectors. Developed countries have more responsibility to cut down their reduction and would need to have emissions close to zero in case of transport and power sectors in the coming decades. Still it is a complex and controversial issue whether such a mitigation effort is technically, economically, and politically feasible. Mitigatory efforts have to be choked out together by experts from agriculture sector, climatologists, growers, environmentalists, and policymakers (Platz et al. 2007).

Adaptation is defined as "initiatives and measures to reduce the susceptibility of natural and human system against actual or expected climate change effects." It deals with how to tackle the impacts of climatic variability: those which are already being observed, those which are anticipated to happen with a high degree of certainty and those with uncertainty but with more frightening effects. Predictions regarding the variability in climatic parameters at the regional and local levels may lack precision but the trends of impact of climate unevenness and change are emerging clearly. Current and future vulnerability assessments are needed for formulating an effective adaptation strategy. An adaptation strategy aims at reducing the vulnerability and increasing the adaptive capability.

Effective adaptation to change in climate requires a cross-sectional approach in order to avoid possible conflicts among different sectors. Adaptation may be costly, but it is much needed to start it now, because it will cost much higher once the effects of climate change get irreversible. Adaptation measures may be foreseen as an opportunity for triggering alternative, innovative, and pragmatic approaches. The need for adaptation certainly arises from the key question of financing. This is particularly problematic for poor developing countries, which do not have the resources to prepare for and respond to these changes. Under the assumption that global emissions will be reduced by half until 2050, UN Development Programme (UNDP) estimated that additional costs for developing countries amount to US$ 86 billion by 2015 (UNDP 2007/2008). The World Bank anticipates the annual climate funding required for a 2 °C trajectory to US$ 28–100 billion for adaptation and US$ 139–175 billion for mitigation. Beyond 2015, the proper level of development support should account for the further cost from climate change if mitigation fails. The nations with good governance and robust diversified economies shall be less vulnerable to shocks of climate change (Seinfeld and Pandis 2006).

The recognition of the fact that some countries especially the developing ones (particularly poor community) will suffer more from vagaries of climate change has added impetus to promote adaptation (Burton 2001). Numerous studies have led stress on the need to pursue adaptation along with the mitigation strategies. Adaptation is considered as a vital step to strengthen the local and regional capacity to deal with the projected and unexpected climate change (Smith et al. 1999). Agricultural systems are dynamic as producers and consumers are constantly responding to alteration in crop and livestock yields, prices of inputs and food,

availability of resources and technological changes. Adaptations can be made at farm level by adjusting sowing and harvesting dates, crop rotation, selection of crop and variety, water for irrigation, use of fertilizers, and tillage practices (Adams et al. 1998). Each adaptive measure can lessen potential losses in yield and can ameliorate yields where climate change is beneficial.

Following measures should be taken to adapt the crops against expected climate change:

- The challenging aspect of adapting crops to expected climate change will be to maintain their genetic resistance against the biotic stress, i.e., pests and diseases. Increasing temperature and variations in humidity affects the responsiveness of agricultural pests and diseases and are likely to introduce new and unpredictable epidemiologies.
- The major expected abiotic stress to crop plants includes heat, drought, salinity, water logging, and inundation. Growth rate is accelerated due to increased temperature but at the expense of photosynthesis, while heat and drought stress may inhibit growth at metabolic level. The harvest index may be reduced if stress occurs at critical developmental stages. Genetic improvement under these circumstances can be achieved by introducing adaptive traits into cultivar of good agronomic background.
- As understanding of physiological and genetic basis of adaptation is improved, this can be expanded in conjunction with molecular approaches to tackle the most challenging aspects of climate change like adaptation to higher temperature without compromising water use efficiency and tolerance to sudden extreme events.
- Genetic manipulation to enhance the specificity of rubisco for carbon dioxide relative to oxygen and to increase the catalytic activity of rubisco in crop plants would increase yield potential.
- Introduction of C4 mechanism in C3 plants can increase yield potential even at warmer temperatures and moderate levels of water deficit.
- Selecting genetic mechanism that enhances nitrogen use efficiency thereby reducing emissions of GHGs.
- Genetic engineering techniques that allow plant roots to release inhibitory compounds to suppress nitrification.
- Practicing reduced or zero tillage in conjunction with crop residue retention can buffer crops against severe weather events.
- Improving the overall environment for the root growth will ensure the optimal expression of genetic potential of the crop plant.
- Diversification of cropping system will aid in preventing the soil-borne pests.
- Cultivation on more robust soil which are less prone to degradation.
- By adopting conservation agriculture techniques which will protect soil from evaporation, wind and water erosion, reduce water runoff, enhanced infiltration thereby reducing inundation and salinity.
- Practicing crop rotation to improve the soil texture and structure (Goldman et al. 2012).

8 Conclusions and Future Perspective

Climate change is unequivocal. Its impacts, vulnerability, and adaptation issues have drawn many scholars from the political, academic, and research sphere. The history of our planet Earth shows us with evident proofs that some time spans of hot and cold periods of climatic changes were naturally exchanging alternatively which affected the entire life on earth. In the last 5–6 decades, humans are the major factor and mainly involved in ups and downs of climatic variations. Because of exclusive usage of fossil fuels, its burning and the resulted increase in carbon dioxide release in the atmosphere generally. Also the climatic changes and variations in their prevailing current stage are actually associated with the main threat of global warming. In the last past three centuries, the average temperature of the world has increased by 0.7 °C. The rise in temperatures is supposed to increase further and thought to be crossing more than 3 °C by the end of this century (Maltais 2012).

The changes in climate have various fearful and terrible faces which would result into flooding, drought conditions, exclusively varying weather events, rise in sea level, and sprouting of new diseases with difficult to find or no cure. This serious and perilous threat may not affect or have any serious influence upon us but our generations to come, our children and grandchildren are at a predictable, eventual and evident risk to pay a high price for our mistakes of today. If preventive measures are not taken into account and done today, then the issue of climate changes will eventually become a struggle for the generations to come (Smith et al. 1999).

Future research thrust on the issue should identify and quantify the immediate and direct impacts of climate change on wealthy nations along with transmission mechanisms of impacts from poor to rich countries. Today's world is more integrated than ever before. Either negative or positive impacts in poor countries soon will be transmitted to the whole world. Transmission mechanisms: from local to national, from national to regional and then global, sector to economy wide, of course, should unambiguously be identified. A loss in agriculture production and productivity may increase population migration from poor to developed countries, which in turn would have political, social, and economic implication (Linda 2012).

The research so far on the arena is more or less concentrated on the impacts of increased temperature on output production and/or factor productivity. But, temperature is only one of the many climate variables. There are few studies on the impacts of climate change via altered precipitation amount and pattern. The economic impacts of increased frequency of extreme weather events such as hurricanes and flooding are less assessed compared to that of temperature. Earlier studies are also more of sector-wise than economy-wide impacts: on agriculture, on human health, on crop production, on livestock production, on forestry, on fishery, on water, and likes. Future studies shall concentrate on economy-wide impacts as it will increase the concern on climate change among stakeholders and will have better policy implication.

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Chapter 9
Importance of Protective Compounds in Stress Tolerance

Aykut Sağlam and Sumira Jan

1 Introduction

Environmental conditions determine plant growth and development. Optimal growth of plants is adversely affected by abiotic stresses such as drought and salt stress (Kintisch 2009). Soil salinity and drought stress result in crop loss affecting about 40 % of the arable lands across the globe (Wang et al. 2003). During last decade, increase in environmental stresses and global warming result in the necessity of developing new crop cultivars that are stress tolerant. Developing tolerant lines for salt and drought stress was more important and convenient owing to their already elaborated tolerance mechanisms reported in various plants (Gregory et al. 2005). Physiological responses to drought, cold, and salt stress are similar resulting in impaired plant growth, altered photosynthetic activity via reduction in the dark reaction of photosynthesis, accumulation of reactive oxygen species (ROS), alterations in ion transport and compartmentalization, faults in the osmotic responses of the cell (Schulze et al. 2002) and changes in metabolite profiles (Shulaev et al. 2008). Low-molecular-weight organic compounds are considered to have protective functions and are accumulated as a consequence of osmotic stress without any metabolic alterations (Bartels and Sunkar 2005). Compatible solutes include organic compounds that serve as tools for osmotic adjustment and protect membranes and proteins from denaturation which reduce impacts of drought stress on plants. They also alleviate ion toxicity resulting from salt stress and maintain ion imbalance. This chapter will focus on importance of osmoprotective compounds for the acclimation to extreme environmental conditions and their role in impeding deleterious effects of environmental stresses.

A. Sağlam (✉)
Molecular Biology and Genetics, Karadeniz Technical University, 61080 Trabzon, Turkey
e-mail: aykut_saglam@yahoo.com

S. Jan
Department of Botany, Faculty of Science, Jamia Hamdard, New Delhi 110 062, India

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2 Osmolytes

Osmolytes are considered as compatible solutes which contribute to cell turgor, protect cellular structures, and alleviate ion toxicity. These solutes mediate osmotic adjustment under drought stress by stabilizing internal potential and maintain osmotic balance (Parida and Das 2005). These protective compounds comprise mainly of amino acids (Pro), quaternary amino acid compounds (alanine betaine, glycine betaine, and proline betaine), amines (polyamines), sugars (glucose, fructose, sucrose, trehalose, raffinose, and fructans), sugar alcohols (mannitol, glycerol, and sorbitol), and sulfonium compounds (choline-*O*-sulfate, dimethylsulfoniopropionate) (Parida and Das 2005; Shulaev et al. 2008; Ahmad and Sharma 2008; Koyro et al. 2012; Dedemo et al. 2013). However, there are contradictory reports suggesting that osmolytes may have alternative protective functions. However, lower concentrations of organic osmolytes in several halophytes indicate that these compounds might not be important for osmotic adjustment (Gagneul et al. 2007). This statement is supported by transgenic tobacco which produces proline at high rates but does not make any osmotic adjustment compared to control tobacco plants, under salt and drought stress (Kishor et al. 1995). In addition, osmoprotectants may also serve in stabilization of redox balance, maintenance of proper protein folding and signaling (Rosgen 2007).

High salinity or dehydration can alter structure of proteins and modify the proteins followed by their denaturation and finally accelerated degradation. However, osmolytes can protect proteins from aggregation or degradation by preserving their native conformations, folding of proteins, and improve their thermodynamic stability so that they can function under stress conditions (Bolen and Baskakov 2001; Street et al. 2006). In addition, osmoprotective compounds have roles in the adaptation process to extreme environmental conditions (Rontein et al. 2002). For instance, high levels of sugars or polyols, quaternary amino acid compounds such as GB, alanine betaine, and proline are produced by halophyte species (Arbona et al. 2010; Lugan et al. 2010). Salt tolerance of halophytic *Limonium* species are related to accumulation of high level of quaternary ammonium compounds such as choline-O-sulfate, GB, and alanine betaine (Hanson et al. 1991). Composition and concentration of these solutes in plants can vary considerably, depending on species and type of the environmental stress (Yancey 2005; Sanchez et al. 2008; Dedemo et al. 2013). For instance, GB is dominant in *Plumbaginaceae* species adapted to dry environments, alanine betaine is apparently more typical in species growing on saline soils, and proline betaine has been detected in plants adapted to arid environments (Hanson et al. 1994; Majumder et al. 2010).

Osmoprotectants are significant for salt and drought stress tolerance in cereals (Garcia et al. 1997; Reguera et al. 2012). High levels of proline and sugar in drought and salt tolerant rice varieties suggest that these protective compounds can contribute to stress tolerance of rice (Roychoudhury et al. 2008). Similarly, glucose, fructose, sucrose, fructan, proline and quaternary ammonium compounds are accumulated in wheat under drought conditions (Bowne et al. 2012; Maevskaya and Nikolaeva 2013).

It has been shown that the accumulation is well correlated with drought tolerance of wheat (Kerepesi et al. 1998; Bajji et al. 2001). Increase in both proline and GB levels of sorghum have been recorded upon water deficit and high salt concentration (Wood et al. 1996; Chen and Murata 2011). However, an accumulation of proline in tolerant sorghum does not contribute to its drought tolerance (Premachandra et al. 1995). Like other extremophile plants, halophytic wild rice *Porteresia coarctata* Roxb. is known to synthesize and accumulate myo-inositol and pinitol for combating saline stress (Sengupta and Majumder 2009; Krasensky and Jonak 2012). Strong correlation between pinitol accumulation and drought tolerance in response to low water potential has been addressed in several tropical legume species (Ford 1984). Several classes of osmolytes such as amines, amino acids, and carbohydrates having roles in salt and drought tolerance of plants will be covenanted individually in the following part.

2.1 Amines

2.1.1 Polyamines

Polyamines (PAs) are low-molecular-weight polycations found in all living organisms and known to be essential for their growth and development. PA levels can be changed by abiotic stresses, such as drought, salinity, and cold (Ahmad et al. 2012a). In addition, a positive correlation between high PA levels and stress tolerance has been recorded (Kovacs et al. 2010; Quinet et al. 2010; Alcazar et al. 2011).

Putrescine (Put), spermidine (Spd), and spermine (Spm) are the most common PAs in higher plants (Ahmad et al. 2012a). PAs are synthesized from arginine and ornithine by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). Putrescine is formed by conversion of agmatine, synthesized from arginine. Spermidine and spermine are synthesized from putrescine by the transfer of aminoporply groups from decarboxylated *S*-adenosylmethionine (dSAM) via Spd and Spm synthases. dSAM is formed by conversion of SAM via a reaction catalyzed by SAM decarboxylase. On the other hand, diamine oxidases (DAO) and polyamine oxidases (PAO) are main PA catabolic enzymes. DOA catalyzes the oxidation of Put to 4-aminobutanal, NH_3, and H_2O_2, while PAO oxidize only higher PAs, such as Spd and Spm.

Protection of membranes and alleviation in oxidative stress are the two functions of PAs (Alcazar et al. 2011; Hussain et al. 2011; Ahmad et al. 2012a) but their functions in stress tolerance are not well understood. Positive role of PAs in stress tolerance has been shown by studies in transgenic plants and various mutant varieties. Putrescine levels of *ADC1* or *ADC2*-deficit mutants which are hypersensitive to stress are lower than wild type (Urano et al. 2004; Cuevas et al. 2008), whereas putrescine levels under drought and freezing tolerance enhance by ADC overexpression (Capell et al. 2004; Alcazar et al. 2010; Alet et al. 2011). Similarly, drought, salt, and cold tolerance of *Arabidopsis* plants increase due to enhanced spermidine content resulting from Spd synthase overexpression (Kasukabe et al. 2004).

Furthermore, tolerance of tobacco plants to salt stress and polyamine levels has been increased by introduction of ODC gene from mouse (Kumriaa and Rajam 2002). In addition, plants turn out to be very sensitive to salinity stress because of Spm synthase deficiency (Yamaguchi et al. 2006).

The application of polyamines (PAs) is also an effective approach for enhancing stress tolerance in plants (Shi et al. 2010). Exogenous application of 0.4 M Spm to soybean plants ameliorates osmotic stress effects by increasing catalase, superoxide dismutase, peroxidase, and polyphenol oxidase activities and modulating levels of plant hormones, ABA and jasmonic acid (Radhakrishnan and Lee 2013). Shu et al. (2013) have examined effects of Spm on chlorophyll fluorescence, antioxidant system, and ultrastructure of chloroplasts in *Cucumis sativus* L. under salt stress. They have found that Spm has reversed effects of salt stress on photosynthetic apparatus. In addition, application of Spm significantly increases superoxide dismutase, peroxidase, and ascorbate peroxidase activities in the chloroplasts thriving under saline conditions. Hence, salt stress in *C. sativus* plants has been mitigated by Spm application.

Exogenous spermidine (Spd) applied to tomato (*Solanum lycopersicum*) cultivars decreases growth and induces increase in free amino acids, ammonium (NH_4^+) contents, and NADH-dependent glutamate dehydrogenase (NADH-GDH) activities. They have suggested that exogenous Spd treatment alleviates disturbances in nitrogen metabolism resulted from salinity-alkalinity stress (Zhang et al. 2013).

2.1.2 Glycine Betaine

Glycine betaine (GB) is the quaternary ammonium compound and methylated derivative of glycine. Along with other quaternary ammonium compounds like -alanine betaine, proline betaine, choline-*O*-sulfate, hydroxyproline betaine, and pipecolate betaine they function as effective compatible osmolytes in halophytes (Ashraf and Harris 2004; Chen and Murata 2008, 2011). Different stress conditions such as osmotic stress (Hanson and Nelsen 1978), salinity (Hanson et al. 1991), and drought (Guo et al. 2009) can induce GB accumulation in plants. The beneficial effects of GB accumulation regarding salt and osmotic stress tolerance have been demonstrated in a number of engineered GB-accumulating plants, including tobacco (Zhang et al. 2008), tomato (Park et al. 2004, 2007), and rice (Chen and Murata 2008). These compounds confer resistance mainly by protecting photosynthetic activity through the maintenance of Rubisco activity and PSII activity (Yang et al. 2008).

Plants are usually very sensitive to environmental stress during reproduction. GB was shown to have a particularly important protective effect on reproductive organs, such as inflorescence apices and flowers during drought and cold stress (Chen and Murata 2008; Sakamoto and Murata 2000). Engineering of GB accumulation has reduced chilling damage on tomato flowers, leading to a 10–30 % increase in fruit production (Park et al. 2004). He et al. (2013) have introduced two genes, glycine sarcosine methyltransferase gene (*ApGSMT2*) and dimethylglycine methyltransferase gene (*ApDMT2*), from the bacterium *Aphanothece halophytica* to maize so that

the engineered plants synthesize more GB than control plants. Thus transgenic maize could be drought tolerant by co-expression of *ApGSMT2* and *ApDMT2*. These data confirm that GB is an osmoprotective compound, which can therefore be explored to improve tolerance to salinity and probably to drought and cold stress.

Activation and protection of the ROS detoxification system is another key component of stress tolerance (Moradi and Ismail 2007). Osmoprotective compounds can scavenge ROS directly, or contribute to the protection of the enzymes involved in the antioxidant system. Increase in antioxidant enzymes activities and alleviation of oxidative damages due to abiotic stresses have been reported in different plant species subjected to exogenous applications of GB (Nawaz and Ashraf 2010; Ahmad et al. 2013). For instance, after exogenous GB applications to *Carapa guianensis* plants, ascorbate peroxidase and catalase activities increase whereas lipid peroxidation has been prevented under water stress (Cruz et al. 2013). Foliar application of 50 mM GB to maize plants reduces adverse effects of salt stress by improving proline, Ca^{2+}, and K^{+} levels and maintaining membrane permeability (Kaya et al. 2013).

2.2 Amino Acids

2.2.1 Proline

The imino acid proline, a common denominator of many stress responses, is accumulated during diverse abiotic and biotic stresses (Kavi Kishor et al. 2005; Koca et al. 2007; Ahmad and Sharma 2008; Ahmad et al. 2012b) such as high salinity (Ben Hassine et al. 2008), drought (Choudhary et al. 2005), oxidative stress (Yang et al. 2009), and intense irradiation (Jan et al. 2012a, b). In plants, proline is synthesized from glutamate in the cytosol and probably also in the chloroplast by delta-1-pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR). P5CS produces glutamate semialdehyde, which is unstable and is immediately converted to pyrroline-5-carboxylate (P5C). P5CR reduces P5C to proline, a reaction that takes place in the cytosol and according to biochemical data also in the chloroplast (Szabados and Savoure 2010; Koyro et al. 2012).

Proline catabolism occurs in the inner-mitochondrial membrane of all eukaryotes. Proline degradation provides electrons and glutamate for mitochondrial usage. Proline dehydrogenase (ProDH), an FAD enzyme localized to the inner-mitochondrial membrane, catalyzes the first oxidizing step of proline to P5C and meanwhile delivers electrons to the mitochondrial electron transport chain (Kiyosue et al. 1996). P5C is further oxidized to glutamate or transported back to the cytosol for proline re-synthesis by the proline cycle (Deuschle et al. 2004; Miller et al. 2009). Proline accumulation during stress protects cellular structures and stabilizes enzymes owing to its antioxidant potential (Kavi Kishor et al. 2005; Mishra and Dubey 2006; Sharma and Dubey 2005). Proline also maintains redox balance, preserve energy source for the stress recovery and functions as protein precursor (Hoque et al. 2008; Islam et al. 2009; Szekely et al. 2008). In addition, proline

synthesis in the chloroplast may allow an efficient oxidation of photosynthetically produced NADPH, which is required for quenching free electrons and nascent oxygen that could otherwise lead to ROS generation (Hare and Cress 1997; Szabados and Savoure 2010).

Studies about mutants and transgenic plants have showed the protective function of proline. Hypersensitive mutant of *Arabidopsis thaliana* with *p5cs1* insertion has confirmed importance of proline in stress tolerance. Proline content of *Arabidopsis* mutant is 90 % lower than the wild type and produces more ROS and lipid peroxidation products (Szekely et al. 2008). However, proline accumulation increases salt and drought tolerance in *P5CS*-overexpressed tobacco, rice, and soybean (Kishor et al. 1995; De Ronde et al. 2004; Kumar et al. 2010). Similarly, *Swingle citrumelo* plants have been transformed with *Vigna aconitifolia P5CS* gene (*VaP5CSF129A*) that improved proline levels and lead to differential expression levels of antioxidant enzymes (de Carvalho et al. 2013). Transgenic plants exhibit improved mRNA levels of ascorbate peroxidase, superoxide dismutase, and glutathione reductase isoenzymes that produce high proline level than non-transgenic plants. de Carvalho et al. (2013) have claimed that high proline level might have a regulatory role on antioxidant enzymes.

Exogenous proline is also effective in stress liberation of plants. Leaves of wild almond (*Prunus* spp.) species exposed to H_2O_2-mediated oxidative stress displayed high levels of proline (Sorkheh et al. 2012). Improved proline levels have decreased lipid peroxidation, membrane electrolyte leakage, and endogenous H_2O_2 content by modulating antioxidant enzymes such as peroxidase, ascorbate peroxidase, and non-enzymatic antioxidant like ascorbic acid that prevented almond species from oxidative stress injury. Similarly, exogenous proline treatment has alleviated salt stress effects by inducing catalase and ascorbate peroxide activities and decreasing endogenous H_2O_2 content in salt-stressed rice plants (Nounjan and Theerakulpisut 2012).

2.2.2 GABA

Adverse environmental conditions cause rapid accumulation of the non-protein amino acid like γ-aminobutyric acid (GABA) to high levels (Kaplan and Guy 2004; Kempa et al. 2008; Renault et al. 2010). Glutamate decarboxylase (GAD) convert glutamate to GABA in the cytosol, then GABA is transported to the mitochondria. Succinate is formed by GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) and involved in the TCA cycle (Shelp et al. 1999; Fait et al. 2008). GABA is closely related with ROS scavenging and carbon–nitrogen balance (Bouche and Fromm 2004; Song et al. 2010; Liu et al. 2011). Enzymes having role in GABA metabolism are induced by salt stress (Renault et al. 2010). Adverse effects of ionic stress increase in GABA-T-deficient *Arabidopsis* mutants. Levels of amino acids (including GABA) increased, while carbohydrate levels have been decreased in these mutants (Renault et al. 2010). Expression levels of genes, which are related to sucrose and starch catabolism increase under salt stress

conditions with simultaneous loss of GABA-T function. Furthermore, compared with wild type, sugar concentration is twofold reduced in *gaba-t/pop2-1* mutant roots. Based on this information, Renault et al. (2013) provide evidence for the implication of GABA in central carbon metabolism regulation in roots under salt stress conditions.

2.3 Carbohydrates

2.3.1 Fructans

When energy demands increase and energy supplies are reduced, plants accumulate carbohydrates as storage substances. These substances are preferred to be rapidly mobilized sugars such as starch and fructans. Main storage carbohydrate of the most plant species is starch, while fructans can be accumulated by several angiosperms grown in the areas with dry periods and seasonal cold (Hendry 1993; Valluru and Van den Ende 2008). High water solubility, resistance to crystallization under freezing temperatures, and fructan synthesis at low temperatures add compensation in accumulation of fructans (Vijn and Smeekens 1999; Livingston et al. 2009). In addition, during freezing and dehydration, fructans can contribute to osmotic adjustment (Spollen and Nelson 1994; Olien and Clark 1995) and stabilize membranes (Valluru and Van den Ende 2008).

Transferring fructose from sucrose to growing fructan chain, fructosyltransferases, 1-SST, and 6-SFT synthesize fructans in vacuole (Vijn and Smeekens 1999; Livingston et al. 2009). Increased fructosyltransferases in transgenic tobacco and rice plants improve levels of fructans that enhance tolerance to drought and low-temperature stress (Pilonsmits et al. 1995; Li et al. 2007; Kawakami et al. 2008). In addition, increases in 1-fructosyltransferase (1-FFT) and fructan 1-exohydrolase (1-FEH) activity in water-stressed *Vernonia herbacea* (Vell.) Rusby plants accumulate about 80 % of fructans in the underground reserve organs, depicting the potential of fructans in maintenance of water content and drought tolerance by osmotic adjustment (Garcia et al. 2011).

2.3.2 Starch, Mono and Disaccharides

Starch, a glucose polymer, serves as a source of soluble sugars and main carbohydrate storage for most of the plants. Environmental changes easily affect starch metabolism. Starch levels are very sensitive to salt and drought stress generally. These stresses cause decrease in starch content and lead to enhancement in soluble sugars in leaves (Todaka et al. 2000; Kaplan and Guy 2004; Basu et al. 2007; Kempa et al. 2008). Under stress conditions, sugars accumulate and function as osmolytes to maintain cell turgour, protect membranes and proteins from stress injury (Madden et al. 1985; Kaplan and Guy 2004). Starch degradation is included by glucan-water

dikinase (GWD) and phosphoglucan-water dikinase (PWD), which catalyze phosphorylation of starch granules. Maltose synthesized from glucans by β-amylases is converted to glucose followed by formation of fructose and sucrose in cytosol (Tetlow et al. 2004; Kotting et al. 2010).

Starch hydrolysis in the leaves under stressed conditions may be related to β-amolytic pathway of starch hydrolysis under normal growth conditions. Decrease in the freeze tolerance of *Arabidopsis sex1* (starch excess 1) mutants, disable to show GWD activity, is an evidence for the relation between β-amolytic pathway of starch hydrolysis and stress conditions (Yano et al. 2005). In addition, during osmotic stress total β-amylase activity has increased, while it has reduced in light-stimulated starch accumulation in wild-type *Arabidopsis*. On the other hand, *Arabidopsis* β-amylase mutant *bam1* (*bmy7*) is hypersensitive to osmotic stress (Valerio et al. 2011). Similarly, a reduction in low stress tolerance of photosystem II has been shown in BMY8 (BAM3) antisense plants, which accumulate high starch levels, have not induced maltose, glucose, fructose, and sucrose accumulation (Kaplan and Guy 2005). Zeeman et al. (2004) have suggested a role of the phosphorolytic starch degradation pathway during stress. After salt and low air humidity exposures to *Arabidopsis* plants deficient in plastidial α-glucan phosphorylase, lesions formation increase in the regions surrounded by cells with high starch levels.

2.3.3 Trehalose

Some desiccation tolerant plants, for example, *Myrothamnus flabellifolius* can accumulate trehalose, the non-reducing disaccharide to high amounts (Bianchi et al. 1993; Drennan et al. 1993). Later, trehalose accumulation has been detected in numerous other plants under different stress conditions such as drought, cold, and high salinity (Pramanik and Imai 2005; Lopez et al. 2008). Stabilization of proteins and membranes can be done by trehalose, which can function as an osmoprotective compound at sufficient levels (Paul et al. 2008). However, trehalose levels of most angiosperms can be increased by abiotic stresses but to moderate level only (Rizhsky et al. 2004; Guy et al. 2008; Kempa et al. 2008).

Trehalose biosynthesis is a two-step pathway in which trehalose-6-phosphate is produced from UDP glucose and glucose-6-phosphate by trehalose phosphate synthase, which is converted to trehalose by the enzyme trehalose phosphate phosphatase (Vogel et al. 1998, 2001). Trehalose is catabolized by trehalase, which converts it to glucose (Goddijn et al. 1997; Brodmann et al. 2002). The importance of trehalose in stress responses has been demonstrated by engineering the trehalose biosynthetic pathway in transgenic plants. Trehalose level has been enhanced by overexpression of bacterial trehalose biosynthetic genes like *otsA* and *otsB* in rice which improve its salt and drought tolerance (Garg et al. 2002). Several other transgenic plants that accumulate trehalose at high levels have been produced. The idea about regulation of stress tolerance can be done by inducing trehalose metabolism and has been proven via studies on genetically modified plants (Ge et al. 2008; Stiller et al. 2008). On the other hand, in another study, modification of trehalase

which is responsible for conversion of trehalose to glucose has showed that trehalase plays a role in the regulation of stomatal closure in plants under drought stress. During water-deficit stress, *AtTRE1* overexpression in *A. thaliana* plants that have low level of trehalose exhibits better resistance to water deficit than *Attre1* mutants that has elevated trehalose contents. High sensitivity of *AtTRE1* stomata to ABA maintains leaf water content by closing more stomata than the mutants (Van Houtte et al. 2013). Exogenous applications of trehalose provide plants with improved tolerance to drought and salt stresses. Trehalose treatments cause increases in transcription of antioxidant enzyme genes such as superoxide dismutase, ascorbate peroxidase, peroxidase, and catalase in salt-stressed rice plants. Trehalose-treated plants recover immediately compared to non-treated plants (Nounjan et al. 2012).

Trehalose is suggested to function as chemical chaperon and has been shown to stabilize membranes and protect proteins in tissues under drought stress (Crowe et al. 1984; Crowe 2007). Trehalose can act as a signal molecule below 1 mg/g fresh weight instead of being a compatible solute (Garg et al. 2002). Therefore, the signaling function of trehalose and trehalose-6P could be more important than the previously suggested chaperone or osmolyte function, although in some tissues such a protective role cannot be excluded (Fernandez et al. 2010).

2.3.4 Polyols

One other class of osmoprotective compounds is polyols or sugar alcohols, which are chemically, reduced forms of aldose or ketose sugars. Water-like hydroxyl groups of polyols forming a sphere of hydration around macromolecules allow them to act as osmoprotectants under low osmotic potential. Polyols have functions as molecular chaperons stabilizing macromolecules. They also prevent membranes and enzymes from oxidative damage by scavenging ROS (Smirnoff and Cumbes 1989; Shen et al. 1997). Compared to sorbitol and galactitol, mannitol is the most common sugar alcohol and is an important photosynthetic product in a number of plant species (Loescher et al. 1992; Rumpho et al. 1983). In some plant species, there has been a correlation between stress tolerance and accumulation of mannitol and sorbitol (Stoop et al. 1996). Increase in tolerance to salinity or water deficit has been observed in *Arabidopsis*, tobacco, poplar, and wheat, which have been introduced mannitol-1-phosphate dehydrogenase (*mtlD*) from *E. coli*, that converts fructose-6-phosphate to mannitol-1-phosphate (Abebe et al. 2003; Chen et al. 2005; Sengupta et al. 2008). Similarly, targeted expression of *mt1D* in tobacco chloroplasts causes an increase in cytoplasmic mannitol concentration in transgenic tobacco plants, this increase, in turn, results in resistance to methyl viologen-induced oxidative stress (Shen et al. 1997). Overexpression of celery M6PR is an alternative way to enhance mannitol biosynthesis and has been shown to be an efficient way to improve salt tolerance of *Arabidopsis* (Zhifang and Loescher 2003). In a halophyte *Prosopis strombulifera*, leaf mannitol content increases during NaCl stress whereas sorbitol content increases after Na_2SO_4 treatment. According to increase in mannitol content during NaCl stress, it has been concluded that

P. strombulifera prefer mannitol for osmotic adjustment, however, sorbitol synthesis during Na_2SO_4 might be related to problems in carbon metabolism due to toxicity of sulfate (Llanes et al. 2013).

Myo-inositol is an essential polyalcohol in plants and eukaryotes for being an important precursor of some lipid signaling molecules and it has potential role in signaling during stress, cell wall biosynthesis, cell death, and plant hormone synthesis. Biosynthesis of myo-inositol starts from D-glucose-6P, which is converted to myo-inositol-1P by myo-inositol-1P synthase (MIPS) (Johnson and Sussex 1995; Majumder et al. 1997). Myo-inositol is produced from myo-inositol-1P by dephosphorylation and is used for the subsequent biosynthesis of all inositol-containing compounds, including phospholipids. MIPS genes were shown to be salt-induced, leading to accumulation of myo-inositol in the halophyte ice plant, but not in the glycophyte *Arabidopsis* (Ishitani et al. 1996). MIPS genes can be regulated by several environmental stress factors such as drought, heat and cold stress, high light and controlled by ABA signals (Yoshida et al. 1999, 2002; Abreu and Aragao 2007; Wei et al. 2010a, b). Phosphorylated derivatives of myo-inositol are important signaling compounds in responses to biotic and abiotic stresses which are involved in numerous regulatory pathway and control diverse aspects of plant development (Nelson et al. 1999). Improved tolerance to salt stress during germination, seedling growth and development has been observed in *Arabidopsis thaliana* that overexpress myo-inositol 1-phosphate synthase gene (*SaINO1*) in halophytic grass, *Spartina alterniflora* (Joshi et al. 2013).

As an important osmoprotectant, a six-carbon alcohol sorbitol is the most preferable accumulated carbon source in some fruit trees of *Rosaceae* family (Tari et al. 2010; Feng et al. 2011; Li et al. 2012). Sorbitol confers tolerance against abiotic and biotic stresses by participating in osmotic adjustment during stress. Sorbitol is synthetized from hexose phosphates like sucrose. Sorbitol-6-phosphate dehydrogenase (S6PDH) is a regulatory enzyme in sorbitol biosynthesis, which catalyzes conversion of glucose-6-phophate to sorbitol-6-phosphate then in turn, sorbitol-6-phosphate is dephosphorylated to form sorbitol by sorbitol-6-phosphate phosphatase (Kanamaru et al. 2004; Liang et al. 2012).

Sorbitol transporter genes are induced by subjecting plants to stress so that plants can accumulate sorbitol. Sour berry, apple, and *Arabidopsis* plants have been scanned for the transporter genes and the genes have been identified in these plants (Gao et al. 2005; Fan et al. 2009). Differential regulation of sugar regulators is maintained through sugar transporters induced in response to varied abiotic and biotic stress (Wormit et al. 2006). Sorbitol accumulation in salt-stressed *Plantago major* has been detected by Pommerrenig et al. (2007). In addition, up-regulation of three sorbitol transporters in apple plants has improved drought tolerance in vegetative tissues with subsequent increment in sorbitol concentration as confirmed by HPLC analysis of leaves, roots, and phloem tissues (Li et al. 2012).

Pinitol is a methylated inositol, which is synthetized from myo-inositol by inositol-o-methyltransferase (IMT1) and ononitolepimerase (OEP1) (Bohnert et al. 1995; Rammesmayer et al. 1995; Sengupta et al. 2008). Pinitol increase has been correlated with improved tolerance of some plants subjected to drought or heat stress.

Increase in the drought resistance of pine seedlings that accumulate pinitol has been determined. The cultivars that acquire higher pinitol content are resistant to drought stress than the low pinitol-producing cultivars (N'Guyen and Lamant 1988). Many studies confirm sucrose as the well-known low-molecular-weight carbohydrate that is accumulated in soybean plants under stress (Yamada and Fukutoku 1985; Fellows et al. 1987). Ford (1984) has reported inadequate increase in sucrose contents and significant accumulation of pinitol in soybean plants under water-stressed conditions. Pinitol accumulation is a characteristic feature of a number of halophytic plants in saline environment and occurs in several glycophytic plants grown under osmotic stress conditions (N'Guyen and Lamant 1988; Gorham et al. 1981; Popp 1984; Paul and Cockburn 1989; Sengupta et al. 2008). Unlike native rice cultivars, pinitol hyperaccumulation has been found in *Porteresia coarctata*, a halophytic wild relative of rice. This pinitol accumulation is controlled by inositol methyl transferase 1 (*PcIMT1*) gene, an essential metabolic response for salt stress (Sengupta et al. 2008). Increased salt tolerance was shown in transgenic tobacco displaying *P. coarctata*, *MIPS* overexpression, and *M. crystallinum* IMT1 gene insertion. These transgenic tobacco plants accumulate more inositol and pinitol that confer improved growth, higher photosynthetic activity, and lower oxidative damage during salt stress (Patra et al. 2010).

3 Conclusions and Future Perspective

Plants being sessile are subjected to diverse environmental stresses that impede their growth and development. Therefore, metabolic adjustment to cope with environmental stress conditions is important considerably for plants. However, this adjustment is brought at different levels in diverse ways making tolerance mechanism even more complex. As each organism, even its varieties exhibit assorted response to array of external stimuli. For instance, changes in cellular metabolism during development and acclimation under adverse conditions are closely related to the developmental stage of a plant. Therefore, there is great necessity to study state of vulnerability at particular developmental stage and metabolic adjustment in stress conditions.

Osmoprotective compounds like sugars and proline could function as metabolic signals and therefore have broader influence on physiological responses and metabolic adjustment to stress conditions. Despite there are many studies about signaling networks, however there is paucity regarding reports about how a metabolic response is induced by an environmental change and what are the roles of osmolytes in stress signaling. Engineering of crop plants via genetic transformation is a promising tool to study the significance of osmoprotective compounds in stress responses and to improve the performance of crop plants under suboptimal conditions. Enhanced accumulation of a metabolite can be achieved via activation of the biosynthetic pathway or inhibition of the catabolic pathway. Furthermore, novel pathways can be established in plants, by introducing genes from other species. Combining advanced

approaches like genomics, proteomics, and metabolomics could increase our understanding of plant stress responses on a global scale and will put forth metabolic bases of adaptation to drought, salinity, or extreme temperatures.

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Chapter 10
Growth Patterns of Tomato Plants Subjected to Two Non-conventional Abiotic Stresses: UV-C Irradiations and Electric Fields

Adriano Sofo, Donato Castronuovo, Stella Lovelli, Giuseppe Tataranni, and Antonio Scopa

1 Introduction

Capabilities for environmental stress perception, signaling, and response of plant species against a broad range of abiotic stressors have a great range of variability.

Ultraviolet (UV) radiation is a component of the solar light and it represents something like 8–9 % of the radiation that naturally reaches the Earth (Frederick 1993). Depending on its wavelength, UV can be divided into three different ranges: UV-A (315–390 nm), UV-B (280–315 nm), and UV-C (100–280 nm). Among them, UV-A represents approximately 6.3 % of the incoming solar radiation and is the least hazardous part of UV radiation; UV-B, even if represents just 1.5 % of the total spectrum, is of particular interest because it can cause a multiplicity of detrimental effects in plants (Hollósy 2002; Jansen and Bornman 2012). Between UV radiations, UV-C is the one with the lower wavelength, or rather with the higher associated energy (Katerova et al. 2009; Nawkar et al. 2013), and it is well known that UV-C has an acute germicidal action on microorganisms in water, on surfaces, and in air (Siddiqui et al. 2011). Indeed, it can induce oxidative results and genetic mutations in plants that in turn have strong negative effects on plant morphology, flowering, pollination, transpiration, and photosynthesis (Murali and Saxe 1984; Booij-James et al. 2000).

The stratospheric ozone layer efficiently filters out most of the detrimental UV radiation shorter than 280 nm but it decreases rapidly at wavelength longer of 280 nm reaching zero at about 330 nm (Hollósy 2002; Nawkar et al. 2013). Therefore, UV-B is not completely shielded by the ozone layer and the UV-A are virtually unaffected by the ozone layer. Fortunately, UV-C is strongly affected by the ozone layer in the stratosphere, so that the amount of this radiation reaching the Earth's surface, except for high mountains, is extremely low (Häder et al. 2007).

A. Sofo (✉) • D. Castronuovo • S. Lovelli • G. Tataranni • A. Scopa
School of Agricultural, Forestry, Food and Environmental Sciences,
University of Basilicata, Viale dell'Ateneo Lucano 10, Potenza, 85100, Italy
e-mail: adriano.sofo@unibas.it

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Nevertheless, in the last decades, human activities have produced dangerous chemicals, such as chlorofluorocarbons (CFC), which have been released into the atmosphere and have contributed to the depletion of ozone protective layer. Therefore, in the future UV-C radiation could increase as the result of stratospheric ozone depletion due to atmospheric pollution. Indeed, the stratospheric ozone layer reduction highlights the ecological implication of increasing UV-B and UV-C radiations on natural ecosystems and on agricultural productions (Jansen and Bornman 2012; Kataria and Guruprasad 2012). This increasingly worsen condition has led researchers to understand the effects of UV radiation on plants and other organisms. In this view, the primary alarm over ozone depletion is the potential impact on human health and ecosystems due to increased UV exposure. This enhanced exposure, especially to UV-C and UV-B, is potentially detrimental to all living beings. In particular, it can be very harmful to plants due to their obligatory requirement for sunlight for survival and their inability to move. It is known that increased UV exposure has been shown to alter the biotic relationships of higher plants, as demonstrated by the changes in plant disease susceptibility and the balance of competition between plant species. The most frequent UV exposure effects on plants are a reduced growth (plant height, dry weight, leaf area, etc.), photosynthetic activity, and flowering (Teramura et al. 1991; Santos et al. 2004; Jansen and Bornman 2012).

In this scenario, characterized by an increasing trend of UV-C flux on Earth, the study of the effect of this radiation on some crops becomes important. There are differences between species as regards to UV radiation sensitivity (Teramura 1983) but actually there is very few information on the effects of UV-C on tomato plants, which is instead considered an important crop in the Mediterranean environments (Albacete et al. 2008).

UV-C irradiation on tomato fruit is studied as postharvest treatment for its effects to delay fungal growth or senescence (Liu et al. 2009), to increase ascorbic acid and total phenolic compounds (Jagadeesh et al. 2009), and to increase lycopene content in tomato fruit (Liu et al. 2009). Tomato fruits exposed to a low level of UV-C (3.7 kJ m^{-2}) showed a delay in fruit ripening and senescence, and an increase of photooxidant products, to which plants react by improving their defense antioxidant mechanisms (Ait Barka 2001; Liu et al. 2009). While the impact of UV-C treatment on tomato photosynthetic characteristics was not studied extensively, in other crops, such as wheat (Li et al. 2007) and pea (Li et al. 2006), negative effects of UV-C radiation on gas exchange were observed. Particularly, UV-C-treated pea seedlings showed a reduced activity of the antioxidant enzymes and an increase of membrane peroxidation, resulting in a lower assimilation activity (Li et al. 2006).

The application of electricity can stimulate the growth of plants to a great extent (Wolverton et al. 2000). This little-known technology, called electro-culture, can accelerate growth rates, increase yields, improve crop quality and plant protection against diseases, insects and frost (Ishikawa and Evans 1990). Electro-culture can also reduce the requirements for fertilizers or pesticides (van West et al. 2002; Wang and Wang 2004). The several approaches to electro-culture include: antennas, static electricity, direct and alternating current, magnetism, radio frequencies,

monochrome and intermittent lighting, and sound. The electricity can be applied to the seeds, plants, soil, water, or nutrients. Particularly, the application of an electric field (EF) can affect directly or indirectly the plants exposed to it, inducing a series of physiological and biochemical responses (Scopa et al. 2009; Berghoefer et al. 2012; Vallverdú-Queralt et al. 2013).

Electric fields (EFs) have been tested in several instances with contradictory results, depending on the strength applied, the substrate in which roots grow, and the plant sensitivity. Several experiments of plants subjected to different types and intensities of EFs have been carried out in liquid media (Wolverton et al. 2000), hydroponic conditions or artificial soil (Nechitailo and Gordeev 2004). Scopa et al. (2009) observed that *Arundo donax* seedlings, exposed to a DC EF of 12.0 V m^{-1} showed a significant increase in growing rate of both shoots and roots. The root meristem architecture (Wawrecki and Zagórska-Marek 2007), as well as the development of lateral roots (Hamada et al. 1992), was proved to be affected by EFs. An EF seems to induce changes in cell membrane potential of the root, although the exact nature of these changes is difficult to predict (Ishikawa and Evans 1990; Berghoefer et al. 2012). Chemiosmotic gradient or/and auxin could play a role in the ultimate establishment of the differential growth pattern that various papers underline (Robinson 1985).

On these basis, this chapter is focused on (a) the possible implications of UV-C irradiation on tomato, one of the most economically important crops of the Mediterranean Area, in order to deepen the ecophysiological response of this species to a changing climate; and (b) the estimation of the effect of a DC EF on developing roots of tomato plants grown in a hydroponic floating system under controlled conditions, in consideration of possible applicative outcomes in plant propagation and cultivation of this important cultivated species.

2 Instrumental Equipment for the Study of the Effects of UV-C and EF in Tomato

2.1 UV-C

In order to assess the effect of UV-C irradiation on tomato plants (*Lycopersicon esculentum* Mill.), the experiments are usually conducted in controlled conditions, using irradiation chambers (0.82 × 0.52 × 0.68 m) coated with aluminum sheets and equipped with an UV-C lamp.

Three seeds are sowed in polypropylene plastic pots filled with a substrate containing an inorganic mineral base of perlite. After few days from germination, seedlings are removed and only the best ones are kept alive to undergone UV-C irradiation. For the whole experiment, except for the irradiation times, plants should be maintained under controlled conditions. On the basis of UV-C irradiation times, plants are divided into different groups.

To analyze the possible photosynthetic activity changes induced by exposure to UV-C, instantaneous gas exchange measurements are carried out on plants before and after the UV-C treatments by a portable open-gas exchange system on the topmost fully expanded leaf. In order to estimate plant color change caused by UV-C radiation, colorimetric leaves measurements are carried out before and after the UV-C treatments using a colorimeter. According to Sugar and Dussi (1998), color changes is evaluated in the CIELAB space system, measuring the color chromatic coordinates L^*, a^*, and b^*.

Inter-knot distance, plant height, and shoot diameters are measured some weeks after the UV-C treatment. Subsequently, the root system of each plant are cleaned and kept in an isotonic water solution to avoid drying. The fresh roots are mounted on slides and observed at different magnifications using a compound optical microscope under transmitted light, and then photographed. Images are analyzed to compare root morphology and evaluate descriptive parameters.

2.2 Electric Field

To assess the effect of an electric field on tomato plants (*Lycopersicon esculentum* Mill.), seeds are firstly sterilized in a solution of 5 % (v/v) NaOCl, rinsed with 95 % ethanol, washed with distilled water, and then put in an inorganic mineral base of sterile sand as solid substrate. The experiments are usually realized in a floating polystyrene vessel with 96 holes. Plants are grown hydroponically in a nutritive liquid medium. After few days from germination, some seedlings are removed and only the best ones are kept alive. Seedlings should be maintained under controlled conditions. The photosynthetic light source is usually a specific fluorescent lamp. Solution volumes are maintained constant throughout the experiment.

Two parallel stainless steel plates are placed in the medium, and they work as electrodes. After some days from the germination, when root length ranged from 1 to 2 cm, seedlings are exposed to a DC EF of 12.0 V m^{-1} with a current intensity of 10 mA, according to Scopa et al. (2009). The EF is applied continuously directly by a 50 Hz voltage set-up transformer, and monitored by a digital multimeter. Plants not subjected to the EF, grown under the same conditions reported above in another identical polystyrene vessel, are kept as controls.

After 4–10 weeks from EF application, tomato plants are randomly selected next to the positive electrode, in the central area, and next to the negative electrode. For each position, plants are taken from different holes of the polystyrene vessel. The root system of each plant is cleaned and kept in an isotonic water solution to avoid drying. The fresh roots are mounted on slides and observed at different magnifications using a compound optical microscope under transmitted light and then photographed. Images are analyzed to compare root morphology and evaluate descriptive parameters. Root apical meristems and root branching per plant are evaluated. Root mean diameter is also measured at 0.02 cm from the tip. The root/shoot ratio and the length per unit root mass (LRM) are calculated.

3 Growth Patterns and Physiological Effects of UV-C and EF in Tomato

3.1 UV-C

The increase of the exposition time to UV-C radiation causes a photo-inhibition of the assimilation activity that could be attributed to phytohormone changes, inhibition of essential enzymatic reactions, and decrease in the uptake and partitioning of nutrients (Teramura and Sullivan 1994). Najeeb et al. (2011) demonstrated that the decrease in photosynthetic performance after UV-C irradiation could be due to the reduction of cell and chloroplast size, accompanied by the disruption of thylakoids and the accumulation of plastoglobuli in chloroplasts. Net assimilation (*A*) deeply decreases in tomato plants exposed to UV-C for 60 and 120 min with respect to the control (Fig. 10.1a).

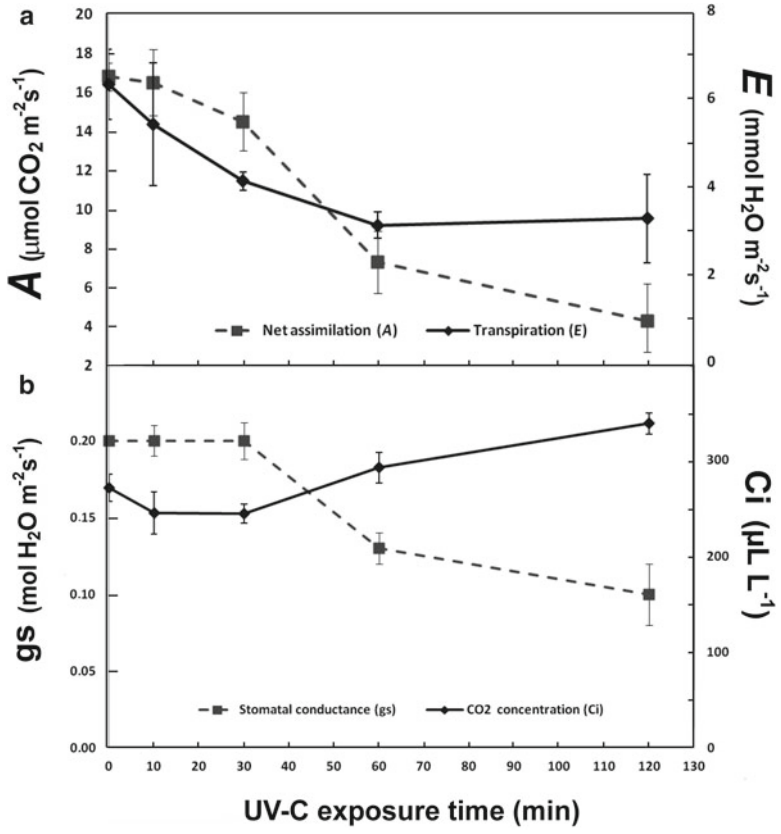

Fig. 10.1 (**a**) Trends of net assimilation (*A*) and transpiration (*E*) in leaves of tomato plants exposed to UV-C radiation for 0, 10, 30, and 60 min, measured 2 h after the UV-C treatment. (**b**) Trends of net assimilation (*A*) and transpiration (*E*) in leaves of tomato plants exposed to UV-C radiation for 0, 10, 30, and 60 min, measured 2 h after the UV-C treatment. Data represents means ($n = 8$) ± standard error

Significant decreases in transpiration (*E*) and stomatal conductance (gs) are also observed (Fig. 10.1). Tomato photosynthetic apparatus is affected by UV-C treatment, as demonstrated by the strong increase in intracellular CO_2 (Ci) up to 338 μL L^{-1}, particularly evident in the 120-min treatment. This is likely due to both the stomatal (gs) and non-stomatal inhibition (*A*) of the assimilation activity (Fig. 10.1). The strong effect of UV-C on photosynthesis reduces the assimilate availability, necessary for plant growth. Indeed, several authors demonstrated that UV-C provokes reduction of carbohydrate content by inactivation of the Rubisco activity in Calvin cycle (Rahimzadeh et al. 2011).

Biometrical measurements, done 4 weeks after the UV-C exposure, point out that a decrease of inter-knot distance occurs in treated plants, in accordance with Bertram and Lercari (1996) and Lercari et al. (2003). The same trend is recorded for plants height and stem diameter. Similar results were found by Najeeb et al. (2011) in UV-C-irradiated *Juncus effusus* plants that showed a significant reduction in plant growth and biomass. As previous authors reported (Hosseini Sarghein et al. 2011), no changes are observed for root morpho-anatomy after UV-C treatments, if compared to control plants, excluding a direct action of UV-C on the hypogeal part of the plants.

Colorimetric characterization demonstrates that, after few hours from the irradiation, the leaves of UV-C-treated plants are characterized by a general color change, while no differences in color among plant groups are detected before the UV-C treatments. In the CIE Lab color space, all the treated plants show similar values of brightness (*L** parameters). Regarding *a** (green–red axis) and *b** (blue–yellow axis) parameters, the values of 10-, 30-, and 60-min UV-C treatments do not differ statistically, but both are statistically lower in the 120-min treatment. Besides, the untreated tomato plants reach the highest values. The same trend is observed for leaf chroma and Hue angle parameters. This colorimetric response was also found by Rozema et al. (1997), who observed a reduction in pigment levels due to increasing exposition time to UV-C radiation.

3.2 Electric Field

Tomato root morphology is strongly affected by the applied EF (Fig. 10.2). Indeed, a significant variation in shoot and root growth rate is observed among the groups of plants grown close to the positive/negative electrode or in the central part of the container (Fig. 10.2). Both the root/shoot ratios of dry weights (R/S) and the LRM of the plants sampled next to the positive electrode show the highest values, if compared to the negative ones. The average length of the main root of the plants ranges from 6 cm next to the negative electrode, to 12 cm in the central part of the container, to 15 cm next to the positive electrode (Fig. 10.2).

The tomato plants grown close to the positive electrode, compared to the plants in the central area and toward the negative one of the container, show pronounced root branching and hair development, and higher root density and length. Under EF

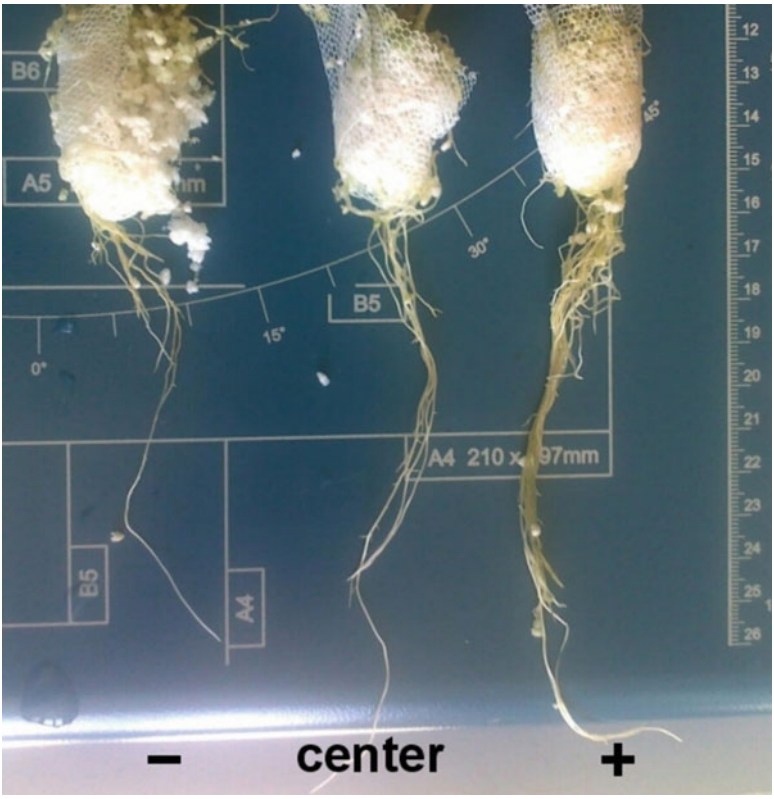

Fig. 10.2 Root morphology of tomato seedlings grown hydroponically under an electric field. Positions: (*left*; –) negative electrode, (*center*) central area, (*right*; +) positive electrode. Scale bars in cm

exposure, root branching increases from 0.3 branches cm^{-1} at the negative electrode to 2.5 branches cm^{-1} at the positive one (Fig. 10.3a). As far as branching is concerned, apical meristems per cm reach the highest counting next to the positive electrode (Fig. 10.3b). The average root diameter of treated plants is less affected by the opposite poles, and it significantly increases in the central area (Fig. 10.3c).

The different growth patterns observed could be related to the different mineral gradients formed by migration of cations and anions in the water solution under the applied EF of 12.0 V m^{-1}. Indeed, chemiosmotic modifications of ion transport, an equivalent of salt influx or salt efflux driven at the expense of an equivalent of electrogenic proton efflux, could occur. The increased ion accumulation seems not to be merely a passive movement under the applied potential, and it is thought that small currents could stimulate active ion pumps or alter the internal distribution of growth-regulating compounds (Black et al. 1971; Robinson 1985). In support of this hypothesis, root morphology, R/S and LRM, and microscopic parameters of all the control plants taken from different vessel position resembles the corresponding

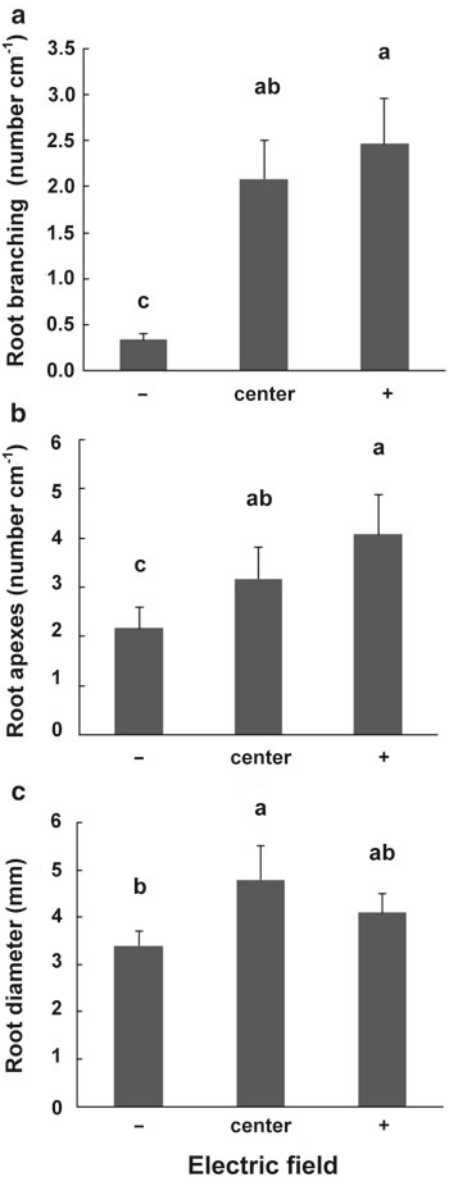

Fig. 10.3 (**a**) Total root branching, normalized to root main axis length (mean per cm ± 20 % error), (**b**) total root apex number, normalized to root main axis length (mean per cm ± 20 % error), and (**c**) mean root diameter at 0.02 cm from the tip (±standard deviation) of tomato seedlings grown hydroponically under an electric field. Positions as in Fig. 10.2. Means ($n = 10$) with different same letters on the columns are significantly different ($P \leq 0.01$) among the positions (−, *center*, +) of both the groups of plants

parameters of the plants under the EF and taken in the central area of the container, suggesting that ion distribution in the container without EF is uniform, as the EF did not cause ion migration nor physiological changes in the membranes of root cells.

An interesting observation could be related to another hypothesis: the root orientation in the growing medium. In fact, the "root direction" of tomato plants is always well defined in the growing solution, as root-growing direction is usually oriented

toward the positive electrode. This curvature is not present in the control plants. Electrotropic curvature in solutions of low electrolyte concentration was already studied using primary roots of maize (Ishikawa and Evans 1990). When submerged in oxygenated solution across which an EF was applied, the roots curved rapidly and strongly toward the positive electrode (Ishikawa and Evans 1990). These responses are controlled by auxin and auxin transport inhibitors (Goldsworthy and Rathore 1985; Ishikawa and Evans 1990). Therefore, electrotropic curvature is probably due to a particular orientation and distribution of membrane proteins or to a different phytohormonal balance under the EF. In this regard, Brown and Loew (1994) determined that EF-directed locomotion caused the lateral redistribution of plasma membrane glycoproteins in fibroblast cells grown in vitro.

4 Conclusion and Future Perspective

An exposition of tomato plants to enhanced levels of UV-C radiation and DC-EF can determine the important and significant alterations in their growth. High UV-C doses (60 and 120 min) determine irreversible damages both at plant physiological and morphological levels, in particular against leaves and shoots, leading the whole plant to death. By contrary, lower irradiations (up to 30 min) allow plants to partially maintain their normal physiological status. Physiological and structural alterations are evident in shoots of tomato UV-treated plants that also exhibit a significant color change, probably due to the photo-oxidation of chlorophylls and other pigments, and a reduced growth (Fig. 10.4a). On the other side, the application of DC-EF in tomato causes significant differences in root development, showing a

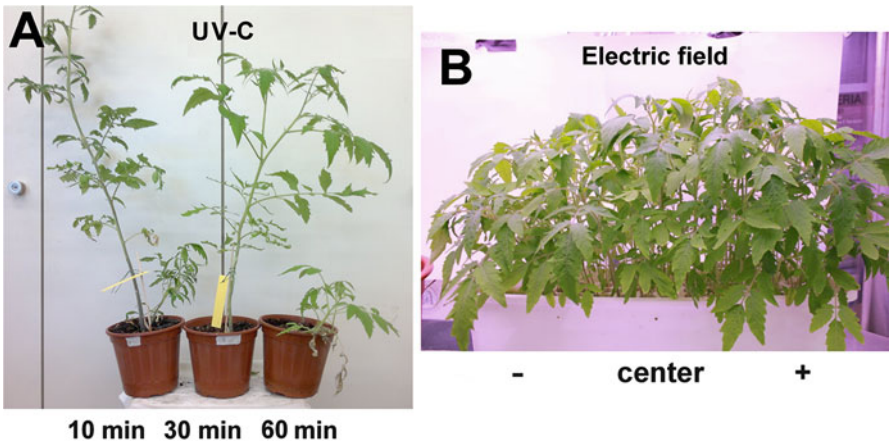

Fig. 10.4 Growth patterns of tomato plants subjected to UV-C radiation (3.8 J m^{-2} at 1 m of distance) and a DC 12.0 V m^{-1} electric field. UV-C exposition times and electric field polarity are indicated in the figure

typical gradient with high developed plants toward the positive electrode (Fig. 10.4b). This plant growth response could be useful in plant nursery techniques. Indeed, a better quality of tomato plants could promote a faster in vitro growth and reproduction of micro-propagated plants, increasing also their survival during the following acclimation phase. Next experimentation should investigate the mechanisms by which the application of a DC-EF, varying in current intensity and voltage, cause the morphological effects in tomato.

The future perspectives foresee physiological, genetic, and molecular investigations and studies on the possible tolerance mechanisms of tomato plants to face UV-C radiation. Furthermore, a better knowledge of electro-culture could make possible the practical use of this unusual abiotic stress in plant propagation and cultivation. The protection of tomato plants against UV-C, combined with the growth-promoting effects of electro-culture, could allow farmers to grow bigger and better crops in less time, with less effort, and at a lower cost.

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Chapter 11
Rhizobacteria: Restoration of Heavy Metal-Contaminated Soils

Seifeddine Ben Tekaya, Sherlyn Tipayno, Kiyoon Kim, Parthiban Subramanian, and Tongmin Sa

1 Introduction

Human use of heavy metals is historically known and dates back to hundreds of years ago. During the era of the Roman Empire, lead acetate was used to sweeten old wine. It was estimated that some Romans may have consumed more than 1 g of lead a day. Lead was used for more than 5,000 years in many aspects like building materials for infrastructures, pipes for transporting water, and as component of pigments for glazing materials. Mercury also was used by Romans to ease toothache and as remedy for syphilis (Jarup 2003). Recently, it has been estimated that around 30 % of earth's lands are contaminated because of the myriad of anthropogenic activities, associated with economic progress, especially in developing countries. Since the industrial revolution, biosphere contamination with toxic metals has increased dramatically classifying the hazards of metal pollution as one of the most important concerns these days. Metals are known, at the same time, to be integral components of microbial life processes. Many were identified as essential nutrients and are required for microbial metabolism, suggesting an evolutionary relationship between microbes and metals. Others are also considered toxic, with no known beneficial roles for microbes. Essential metals play roles as catalysts of biochemical reactions, as components of protein structures, as bacterial cell wall stabilizers, and for maintaining osmotic balance. Nonessential metals are toxic to microorganisms even in small quantities, which are sufficient enough to influence microbial population in the soil. Such conditions may however, favor the natural selection of resistant microorganisms. Such resistance may be the result of intrinsic mechanisms under the influence of certain environmental agents.

S.B. Tekaya • S. Tipayno • K. Kim • P. Subramanian • T. Sa (✉)
Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea
e-mail: tomsa@chungbuk.ac.kr

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Accumulation of high soil metal concentration, regardless of whether they are essential or not, leads to severe damages to soil fertility and biological structure. One peculiar feature of heavy metals is that they cannot be fully destroyed but only transformed from one oxidation state to another. Conventional methods such as thermal treatment, landfill, and electroreclamation are not effective because of their destructive effects to environment, high cost, and low efficiency. Such techniques are nonetheless widely employed in developed countries. It has therefore become a necessity to exploit new suitable methods for cleaning up soils. Microorganisms and microbial processes have attracted wide interest as tools for bioremediation because of the panoply of mechanisms they are able to adopt to cope with metal toxicity in the soil. This is in addition to the advantage of being cost-effective.

Among soil bacteria, rhizobacteria attract more and more attention because of their symbiotic contribution to their host plants. These bacteria are able to colonize plant roots and can form symbiotic associations with their host or be free living. Their contribution to the host plant are in the form of a number of synthesized compounds that help in the uptake of nutrients as well as biocontrol agents against phytopathogens (Zhuang et al. 2007; Martins et al. 2013). Many plants have been exploited for their abilities to adsorb metals and thus, offer one more potential application in bioremediation. In this context, plant association with rhizospheric metal-resistant bacteria is crucial for optimal functioning. Rhizospheric bacteria are studied for their role in mobilizing metals and making them available for their hosts, or simply alleviating the metal stress imposed on the host. Plants and their symbiotic microbes are characterized by complex interactions, which will be covered in this chapter. It is also known that because of the complex soil properties and the chemical instability of the metals, their total cleanup is still a subject of concern, especially with the limitations of biological cleaning agents. Besides this, plants have always shown limited access for soil metals and even essential nutrients. The tolerance level of bacterial symbionts to the concentration of surrounding metals is often limited. Moreover, after metal uptake, eventual bacterial lysis can result to the release back of accumulated metals into the soil.

To overcome these limitations, scientists have started exploiting the genomic arsenal of both eukaryotes and prokaryotes to engineer organisms with optimized biological efficiency for soil remediation. Genetic manipulation was done to increase the ability of soil microbe to bind metals, through use of proteins such as metallothionein, phytochelatin, and other similar molecules, targeted for their high affinity to immobilize metals (Zhuang et al. 2007). On the other hand, exploiting specific taxonomic groups, and expressing higher competence among diverse rhizospheric bacterial populations can also be promising. It is known that actinobacteria are the main microbial sources of active metabolites. Doumbou et al. (2011) reported their superiority in agroactive compound production, as well as their plant growth-promoting activities. Furthermore, it has been shown that in metal-contaminated lands, they are particularly resistant and make up one of the most persistent taxa. Studies on their metal resistance mechanisms are emerging. In this chapter, we give an overview of their metal resistance potential as well as their ability to promote plant growth.

2 Metals and Metalloids

The definition of heavy metals has always been associated with contamination and toxicity to humans and the environment, thus, encompassing heavy metals and metalloids known also as semi-metals (Beolchini et al. 2013). The most commonly used definition of heavy metals is based on the density of their elemental form, generally above 7 g mL^{-1}. Many authors suggested definitions based on physicochemical concepts, which however, cannot make logical sense since there is no connection between those physicochemical properties and density, toxicity, or ecotoxicity (Colin et al. 2012). Metalloids such as selenium and arsenic have often been also included in the group of heavy metals because of their toxicity. They do have physical properties similar to metals, but different chemical behavior, closer to nonmetals (Duffus 2002).

In terms of their functions in living organisms, heavy metals may be divided into two groups. Some are considered trace elements because they are essential in low concentrations for the maintenance of important biological processes. These are often components of enzymes and as cofactors. At higher doses, they become toxic, often results in the production of radical oxygen species. Examples of trace elements are iron, zinc, copper, manganese, cobalt, nickel, and chromium. Metals like cadmium, mercury, and lead are classified as nonessential or with yet no known beneficial function, and are therefore toxic even at low concentrations (Valls and Lorenzo 2002). According to Jarup (2003), arsenic, mercury, lead, and cadmium present the most important threat to humans.

2.1 Arsenic

Arsenic is considered as metalloid and occurs in rocks, soil, water, and air. Smelter activities of nonferrous metals and use of fossil fuels for energy production are the most common anthropogenic causes of arsenic release into environment, especially soil (Stolz et al. 2006). In natural conditions, arsenic enters into terrestrial and aquatic ecosystems via many possible natural processes such as volcanic emissions, biological activities, and weathering reactions (Purakayastha 2011). Arsenic has four oxidation states: arsenate [As(V)], arsenite [As(III)], elemental As(0), and arsenide [As(III^{-})]. Arsenate and arsenite, in their inorganic form, are soluble with $H_2AsO_4^-$ and $HAsO_4^{2-}$ in aerobic conditions and $H_3AsO_3^0$ and $H_2AsO_4^-$ in anaerobic environments (Stolz et al. 2006). Stolz et al. (2010) described the alien-like features of certain microorganisms take up arsenic for essential life processes. This was also in conjunction with findings that described the use of arsenic instead of phosphate by certain anaerobic bacteria for metabolic processes (Wolfe-Simon et al. 2011). Arsenic is mainly toxic because it interferes with metabolic processes involving the major bioelement, phosphorus due to their closely analogous structures (Nies 1999).

2.2 Mercury

It was reported that mercury has the strongest toxicity among heavy metals (Nies 1999). Toxicology studies showed that methyl mercury is very toxic to human embryo and fetus. Most frequent anthropogenic sources of mercury are chlor-alkali, paints, disinfectants, pharmaceuticals, and pulp and paper industries. It can also be released by burning of fossil fuels in the environment (Wang et al. 2004). Asian countries, due to their continuous economical growth and population increase, are considered one of the major sources of mercury pollution, with 28 % emission of global mercury. The most dominant form of atmospheric mercury is Hg^0 which can be transported for long distances of over 1,000 km and has a residence time in the atmosphere of 0.5–2 years. It can however be converted to methyl-mercury and introduced to the food chain causing threat to human health (Li et al. 2009; Dash and Das 2012). At the microbiological scale, bacteria resist mercury by binding it to thiols groups, limiting the access of mercury to the cell by forming a permeability barrier or via mechanisms controlled by the *mer* operon (Barkay et al. 2003; Schelert et al. 2004).

2.3 Lead

The biological availability of lead is low, suggesting that it will not pose that much toxic threat to microorganisms in normal levels. In fact, Nies (1999) described lead as a heavy metal which did not deserve its bad reputation. Lead is widely used as a fuel additive and its toxicity to humans and animals is often associated with neuropsychiatric symptoms and damages on the reproductive and other bodily functions. It has been shown that bacteria can resist lead contamination by precipitation of lead phosphate within the cell or by using metal ion efflux system consisting of P-type ATPase or CadA P-type ATPase as well. This efflux mechanism was observed among *Ralstonia* sp. (Rensing et al. 1998).

2.4 Cadmium

Cadmium is the best-known heavy metal and is classified as nonessential and toxic. Furthermore, its toxicity to living beings is high and it has no reported beneficial role. In plants, cadmium can decrease the concentration of many micronutrients (Kumar 2012). The effect of cadmium in plants can be attributed to its disturbance of oxidation-related processes because of its low redox potential. Cadmium is known for its affinity to the SH groups of proteins, thus affecting their properties and functions (Sandalio et al. 2001). Among bacteria, cadmium resistance is driven by the efflux system which is mostly present in gram-positive cells. In gram-negative bacteria, its

removal is related to the *czc* and *ncc* systems. It is important to mention that among cyanobacteria, cadmium can be immobilized by the metallothionein protein, not described in any other prokaryotes but expressed among eukaryotes (Nies 1999).

3 Soil Microbes and Metals Interactions

Metals released in the environment are indestructible and their biodegradation is never evident. At high concentrations, their impact on soil microbial communities can be damaging, expressed in total biomass reduction, community structural changes, and elimination of some specific taxa with crucial roles in the rhizosphere (Giller et al. 1998; Gray and Smith 2005). A high concentration of metal ions can totally inhibit microbial metabolic activities, such as cell division; cause protein denaturation, cell membrane disruption, inhibition of enzyme activities, DNA damage, and transcription inhibition. The most known metals to affect those metabolic processes are Cd, Zn, Hg, Pb, and Ni (Khan et al. 2010). However, some metals, such as Fe, Zn, Cu, Mn, Co, Ni, and Cr are acknowledged to be useful for microorganisms. At low concentrations, they can be incorporated into enzymes and cofactors but they become toxic at higher concentrations by releasing reactive oxygen radicals through Fenton reactions (Valls and Lorenzo 2002).

Microbes can interact with metals existing in the surrounding environment by binding them to the cell surface or transporting them inside the cell where they may exercise some functions related to cell metabolism. It has been shown that some microorganisms are able to reduce metal ions such as Hg^{2+} and Ag^{+} to Hg^{0} and Ag^{0} thus providing a perfect model for total metal removal from the soil (Ehrlich 1997). Recent study reported the ability of nitrogen-fixing rhizobacteria to resist high mercury concentrations in soils holding their host plants. Ruiz-Diez et al. (2012) reported the resistance of *Rhizobium radiobacter*, *Bradyrhizobium canariense*, *Ensifer medicae*, and *Rhizobium leguminosarum* with respective MICs of 30, 12.5, and 6 μM. The mechanism of resistance remains unclear since there is a lack of data on nitrogen-fixing bacteria and metal tolerance. Giller et al. (1998) in their review for metal toxicity reported the inability of N-fixing bacteria to establish proper nodule activity under heavy metals stress. Tolerance of rhizobacteria to metals has been widely studied, albeit relatively recent and still attracting interest among researchers (Kumar and Patra 2013). Lakzian et al. (2002) reported the resistance of N-fixing bacteria to zinc, while other studies showed that rhizospheric bacteria can resist different cadmium amounts in the soil, and alleviate the stress imposed by the metal on the host plant (Robinson et al. 2001; Dell'Amico et al. 2008). Accumulation of metals by soil microorganisms is not "free of charge" since the mechanism of tolerance will require higher energy expenditure. Soil bacteria under metal stress will turn the energy provided for growth to cell maintenance (Giller et al. 1998). Accumulation can occur through metabolism-independent, passive or metabolism-dependent mechanisms.

3.1 Active Uptake of Metals

Essential metals for bacterial metabolism are taken up actively, in the same way toxic metals can be taken up when mistaken for essential metals. The amount of metal uptake depends on both the element and the microorganism (Avery 1995). This active uptake is energy consuming, occurs slowly and may depend on specific transport systems. A rapid accumulation which slowly continues after the rapid phase is also an indication of active uptake (Ledin 2000; Haferburg and Kothe 2007). It has been shown that metals which are accumulated by active processes are localized in cellular parts distinct from those holding passively the adsorbed metals. *Pseudomonas* sp., widely known for their plant growth-promoting properties, have been shown to actively accumulate chromium and mercury in their membrane fractions. Cells growing in presence of Hg (under experimental conditions) showed fragile cell surface structure compared to non-exposed strains (Horitsu et al. 1978; Kong et al. 1994). It was also shown that Ni accumulation in *Bradyrhizobium japonicum* occur with an active process involving Ni-binding soluble protein which transfer Nickel ions to the intracellular side (Maier et al. 1990).

3.2 Passive Uptake of Metals

The passive transport of metals can be defined as its biosorption/sequestration on living or dead bacterial cells. This process is therefore attributed to the bacterial surface properties, defined by its charge and the nature of the metal group linked to its surface (Barkay and Schaefer 2001). Passive metal uptake, also called biosorption is facilitated by panoply of interactions such as hydrophobic interactions, electrostatic interactions, microprecipitation, adsorption, and ion exchange. In this mechanism, metals tend to bound to the phosphoryl groups of lipopolysaccharides (Langley and Beveridge 1999). Adsorption of lead, cadmium, copper, and zinc by plant growth-promoting rhizobacteria (PGPR) was demonstrated in several studies. Some examples include the bacteria *Azotobacter chroococcum* and *Bacillus megaterium*, which improved growth of maize and Indian mustard under metal stress by binding Cd^{2+} and Pb^{2+} to their cell walls (Huang et al. 2000; Wu et al. 2006, 2009). Generally, the ability of these bacteria to passively bind to metal ions is estimated by using Langmuir and Freundlich models which suggest that maximum adsorption will occur if a saturated monolayer of solute molecules is present in the adsorbent surface (Ledin 2000).

3.3 Mobilization and Immobilization

Bacterial interactions with metals can be summarized according to their mobilization and immobilization capacities (Fig. 11.1). Mobilization consists of the speciation of metals into soluble forms through dissolution of their metal compounds such as

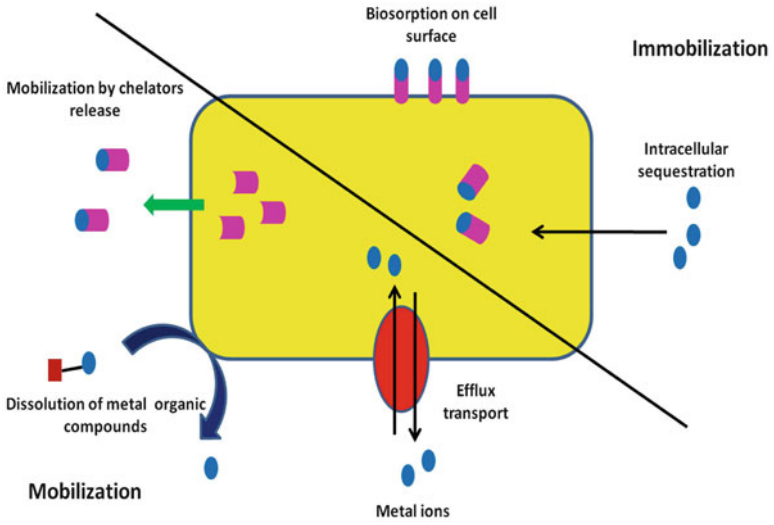

Fig. 11.1 Different mechanisms of metal–microbe interactions

oxides, phosphates, sulfides, and others. Acidification of the environment resulting in metal desorption by protonation of binding site can be made by bacteria through maintenance of charge balance, H^+-ATPase proton efflux, or carbonic acid formation (Gadd 2008). Microbes have the ability to form complexes with metals in solution by releasing extracellular metabolites such as polysaccharides, diffusible pigments, organic acids, and siderophores. Siderophores are considered one of the most important compounds released by rhizobacteria (Neilands 1995; Sharma and Johri 2003; Palanché et al. 2004). In the soil, bacteria, plants, and other organisms compete for Fe uptake, which generally, is not sufficiently soluble at pH levels suitable for life. Siderophore-producing bacteria can easily assimilate Fe ions, as well as other metals such as manganese, chromium(III), and magnesium (Gadd 2008). Organic acid production coupled with siderophores can also contribute to pH change, increasing chelator effects, which lead to metal mobilization. *Pseudomonas fluorescens* was described to be capable of leaching uranium by producing a chelator called pyoverdine (Kalinowski et al. 2004). Reduction of Hg^{2+} to Hg^0 by mercuric reductase also results in the mobilization of this metal by allowing its diffusion out of the cell (Silver 1998).

Metal immobilization, on the other hand, can result from a number of processes which reduces the external free metal concentration. Solubilization increases under certain conditions that shifts equilibrium and allow the release of more metals into solution (Gadd 2004, 2008). Biosorption previously described as passive metal uptake is considered as a metal immobilizating process. Immobilization can also occur through the action of specific metal binding compounds, such as organic acids, alcohols, or macromolecules like humic acid, fulvic acid, and polysaccharides in addition to extracellular polymeric substances (Sayer and Gadd 2001).

Mobility and toxicity can also diminish when the metal is reduced to a lower redox state (Finneran et al. 2002). Many rhizobacteria have been reported to reduce a wide range of heavy metals. *Ochrobacterium* and *Bacillus cereus* have been reported to reduce Cr(VI) to Cr(III) (Faisal and Hasnain 2006), *Bradyrhizobium japonicum* can also reduce arsenic, decreasing its absorption by soybean plants, which leads to growth enhancement (Reichman 2007).

4 Mechanisms of Metal Cleaning by Rhizobacteria

PGPR can remove heavy metals by mobilization, immobilization, or transformation to less toxic forms. Those mechanisms include exclusion, extrusion, accommodation, and biotransformation (Nies 1999; Umrania 2006).

4.1 Metal Exclusion

In the exclusion process, the metal ions are kept away from the target site. Creation of permeability barrier by alterations in the envelope, membrane, and cell wall of microorganisms is a tentative process of protecting major cellular components. Such alterations usually results from a single gene mutation which is enough to alter the membrane permeability to metal ions (Rouch et al. 1995). Also bacteria that possess an external coating of polysaccharide can protect their sensitive cellular components from contact with metal ions. *Pseudomonas putida* and *Arthrobacter viscosus* are able to bind Cd^{2+} to their exopolysaccharide coat. This binding depends to a major part on the pH of the medium, which should be between 4 and 9 (Bruins et al. 2000). Bacteria can also release some chelator compounds which bind to the metal outside the cell. Melanin presents a good example of a cation chelating property through the anionic function of the carboxyl group and the deprotonated hydroxyl group in the compound (Riley 1997).

4.2 Metal Extrusion

Extrusion is a mechanism of active efflux driven by membrane potential which allows bacteria to avoid the toxic effect of metals (Canovas et al. 2003). It is the largest metal resistance system which is encountered in the environment. The extrusion system resistance is generally encoded by plasmid genes that encode for transport proteins, and rarely seen within chromosomal DNA. The cadmium extrusion system in bacteria were found to involve proton exchange mechanisms, while arsenate and arsenite elimination involves ATP anion extrusion pumps (Rosen et al. 1985). Rosen (2002) defined two extrusion systems among bacteria: the carrier-mediated efflux, described from *Bacillus subtilis* and the ATPase translocating system.

4.3 Bioaccommodation

Bioaccommodation refers to the intracellular sequestration of metals by protein binding, where the metal is maintained in the cytoplasm away from major cellular components. This phenomenon is encountered among *Pseudomonas* sp. and target essentially cadmium, copper, and zinc through synthesis of proteins after gene induction from high levels of the previously cited metals. Those proteins are known by their possession of cystein residues that act as sink for the excessive toxic metals. *Pseudomonas putida* shows such characteristic cystein-rich protein production. Likewise, some cyanobacteria synthesize eukaryotic-like metallothionein, also rich in cystein residues (Silver and Phung 1996).

4.4 Biotransformation

Biotransformation refers to the reduction of the metals to less toxic forms. Such kind of detoxification pathway is generally mediated by enzymes which generate reduction, oxidation, methylation, and alkylation reactions. In this discussion of metal removal processes, we excluded description of the enzymes involved in immobilization and precipitation. The best-studied examples of such reactions are those related with mercury and arsenic (Valls and Lorenzo 2002). Mercuric reductase, which is coded for by the *mer*A gene and catalyzes the conversion of Hg^{2+} to the volatile Hg^0, has been observed in *Pseudomonas putida*. This bacterium was able to volatize more than 90 % of the metal in a 40 mg L^{-1} solution within 24 h (Okino et al. 2000). Transformation of As(V) or As(III) through methylation reaction allows its volatilization under dimethyl or trimethyl-arsine form. The enzymes involved here remain not well studied; however, it is known that ArsM, a methyl transferase, generates trimethylarsine in *Rhodobacter sphaeroides* (Stolz et al. 2010). Also, redox transformation through reduction and oxidation processes can lead to the mobilization of metals, metalloids, and organic compounds (Gadd 2008).

5 Rhizobacteria as Tool for Heavy Metal Cleaning

5.1 Plant Growth-Promoting Rhizobacteria

The rhizosphere holds a high density of microbial population, resulting from the mutually beneficial plant root-microbe association. Rhizospheric bacteria that exercise beneficial effects on their host plants are termed PGPR. They constitute around 2–5 % of the microbial pool in the rhizosphere (Solano et al. 2008). According to their functions, these microbes can be divided into two categories: free-living bacteria and symbiotic bacteria. Plant root exudates provide nutrition in forms of small molecules of amino acids, sugars, and organic acids for the associated

microorganisms which in turn promote the plant growth. The result of this beneficial association increases microbial activity in the rhizosphere (Khan 2005). It has been even reported that microbes tend to migrate from the bulk soil to the rhizosphere where they aggressively colonize plant roots (Ma et al. 2011). A mechanism of plant growth promotion depends on the release of metabolites which may be phytohormones, such as gibberellins, auxins, and cytokinins. The effect of these substances to the plant is generally concentration-dependent (Dimkpa et al. 2009). Substances whose activities can result to pathogens suppression, mineral uptake improvement, nitrogen fixation, and tolerance to abiotic stress are also secreted by rhizobacteria in the form of enzymes, osmolytes, siderophores, organic acids, nitric oxides, biosurfactants, and antibiotics (Sikora et al. 2007; Belimov et al. 2008; Ma et al. 2011). PGPR that are capable of fixing atmospheric nitrogen and making it available to plants are known as diazotrophic bacteria.

Many genera have been isolated from plant species such as rice, sugarcane, sorghum, corn, pineapple, and coffee. *Azospirillum* was the first rhizobacteria used for plant growth promotion along with *Azoarcus*, *Azotobacter*, *Burkholderia*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum*, and *Paenibacillus* (Solano et al. 2008). These bacteria can also synthetize siderophores which sequester iron from the soil and provide it to the plant cell, which then uptake the whole iron–siderophore complex. PGP bacteria can also make some minerals more available for the plant by facilitating solubilization of these minerals, the best example of which is phosphorus. Another capacity is the synthesis of ACC deaminase, which can reduce plant stress ethylene levels. The indirect process for plant growth promotion is through the function of rhizospheric bacteria as biocontrol agents against phytopathogens by antibiotic production, depletion of iron from the rhizosphere, induction of systemic resistance, and synthesis of lytic enzymes that attack pathogen cell wall, and competition with pathogens for binding sites on roots (Glick 2010). Endosymbiotic plant rhizobacteria are defined as beneficial colonizers that do not cause symptomatic infections. They generally reside in the apoplasm or symplasm and are able to induce physiological changes that promote the growth of the plant. This beneficial effect is believed to be stronger than that from many free-living rhizobacteria, especially under stress conditions (Conrath et al. 2006; Hardoim et al. 2008). Once in the plant, endophytes stimulate growth through several mechanisms, especially by accumulation of osmolytes, stomatal regulation, reduction of membrane potential, and change in phospholipid content of the cell membrane (Compant et al. 2010). Other rhizobacteria can enhance plant growth by one or combinations of modes of action like providing N to plant after N_2 fixation, increasing root surface area, allowing other symbiotic associations, iron uptake by the IAA precursor anthranilic acid instead of siderophores (Khan et al. 2009).

Microbial IAA promote plant growth by increasing lateral root proliferation which will allow uptake of nutrients and minerals resulting to increased root exudation and further increase of bacterial proliferation (Lambrecht et al. 2000). IAA also help plants to overcome abiotic stress by expanding the root and shoot length, like that of wheats exposed to high level of salt (Egamberdieva 2009). ACC deaminase is another enzyme released by rhizospheric bacteria that acts as a plant growth promoter by functioning as a “sink” for ACC and lowering ethylene level in

developing or stressed plants (Glick et al. 1998). Ethylene is required for early stages of plant development. Being crucial for seed germination, its rate increases during germination and seedling growth and is also produced during plant responses to stress (Glick 2003). However, high amount of ethylene results in the inhibition of root elongation. ACC deaminase cleaves ACC, the ethylene precursor, to produce ammonia and α-ketobutyrate. This enzyme has been detected in many soil microorganisms including fungi and yeasts (Minami et al. 1998; Ghosh et al. 2003; Glick 2003). Another plant growth promotion ability provided by rhizospheric bacteria is the uptake of iron which is crucial for the plant metabolism. Iron functions as cofactor for a number of enzymes, important in many biological processes such as respiration, nitrogen fixation, and photosynthesis (Solano et al. 2008).

Plants have their own chelating agents called phytosiderophores that bind Fe(III). Their affinity to Fe ions is however, lower compared to microbial siderophores. This can be explained by differences in their requirements for Fe(III). Rhizospheric bacteria can promote plant growth in the presence of soil pathogens by binding iron metals in the rhizosphere, depriving the phytopathogens of this essential mineral. This competitive mechanism prevents pathogen proliferation in favor of the host plant. *Pseudomonas fluorescens* is one of the rhizospheric bacteria known mostly for its siderophore production in the forms of pyochelin and pyoverdine (Solano et al. 2008). Phosphorous is one of the most limiting nutrients, which are absorbed by plants only in its soluble form. In the soil, however, phosphorus is mostly available in its organic form, which represents 30–50 % of the total soil phosphorus. Soil microorganisms are able to mineralize it so that it becomes available to plants in soluble form. The most abundant form of phosphorus accessible for microorganisms is the Ca-P complex but other bacteria can also solubilize Fe-P, Mn-P, and Al-P. There are two possible mechanisms of phosphate solubilization by bacteria: release of organic acids that interact ionically with phosphate cations resulting to their release; and release of phosphatases that cleave bound phosphate groups and liberate them in soluble forms (Fig. 11.2).

5.2 Rhizobacteria and Heavy Metals

Many rhizobacteria have been reported as beneficial for their host plants in metal-contaminated soils, e.g. *Achromobacter*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, and *Serratia* (Gray and Smith 2005). Obviously, one condition for protecting plants from metals toxicity is resistance of the rhizobacteria to those metals. Van der Lelie et al. (2000) reported some highly metal-resistant rhizobacteria such as *Alcaligenes eutrophus*, isolated from diverse biotopes in contaminated Belgian lands to desert soils in the Congo Republic. The resistance of these microorganisms is generally attributed to the presence of megaplasmids. For instance, *Cupriavidus metallidurans*, formerly known as *Ralstonia metallidurans* carries the megaplasmids, pMOL30 and pMOL28. Presence of pMOL30 increases resistance to zinc by 50-folds, cobalt by 33-folds and cadmium by sevenfolds, while pMOL28 mediates resistance to nickel and increases resistance to cobalt by 16-folds.

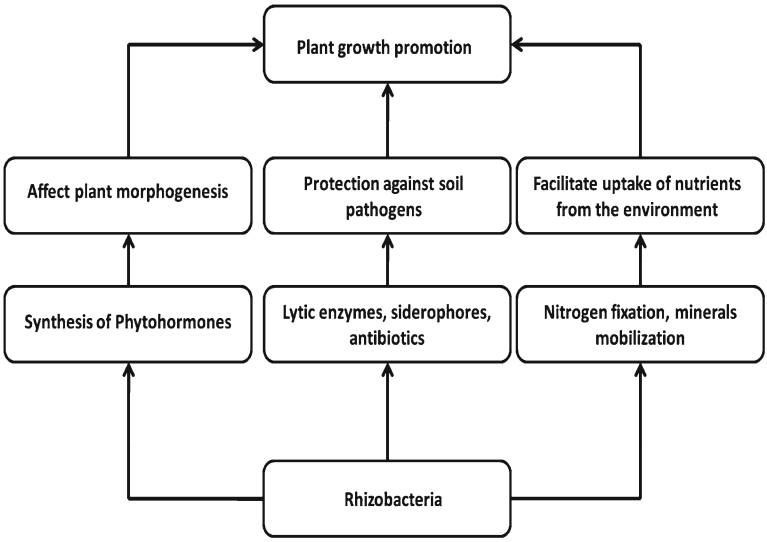

Fig. 11.2 Rhizobacterial mechanisms for plant growth promotion

Cobalt, zinc, and cadmium resistance genes within the pMOL30 plasmid are part of the *czc* operon (Nies 2006). Enhancement of plant growth by the rhizospheric bacteria, *Ralstonia eutrophus* CH34 through alleviation of metal stress has been reported. It was found that under high concentrations of cadmium or zinc, the rhizobacterium was able to decrease the metal concentration up to 99 % in the late log phase. This decrease is coupled with pH increase of up to 9 and precipitation and sequestration of metals (Van der Lelie et al. 2000).

In other studies, the beneficial effect of *Methylobacterium oryzae* and *Bulkhorderia* sp. on the growth of tomato under nickel- and cadmium-treated soils was also observed. These two strains significantly promoted the plant growth by reducing toxicity of the two metals. These bacteria are capable of reducing translocation of metals into shoots and synthesizing phytohormones and ACC deaminase which enhance plant growth (Madhaiyan et al. 2007). It has been proven that rhizobacteria may acquire resistance when grown in metal-contaminated soils. Because of the symbiotic nature of the relationship, these nodule bacteria, which increase metabolic activities due to collected nutrients from the root exudates, can in turn assist in resisting the metal toxicity (Zhuang et al. 2007).

5.3 Rhizobacteria-Assisted Phytoremediation

Phytoremediation is the use of plants to extract, detoxify, or sequester pollutants from shallow soil and water (Alkorta and Garbisu 2001). It is a well-appreciated

Table 11.1 Effect of some rhizobacteria on plants under metal stress

Bacteria	Mechanism	References
Acinetobacter sp.	Enhancing uptake of iron and zinc via IAA production	Lippmann et al. (1995)
Pseudomonas aeruginosa	Secretion of pyoverdine and pyochelin which enhance chromium uptake by maize	Braud et al. (2009)
Streptomyces tendae	Siderophore production to enhance cadmium uptake by sunflower	Dimkpa et al. (2009)
Bacillus sp.	Lipopeptide production increasing growth and cadmium uptake by tomato	Sheng et al. (2008)
Gluconacetobacter diazotrophicus	Mobilization of zinc via 5-ketogluconic acid	Saravanan et al. (2007)
Bulkhorderia caribensis	Iron solubilization through gluconic acid production	Delvasto et al. (2009)
Pseudomonas aeruginosa	Mobilization of copper through rhamnolipid production	Venkatesh and Vedaraman (2012)
Azotobacter spp.	Decreasing uptake of cadmium and chromium by *Triticum aestivum*	Joshi and Juwarkar (2009)
Achromobacter xylosoxidans	Increasing uptake of nickel and chromium by *Brassica juncea* by promoting its roots and shoot length	Ma et al. (2009)
Pseudomonas tolaasii	Enhancing cadmium uptake by *Brassica napus* through root elongation promotion	Dell'Amico et al. (2008)
Sanguibacter sp.	Increasing cadmium translocation and uptake, increasing of shoot and root dry weight within *Nicotina tabacum*	Mastretta et al. (2009)
Methylobacterium oryzae	Decreasing ethylene emission and uptake of nickel and cadmium, enhancing of plant growth within *Lycopersicon esculentum*	Madhaiyan et al. (2007)
Rhodococcus erythropolis	Enhancing plant growth in presence of Cr^{6} and reduction of Cr^{6} to Cr^{3+}	Trivedi et al. (2007)

method compared to traditional techniques due to its benefits in landscape preservation, improvement of soil microbial activities, which eventually plays a crucial role in maintaining healthy ecosystem (Cunningham et al. 1995). Phytoremediation includes five major mechanisms: (a) phytoextraction, which consists of the uptake of metals into harvestable parts of the plant; (b) phytodegradation, the degradation of the metal by the plant and its associated microbes; (c) rhizofiltration, the absorption of metals from contaminated waters by roots; (d) phytostabilization, consisting of the immobilization and reduction of the toxicity and bioavailability of metals by plant roots and their associated microorganisms; and (e) phytovolatilization, the volatilization of the metal by the plant into the atmosphere (Khan 2005). It is known that plants and microbes communicate through chemicals in root exudates, such as organic acids, amino acids, and phenolic compounds. This method of interaction became the basis of microbe-assisted phytoremediation in metal-contaminated soils (Table 11.1).

Bacteria can enhance plant remediation capacities and reduce the metal phytotoxicity. Plants stimulate bacterial growth through root exudates released in the soil.

Regardless of whether the plant possesses affinity to the metal contaminants, metal degradation by associated metal-resistant rhizobacteria is possible thereby, reducing phytotoxicity in the soil. In the context of soil decontamination, it is also possible for the plant to secrete low-molecular-weight compounds that act as chelating agents, which enhance the phytoavailability of soil metals. In fact, phytoextraction is considered a cost and environmental friendly procedure for heavy metal removal in contrast to physico-chemical processes, which are expensive and harmful to the soil structure. Many plant species have the ability to accumulate metals without alteration of their growth and development. However, some disadvantages need to be mentioned, such as the small biomass and the slow growth of those plants, which require few years before significant reduction in metal content can be achieved. Low availability of metals in soil can also limit the efficiency of phytoremediation, and high metal levels can affect the plant efficiency. Phytoremediation alone, therefore, may be inefficient. The use of metal-tolerant and fast-growing grasses of no interest in the food industry like the vetiver and hemp capable of growth in a wide range of ecosystems are good candidates for phytoremediation.

Rhizospheric microorganisms that affect metal bioavailability by altering soil pH and releasing chelators were reported to have been used as plant partners for accelerating the phytoremediation process and improving the plant growth by sequestration of heavy metals (Naees et al. 2011; Abou-Shanab et al. 2005). Plant biomass is important and proportional to the metal phytoextraction capacity. In this way, the application of plant growth-promoting substances such as auxins and cytokinins has positive effect on phytoremediation by increasing biomass of tolerant non-hyperaccumulating plants. Application of PGPR that produce IAA, such as *Acinetobacter*, enhances the uptake of iron and zinc including minerals such as calcium, potassium, and phosphorus by the host plant. However, the use of PGPR gives limited results when there is a lack of nutrients. Application of fertilizers to enhance biomass and increase metal extraction is needed. Investigation of ideal plant-PGPR-soil type combination is also required for efficient results (Khan 2005). Bacterial inoculation has been so far effective in enhancing the metal uptake and plant growth under metal stress, when the metal concentration is low. Selected microorganisms are generally metal-resistant and express plant growth-promoting traits such as IAA, ACC deaminase, and siderophore production. These three plant growth-promoting traits have been highly exploited, with the intention of taking advantage of the bacteria's ability to proliferate under such conditions, at the same time, improve plant growth. Mechanisms of actions of these substances under metal stress are still not fully understood and most data relate only to their basic mechanisms of action (Glick 2010). Siderophores, for example, are able to bind a multitude of metals other than iron in the soil. Their roles in heavy metal phytoextraction are attributed to their ability to complex with unavailable forms of metals and solubilize them. *Pseudomonas aeruginosa*, for example, enhances bioavailability of chromium for maize through pyoverdine and pyochelin production. Another example is *Streptomyces tendae*, which enhances availability of cadmium for sunflower (Braud et al. 2009; Dimkpa et al. 2009).

The mechanism of metal uptake cannot be generalized in a simple statement due to differences in plant properties and their affinity to heavy metals. Therefore, each

case needs to be studied. Yet with the example of siderophores, numerous observations were made, where siderophore-producing microbes enhanced plant growth but did not allow uptake of metal by the plant. For instance, one strains of *Pseudomonas aeruginosa* reduced uptake of cadmium in *Cucurbita pepo* and *Brassica juncea*, while another *Pseudomonas* strain, which also produces siderophores, enhanced growth but reduced nickel uptake in chickpea (Rajkumar et al. 2012). Other than those "classic" substances produced by rhizospheric bacteria, other substances that may also participate in phytoremediation process have been broadly described. Sheng et al. (2008) described the production of biosurfactant by bacteria, which may help enhance bioavailability of the metal for phytoremediation. The roles of organic acids in metal complexation and increase of mobility for plant uptake have also been studied. These compounds, produced by plant-associated microbes are usually low-molecular-weight compounds (300 Da maximum) with one or more carboxyl groups. They have attracted attention because of their ability to solubilize heavy metals and cause mineral nutrients mobilization in the rhizosphere. Organic acids have the ability to bind metal ions through complexation reactions. The stability of the complex acid metal depends on the nature of organic acid, the number and position of its carboxyl groups, the binding of the heavy metals, as well as the soil pH. It has been shown that *Gluconacetobacter diazotrophicus* can solubilize zinc in vitro by releasing 5-ketogluconic acid (Saravanan et al. 2007). *Bulkhorderia caribensis* showed ability to solubilize P and Fe using gluconic acid coupled with exopolysaccharide production and biofilm formation (Delvasto et al. 2009).

6 Bacterial Engineering: Biotechnological Innovations to Overcome Toxicity

Lovley and Lloyd (2000) said, in their editorial for Nature Biotechnology: "Microorganisms are not alchemists, no matter how a microorganism acts upon a toxic metal, the metal is not destroyed." This section relates to our previous discussion in this chapter, dealing with the difficulty of cleaning metal pollution in the soil. In fact, among all soil contaminants emanating from anthropogenic activities, heavy metals are believed to be the most hazardous and damaging to the ecosystem, and the most complex to remove. Microbial application to remove metals from soils is dependent on many biotic and abiotic factors, such as plant–microbe interaction and soil pH, which limits the efficiency of the remediation process employed. To overcome these obstacles, scientists resort to genetic manipulation and engineering of interesting microbes to enhance their efficiency.

One prominent study in this context was published by Valls et al. (2000) in Nature Biotechnology, wherein they succeeded in cloning the eukaryotic metallothionein from mouse to the soil bacterium *Ralstonia eutrophus*. This surface protein considerably increased cadmium biosorption by the microorganism and showed growth improvement of inoculated, cadmium-sensitive tobacco plants compared to the one growing in presence of the wide-type bacterium strain.

However, it was not possible to reproduce such effect, when the gene was cloned into other bacterial groups. It did work nicely on the gram-negative bacterium, *E. coli*, which was initially used as cell host before looking for a suitable soil bacterium for use in bioremediation. The study that employed this cloning technique aimed to concentrate as much cadmium ions and reduce these to their elemental state in the field scale. This immobilization process could be a very good solution for long-term application.

Phytochelatin is another molecule which can bind heavy metals. It consists of a short peptide composed of $(Glu\text{-}Cys\text{-}Gly)_n$, where *n* ranges from 1 to 11. Its affinity to metal was recognized to be stronger than metallothionein due to the repeating Glu-Cys moieties. The phytochelatin synthase gene was cloned from *Arabidopsis thaliana* into the rhizobacterium, *Mesorhizobium huakuii*. The transformed bacterium should assist in the accumulation of cadmium by its symbiont, *Astragalus sinicus*, known to form nitrogen-fixing root nodules. Together, this presents an efficient cadmium cleaning system in addition to the nitrogen fixation ability (Sriprang et al. 2003). However, the γ-carboxylamide bond between the glutamine and cystein indicates that these two residues must be synthetized enzymatically, which can be an obstacle to the microbial-restricted metabolic machinery, in case of wider application expectation. As an alternative, synthetic phytochelatin was proposed, which can be synthetized from a synthetic DNA template and which expresses the same affinity as the natural one (Cindy et al. 2006).

Due to their thick cell wall, gram-positive bacteria are more suitable to face field conditions when applied. Yet studies regarding genetical engineering of these bacteria for remediation applications are limited. Samuelson et al. (2000) succeeded in expressing surface peptide on *Staphylococcus* spp., able to bind mercury and cadmium. For improvement of plant phytoextraction, enhancement of endophytic potential by genetic manipulation was also studied. Engineered *Bulkhorderia cepacia*, for resistance to nickel, increased metal uptake by 30 % in the roots of its host, *Lupinus luteus*. Transgenic plants, expressing bacterial reductases were also able to volatilize mercury and selenium and accumulate arsenic in their shoots (Nele et al. 2009). Researches on recombinant microorganisms are increasing, but at the same time, facing legislative and public opposition against their use, although even indigenous microorganisms are naturally manipulated in the environment (Goodnight 2000). Assessment of the risks involved in introduction of recombinants should be interpreted through logical studies involving lab scale microcosm and observations concerning the possible ecosystem alteration upon their introduction.

7 Actinobacteria: A Microbial Power to Exploit for Metal Remediation

In this part, we aim to discuss the importance of an interesting phylum of soil prokaryotes for heavy metal cleaning. As concluded by Khan et al. (2009) in a similar discussion, most PGPR cannot effectively perform in extreme environments. Actinobacteria

is a prokaryotic phylum known for its wide range of metabolic activities, which enable them to establish populations in all types of environmental conditions, including pollution. Here, we discuss the potential of actinobacteria as PGP candidates for metal remediation. One of primary reasons for choosing this phylum is related to their wide metabolic capacity. In fact, 90 % of commercialized antibiotics and two-thirds of biological active compounds come from actinobacteria (Hamaki et al. 2005). The extremophilic characteristics of actinobacteria are well reported and many genera encountered in soils were associated with stress resistance. We can mention the examples of *Geodermatophilus*, *Blastococcus*, *Modestobacter*, *Rhodococcus*, and the well-studied *Streptomyces* (Larkin et al. 2005; Gtari et al. 2012). Many studies of metal-stressed soil bacterial communities demonstrated that actinobacteria is a major active group. Gremion et al. (2003) showed the dominance of actinobacteria compared to α proteobacteria in the bulk and rhizosphere of contaminated soils, using 16s rDNA and rRNA analyses. Also, many other studies showed actinobacteria as a consistently dominant group together with α proteobacteria in metal-contaminated lands (Lazzaro et al. 2008; Karelova et al. 2011; Tipayno et al. 2012).

7.1 Plant Growth Promotion Abilities Among Actinobacteria

It is known that actinobacteria are potential sources of bioactive compounds, but their agroactive potential is also considerable. In fact, 60 % of herbicides and insecticides produced are from genus *Streptomyces* (Doumbou et al. 2011). Aldesuquy et al. (1998) published one of the first reports on plant growth promotion by *Streptomyces*, where they showed its ability to increase shoot length and mass in wheat (Table 11.2).

The hormone analysis revealed the production of gibberellins, cytokinins, and auxins by *Streptomyces rochei* and *Streptomyces olivaceoviridis*. Some other examples can be mentioned such as polyoxin production against fungal phytopathogens by *Streptomyces cacaoi* (Copping and Duke 2007). Also, *Streptomyces kasugansis* was shown to control the rice blast agent, *Pyricularia orizae* and *Pseudomonas* diseases in many crops (Schluenzen et al. 2006). Isolated endophytic S*treptomyces* from plant roots were able to synthetize plant growth-promoting compounds such as zeatine, indol acetic acid, and gibberellic acid, as well as demonstrate antagonistic activities against the plant pathogen, *Pseudomonas savastonii* (Sardi et al. 1992; Solans 2007; Ghodhbane-Gtari et al. 2010). Other studies were carried out on other genera of actinomycetes for plant growth promotion, like the hyperparasitism of *Nocardiopsis dassonvillei* against *Fusarium oxysporum* (El-Tarabily and Krishnapillai 2006). Biological control of *Fusarium oxysporum* and *Sclerotinia minor* and phosphate solubilization activity of *Micromonospora* sp. have also been reported (El-Tarabily et al. 1997, 2000). *Rhodococcus* spp. living in plant rhizosphere use ACC as a nitrogen source through production of ACC deaminase. Thus, ACC content in plants decreases leading to ethylene accumulation reduction (Arshad et al. 2007).

Table 11.2 Plant growth promotion activities among actinobacteria

Strains	PGP characteristic	References
Streptomyces sp.	Siderophore production	Lee et al. (2012)
Rhodococcus sp.	IAA production	De Carvalho Costa and Soares De Melo (2012)
Rhodococcus erythropolis	Enhancing plant growth under Cr^{6+} toxicity	Patel et al. (2012)
Frankia sp., *Actinoplanes* sp., *Micromonospora* sp., and *Streptomyces* sp.	Production of IAA, gibberellin, and zeatin	Solans et al. (2011)
Streptomyces and non-identified non-*Streptomyces* strains	Egg hatching of the nematode *Meloidogyne incognita*	Ruanpanum et al. (2010)
Actinomadura glauciflava, *Nonomuraea rubra*, and *Nocardia alba*	Protease activity, ammonia IAA, and siderophore production	Nimnoi et al. (2010)
Leifsonia soli	Plant growth promotion by ACC deaminase production	Madhaiyan et al. (2010)
Microbacterium azadirachtae	IAA production, P-solubilization, ACC deaminase activity, and sulfur oxidation	Madhaiyan et al. (2010)
Streptomyces spp.	Production of zeatin, gibberellic acid, IAA. Antagonism against *Pseudomonas savastonii*	Ghodhbane-Gtari et al. (2010)
Actinoplanes campanulatus, *Micromonospora chalcea*, and *Streptomyces spiralis*	Reduction of root crown rots induced by *Pythium aphanidermatum* among cucumber	El-Tarabily et al. (2010)
Actinomadura sp.	Production of antifungal compounds, IAA, and siderophores	Khamna et al. (2009)
Micromonospora aurantiaca	Strong antagonistic activity against *Pythium ultimum* and *Fusarium oxysporum*, IAA, and P-solubilization activity	Hamdali et al. (2008)
Streptomyces cacaoi	Antagonism against fungi	Copping and Duke (2007)
Streptomyces kasugaensis	Antagonistic activity against *Pyricularia orizae*	Schluenzen et al. (2006)
Micromonospora carbonacea	Cell wall degradation of *Sclerotina minor*	El-Tarabily et al. (2000)
Streptomyces olivaceoviridis and *Streptomyces rochei*	Auxin, gibberellin and cytokinin production	Aldesuquy et al. (1998)
Micromonospora endolithica	P-Solubilization activity	El-Tarabily et al. (1997)

7.2 Actinobacterial Abilities for Restoration of Heavy Metal-Contaminated Soils

As exceptional metabolic machineries, actinobacteria can offer a suitable alternative for metal removal from soils. It was estimated that of the entire actinobacterial secondary metabolite arsenal, only a tiny fraction has been discovered. This may be due to the unbalanced research focus on the medical field. According to the bulk

data existing on the actinobacteria resistance to metals and metalloids, tracing novel secondary metabolites pattern is still possible and promising. Their filamentous nature alone, similar to that of fungal hyphae, presents them as good heavy metal accumulators (Panday et al. 2004). *Streptomyces galbus*, a strong producer of antifungal metabolites, significantly increased production in presence of copper, zinc, or iron (Paul and Banerjee 1983). It has been also reported that *Arthrobacter mysorens* can promote plant growth in soils contaminated with cadmium and lead (Francis et al. 2010). *Microbacterium arabinogalactanolyticum* promote nickel accumulation by *Alyssum murale*, while the nitrogen-fixing actinobacterium, *Frankia* sp. significantly increase yield of their host *Alnus glutinosa* in the presence of nickel (Wheeler et al. 2001). Richards et al. (2002) described also the resistance of *Frankia* isolates to a set of heavy metals for the purpose of checking the gene circulation process between them and their host plants. Future investigations into the potentials of *Frankia* for bioremediation need to provide resolution to the difficult culture and maintenance requirements.

Rhodococcus erythropolis, a psychrotrophic actinobacteria known for its plant growth-promoting traits, can reduce chromate at temperature lower than 10 °C (Trivedi et al. 2007). It has been shown that a particular family of actinobacteria may be using metal efflux-like mechanisms for their well-known ability to tolerate antibiotics. An example is the ABC transport system for antibiotics, which can also be used as efflux pump for many metals (Borges-Walmsley et al. 2003). As biological factories for pigment production, such as eumelanin, actinobacteria belonging to genera *Geodermatophilus*, *Modestobacter*, and *Blastococcus* showed phenomenal resistance to metals. *Geodermatophilus obscurus*, *Modestobacter multiseptatus*, and *Blastococcus saxobsidens* can grow under 30 mM lead, while *Blastococcus saxobsidens* showed phenomenal growth in 85 mM of $AsO4^{3-}$ (Gtari et al. 2012). Mechanisms of resistance to metals among those strains with extremophilic properties have not yet been elucidated. However, massive melanin production in this group suggests the possible chelator activity of these pigments that can keep metals outside the cell (Fogarti and Tobin 1996; Nosanchuk and Casadevall 2003). Pigments released to the cell's environment have beneficial effects not only on the producer microorganism but also on adjacent cells. This was confirmed for many *Streptomyces* species (Schmidt et al. 2005). Furthermore, resistance of *Streptomyces* to Cr and its ability to reduce Cr(VI) to Cr(III) was reported to be dependent on the carbon source. *Streptomyces termocarboxydus* was shown to increase the reduction rate when glycerol is the carbon source (Marta et al. 2011). For Cd, TEM study showed Cd^{2+} localization in the cell wall of *Streptomyces tendae* suggesting a passive mechanism for uptake (Sineriz et al. 2009).

8 Conclusions and Future Perspective

Heavy metals threat is continuously increasing and strategies to overcome it is a primary concern, especially in developing countries. Among all adopted strategies for metal removal, biological means are the most suitable and cost-effective.

In agricultural lands, prokaryotic populations called rhizobacteria, occupying the rhizosphere region of the roots, are widely exploited for their role in plant growth promotion. Research on these bacteria showed the involvement of plant growth-promoting metabolites such as IAA, siderophores, and ACC deaminase (Glick 2003). In metal-contaminated lands, these rhizobacteria have been exploited to enhance metal uptake by plants for phytoremediation processes, or to alleviate metal stress among sensitive crops. In this chapter, we enumerated the panoply of mechanisms used by microorganisms to cope up with metal stress, and mobilize their plant growth promotion traits in association with their host plant to overcome metal stress. Plant–microbe systems have shown great potential in reducing metal contaminants in soils but their efficiency during application remains inadequate. An important factor here is metal tolerance limitation. For this reason, scientists have resorted to microbial engineering to improve the potency of these biological systems. Relevant results have shown increased ability for metal uptake by microorganisms after introducing foreign proteins, such as metallothionein and phytochelatin (Valls et al. 2000; Sriprang et al. 2003).

In this book chapter, we turned our attention to actinobacteria as a rhizospheric taxon of great potential, due to its high metabolic activity and dominance in metal-contaminated lands. Investigating the hidden metabolic power of actinobacteria will offer a considerable contribution for cleaning of metal-contaminated soils. Difficult to eliminate as they are, heavy metal biomineralization to a solid inactive phase offers a more effective alternative to mere inoculation of metal-tolerant strains, regardless of the mechanism they employ. In consortium with genetic and molecular technologies, discoveries such as the first published report on *Streptomyces* bioemulsifier under chromium contamination can continue to rise (Colin et al. 2013). Schutze et al. (2013) recently were already able to show biomineralization of copper, nickel, and manganese by *Streptomyces*. We suggest that more thorough studies be done in this area, to uncover or develop multiresistant and multifunctional microbes, like *Cupriavidus metallidurans*, that would offer solutions to metal remediation concerns.

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Chapter 12
Potassium and Sodium Transport Channels Under NaCl Stress

Igor Pottosin, Ana-María Velarde-Buendía, and Oxana Dobrovinskaya

1 Introduction

Over 800 million hectares or ~7 % of the world's total land area is affected by salinity (Munns 2005). Secondary salinity due to non-optimal agricultural practices according to estimate will affect up to 50 % of cultivated land by 2050. At the same time, increased population will require a 50 % increase of agricultural production (Shabala and Cuin 2007). Thus, there are not only billions of dollars losses annually which are produced by salinity, but a strategic challenge for the humankind. Almost all crops are glycophytes, so that their growth is suppressed by salinity. Traditional breeding had only limited success. It is obvious that salt tolerance is not relied on one or few key elements but represents a rather complex trait, where interactions between different elements and pathways all count. Still, there is a hope that smarter biotechnology approaches, focusing on the overexpression of working circuits rather than single elements can be a better solution.

Salinity implies low water potential in the soil, so it comes hand-to-hand with drought. Indeed, salt stress is hyperosmotic in nature and represents similar challenges (turgor loss, changes in operation of biomolecules due to alteration of their hydration shells). Yet, salinity is not restricted to this and plant responses to salt are fundamentally different from responses to water stress alone, although sharing some characteristics. Salt stress has first of all the ionic basis. Most commonly,

I. Pottosin (✉)
Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Av. 25 de julio 965, Villa de San Sebastián, 28045 Colima, Colima, México

School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, TAS 7001, Australia
e-mail: pottosin@ucol.mx

A.-M. Velarde-Buendía • O. Dobrovinskaya
Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Av. 25 de julio 965, Villa de San Sebastián, 28045 Colima, Colima, México

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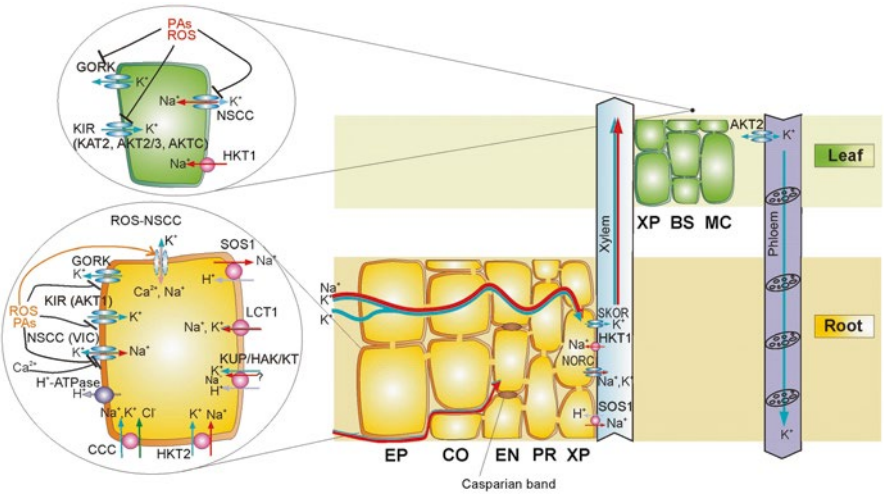

Fig. 12.1 Principle pathways for uptake, efflux, and translocation of K^+ and Na^+ in planta. In roots, symplastic (upper routes) and apoplastic pathways for K^+ and Na^+ transport are drawn. Carriers and channels, involved in the uptake of K^+ and Na^+ from the soil are plotted at expanded scale for a typical epidermal cell. NSCC does not imply a single channel type, but refers to a variety of nonselective channels, which may coexist in the same tissue (see text for the discussion of possible candidates). Channels and carriers, involved in K^+ and Na^+ loading into the xylem, are specified directly in xylem parenchyma cells. A generalized leaf cell (not a guard cell) with Na^+ and K^+ channels and transporters is shown, and weakly rectifying K^+ channel AKT2, participating in the phloem K^+ recirculation is indicated in separate. Remodeling of the plasma membrane conductance by some factors, related to the stress, like reactive oxygen species (ROS) and polyamines (PAs), is also depicted. *EP* epidermis, *CO* cortex, *EN* endodermis, *PR* perycycle, *XP* xylem parenchyma, *BS* bundle sheath, *MC* mesophyll

salinity implies a sodicity, i.e. high Na^+ in external medium, although some important crops, like grapevine or citruses, are relatively Na^+-resistant, but highly sensitive to elevated cytosolic Cl^-. Uptake of Cl^- in parallel with Na^+ is inevitable for the sake of the charge balance, and massive uptake of solute ($Na^+ + Cl^-$) is necessary for the compensation of the decrease of water potential in salinized soil. Once taken up, Na^+ and Cl^- need to be treated properly. The expulsion of both ions can be only temporal or partial solution, because this inevitably provokes futile and energy-costing NaCl cycling. Yet within plant, exclusion of toxic ions by metabolically active tissues and their relocation to less vulnerable or less metabolically important ones, along with their intracellular sequestration into vacuoles may serve as a working strategy for the stress resistance. In this paper, we will focus solely on the cation transport, whereas interested readers may found the update on the Cl^- transport in relation to salt resistance in the excellent review by Teakle and Tyerman (2010).

Root hairs and epidermis are the first plant tissues to encounter the elevated salinity. Na^+ may be transported then up to the Casparian band of the endodermis both via apoplastic or symplastic pathways (Fig. 12.1). There it is forced to cross the

plasma membrane, albeit some important crops (rice) display interruptions in the endodermis, so that Na^+ loading into xylem via entirely extracellular (apoplastic) pathway is possible (Munns and Tester 2008; Kronzucker and Britto 2011; Horie et al. 2012). Moving with a transpiration stream and being partly absorbed by stems, Na^+ eventually enters leaves and there exerts its toxic effects on the photosynthesis. Leaves are the organs which accumulate the highest amount of Na^+ in planta (Conn and Gulliham 2010), because the removal of Na^+ to phloem is limited (Munns and Tester 2008). This has dramatic effects on the overall plant economy and growth. On the contrary, some (mostly, dicotyledonous) halophytes can efficiently expel Na^+ from leaves via transformed trichomes, salt bladders, or salt glands (Flowers and Colmer 2008; Shabala and MacKay 2011). Contrary to Na^+, K^+ appears to be a rather mobile ion, and it can be loaded into phloem and translocated to actively grown tissues like shoots and root apices (Marschner 1995). The recirculation of K^+ obviously uses pathways, which are selective for K^+ over Na^+, like weakly inward rectifying K^+ channels AKT2/3, participating in phloem loading and unloading (Marten et al. 1999; Gajdanowicz et al. 2011).

Redistribution of Na^+ and K^+ within plant under salt stress depends on the degree of cell connections and, when exchanged apoplastically, on the relative expression of different cation and K^+ channels and transporters, and on energy considerations (electrochemical gradients for both ions across tonoplast and plasma membrane for each cell types and in case of transporters also on the nature of co-transported ion and on its gradient). Some routes of Na^+ and K^+ transport across plasma and intracellular membranes are relatively selective for K^+, but most of them are not. Plants in their plasma membranes are expressing a great variety of ion channels and transporters, only weakly differentiating between Na^+ and K^+. These are potentially suitable for low-affinity Na^+ influx under salinity as well as for Na^+-induced K^+ efflux. The open question is that which routes are de facto major contributors, and how their relative contribution varies between different species and tissues. In addition, salt stress and related factors, like increased levels of reactive oxygen species (ROS) and polyamines (PAs), tend to remodel the plasma membrane ionic conductance. In particular, ROS are capable to induce novel nonselective conductance permeable for Ca^{2+} (Pei et al. 2000; Demidchik et al. 2003; Foreman et al. 2003) and both ROS and PAs are capable to suppress some constitutively expressed plasma membrane K^+ channels and nonselective cation channel (NSCC). Tonoplast represents a simpler system than plasma membrane, expressing only two nonselective (FV and SV) and one K^+ selective (VK) channels; their properties are remarkably similar between different tissues and species (Pottosin and Muñiz 2002; Pottosin et al. 2003; Pottosin and Schönknecht 2007). Yet, salt stress and stress-related factors may cause changes in channels' expression and regulation also in tonoplast, thus, remodeling its cation conductance and Na^+/K^+ selectivity. In this chapter we will consider, what is currently known on the properties of K^+ and nonselective Na^+-permeable channels and transporters in vacuolar and plasma membrane, as well as on the changes of their activity, induced by salt stress or related metabolites, like ROS and polyamines.

2 Thermodynamics of the Symplastic Na^+ Transport and Tissue Na^+ Distribution

Roots absorb Na^+ via symplastic and apoplastic pathways (Fig. 12.1), but their relative contributions are not well established. Yet apoplastic pathway may play an important role in some species. An important example is rice, which displays interruptions in endodermis so that Na^+ may be uploaded to xylem via the "apoplastic bypass." Deposition of silicon, which decreased apoplastic transpiration rate fivefold, efficiently decreased Na^+ uptake by rice seedlings and increased their salt resistance (Gong et al. 2006). Similar effects of silicon on Na^+ uptake were reported for sugarcane and canola (Ashraf et al. 2010; Farshidi et al. 2012). However, more than a half of Na^+ reaches shoot via symplast (Wu and Wang 2012) and the bypass pathway in rice may be strongly reduced by Ca^{2+} (Anil et al. 2005).

Symplastic pathway implies that Na^+ needs once to cross the plasma membrane. Potentially, it can occur at any cells layer on the way from root surface to the xylem, but basing on the available data on ion distributions this may happen already at the level of root hairs/epidermis. Plant cells possess a very negative membrane potential difference (PD). In root hairs and root epidermis, the PD is ranging between −100 and −200 mV (cytosol negative) under normal law salt conditions (Mertz and Higinbotham 1976; Lew and Spanswick 1984; Lew 1991; Hirsch et al. 1998). The equilibrium potential for Na^+, E_{Na} is given by the Nernst equation:

$$E_{Na} = RT / F \ln\left\{\left[Na_o\right]/\left[Na_i\right]\right\}$$

where $[Na_i]$ and $[Na_o]$ are cytosolic and extra-cytosolic Na^+ concentrations, T is absolute temperature, R and F are universal gas and Faraday constants, respectively.

For 20 °C, this equation can be rewritten as:

$$E_{Na} = 58\ mV \lg\left\{\left[Na_o\right]/\left[Na_i\right]\right\}$$

$[Na_i]$ or cytosolic Na^+ concentration is normally low (10 mM or less), when plants are grown in non-saline conditions. For a long time, it was believed that plants may not tolerate $[Na_i]$ >30 mM (Munns and Tester 2008). Yet under salinized conditions, values up to 200 mM were reported for roots of some plants, and even higher concentrations may be found in leaves (Kronzucker and Britto 2011). In all cases, however, $[Na_i]$ may not exceed external Na^+, $[Na_o]$. Therefore, E_{Na^+} is *positive* at all conditions, so that Na^+ *influx* into cytosol from the exterior will be passive unless PD would become positive, which was rarely reported for plants, although external salt may induce large membrane depolarization. This depolarization may be transient or long (up to days) lasting, depending on the plant species (see Sect. 4). Conversely, Na^+ extrusion to the external medium needs to be active. In plants, Na^+/H^+ antiporter (SOS1) is believed to be a principle mediator of the active Na^+ efflux across the plasma membrane (Zhu 2003).

For an electroneutral Na^+/H^+ antiporter, the condition of the net Na^+ efflux is met when

$$\lg\left\{\left[Na_o\right]/\left[Na_i\right]\right\} < \Delta pH$$

For normal soil pH range 5.5–6.5 and typical cytosolic pH of 7.2–7.4, this implies that SOS1 can lower the cytosolic Na^+ concentration by 1–2 orders of magnitude compared to the external Na^+. However, in increasingly alkaline soils its operation as a Na^+ efflux pathway will be handicapped. SOS1, which is preferentially expressed at xylem/xylem parenchyma boundary (Shi et al. 2002), likely participates in the long-distance transfer (Fig. 12.1), in particular, in the xylem loading (De Boer and Volkov 2003). Given that the xylem vessel space is by 60 mV more positive as compared to the interior of xylem parenchyma, the requirement for active transport stays unless more than tenfold gradient of Na^+ is built up between these two compartments (Munns and Tester 2008).

However, there are scenarios which consider that at stress conditions potential difference across xylem parenchyma plasma membrane drops and cytosolic Ca^{2+} increases. This causes the activation of NORC (nonselective outward rectifying channels), and they in turn clamp PD close to zero and may mediate Na^+ and Cl^- uploading, which will be passive under these conditions (Wegner and De Boer 1997). Indeed, under strong salt stress, the PD in cortical cells reaches the values close to zero or even positive (Hua et al. 2008). Basing on the trans-root potential (TRP) values before acute application of salt shock and after TRP relaxation to a steady-state value, potential difference in parenchyma of the intact stele in maize and barley is by some 20 mV more positive as compared to the cortex, favoring the above-mentioned scenario (Wegner et al. 2011). For K^+ it appears that passive transport accounts for more than a half of K^+ into xylem at any condition (Gaymard et al. 1998; Lacombe et al. 2000).

Na^+ is not equally distributed between different cell types within a root. A common pattern, observed in salinized roots of durum wheat or maize, displays major salt (Na^+ and Cl^-) accumulation in epidermis and the lowest Na^+ (highest K^+) concentration in the inner cortex (Hajibagheri et al. 1987; Läuchli et al. 2008). These data support the dominance of symplastic pathway for Na^+ uptake and that most Na^+ ions cross plasma membrane already in the root epidermis. Another control point in salt uptake is xylem parenchyma. At the boundary of xylem and xylem parenchyma Na^+ can flow in both directions, so that when Na^+ retrieval from xylem dominates it results in a higher Na^+ content in xylem parenchyma than in endodermis; the latter normally does not represent a barrier for Na^+ transport (Läuchli et al. 2008). It should be noted that radial decrease of Na^+ content from epidermis to inner root tissues is also observed in halophytes, but reflects a contrasting strategy in Na^+ handling. Whereas glycophytes, even tolerant ones like barley, restrict Na^+ loading to the xylem, many halophyte species facilitate xylem loading and transport of Na^+ (and Cl^-) to the shoot, resulting in contrasting, low in xylem parenchyma and high in the shoot, Na^+/K^+ ratio (Storey et al. 1983b).

It is thought that Na^+ accumulation in leaves generally (with some exceptions, like bread wheat) lacks tissue specificity (Munns and Tester 2008). Yet in barley leaves, a much higher accumulation of Na^+ (in parallel with K^+ and Cl^-) was observed in the epidermis as compared to the mesophyll (Karley et al. 2000a). At the same time, plasma membrane expressed similar sets of inward rectifying Na^+ and K^+-permeable channels. However, activity of Na^+-permeable channels is higher in epidermis. Therefore, preferential accumulation of Na^+ in epidermis may be due to the channel-mediated uptake of this ion from apoplast. Symplastic relocation, from xylem via bundle sheath, may play a limited role, because it would result in almost equal Na^+ distribution between different cell types (Karley et al. 2000a, b). Yet studies on a different barley variety did not reveal any difference between Na^+ and K^+ contents in mesophyll, whereas imposed salinity caused Na^+ increase and K^+ decrease in both tissues, less and more prominent for Na^+ and K^+, respectively, in mesophyll (Fricke et al. 1994, 1996). Thus, Na^+ distribution and relocation pathways are sensitive to different growth conditions and to varietal differences. As an average, under non-stressed conditions, monocots (Poales) as well as dicots accumulate Na^+ mainly in epidermis (Conn and Gulliham 2010). It should be noted that widely accepted view on the strict correlation between Na^+ leaf content and decrease in photosynthesis (Munns and Tester 2008) may be not valid, at least for some species. For barley and durum wheat, maintenance of high cytoplasmic K^+ in mesophyll turns to be far more important for salt tolerance (Cuin et al. 2003; James et al. 2006).

Guard cells are isolated from the symplast and can take up ions only via apoplastic way. In some halophytes (*Cakile maritima*) under salt stress, stomata exchange K^+ for Na^+, without loss of function (Eshel et al. 1974). Yet *Atriplex tripolium* and *Aster subcoeruleus* possess an efficient (still unknown) mechanism to exclude or restrict Na^+ from guard cells (Perera et al. 1997; Robinson et al. 1997).

3 Routes for Na^+ Entry and K^+ Transport in Plasma Membrane

Plasma membrane in plants expresses two major K^+-selective currents, contrasting in their voltage dependence: a time-dependent inward rectifier (KIR), normally activating at potentials more negative than −100 mV, and outward delayed rectifier, KOR (Fig. 12.1).

These currents are encoded by K^+ channels genes, belonging to the *Shaker* family. High $K^+/Na^+ > 50$ selectivity (Amtmann et al. 2004), Na^+-induced membrane depolarization above E_K (see below), and salt-induced down-regulation of KIR in roots (Fuchs et al. 2005) makes its contribution for K^+ and Na^+ transport under salt stress highly improbable and will not be considered here. The only KIR channel, which may conduct outward current, once switched to a "leaky" mode of activity by dephosphorylation plus yet unknown amino acid modification is AKT2 (Michard et al. 2005; Sandmann et al. 2011). It is expressed in leaves

(guard cells and phloem tissues) and in the root stele. In guard cell it usually forms heteromeric complexes with KAT2, thus, expressing currents with intermediate properties (Xicluna et al. 2007). In a "leaky" mode AKT2 can mediate both phloem loading and unloading (Fig. 12.1), but nothing is known on its role under salt stress. However, it is hypothesized that under low ATP conditions, the energy, stored as $\Delta\mu K^+$ between phloem sieve elements and apoplast may be used via AKT2 for assimilates (sugars) reloading to phloem (Gajdanowicz et al. 2011).

On the contrary, KOR is activated by membrane depolarization and displays a lesser, $K^+/Na^+ \sim 10$, selectivity (Roberts and Tester 1997; Amtmann et al. 2004). These channels may mediate some Na^+ influx (yet not proved directly) but, indisputably more importantly, they contribute greatly to the Na^+-induced K^+ efflux in roots (Shabala et al. 2006, 2010; Chen et al. 2007a; Cuin et al. 2008). KOR are mediated by *GORK*, expressed in root epidermis and hairs, and in guard cells (Ivashikina et al. 2001; Hosy et al. 2003) and *SKOR*, expressed in stele (Gaymard et al. 1998; Lacombe et al. 2000). An interesting and unusual property of both KORs is their regulation (voltage-dependent inhibition) by external K^+. Mechanisms of voltage- and K^+-dependent gating of plant *Shaker* channels are described elsewhere (Johansson et al. 2006; Dreyer and Blatt 2009). For our purpose, however, it is important to mention, that a combination of gating by voltage and external K^+ makes KOR a genuine outward rectifier, which only opens at voltages *above* the E_K, thus promoting exclusively the *efflux* of K^+ from the cell. Moreover, gating by external cations followed the selectivity sequence for permeation, so that high external Na^+ caused little effect on the KOR voltage gating (Blatt and Gradmann 1997).

And there are a great variety of loosely classified, so-called NSCCs, only weakly discriminating between Na^+ and K^+, some are permeable also to divalent cations (Ca^{2+}) or even anions. Basing solely on the NSCC voltage dependence, there are hyperpolarization-activated NSCC (HA-NSCC or HACC), depolarization-activated NSCC (DA-NSCC or DACC), and relatively voltage-insensitive NSCCs (VI-NSCC or VICC) (Demidchik and Maathuis 2007). Solely in barley root epidermis there are at least four distinct NSCCs, differed by their voltage dependence (VICC or DACC), time-dependent or instantaneous activation modes, or displaying the mixture of both (Velarde-Buendía et al. 2012a). One of these currents is time- and voltage-dependent outward rectifier (NORC), which is also found in xylem parenchyma and is relatively well studied (Wegner and Raschke 1994; Wegner and De Boer 1997). NORC is almost equally permeable to Na^+, K^+, and anions, so, as already mentioned, once activated, it may mediate loading of all these ion species to the xylem (Wegner and De Boer 1997). Further classification of NSCCs is based on their regulation by intra- and extracellular factors. For the purpose of this review, it is important to mention ROS-activated NSCC, which are considered in detail in Sect. 7.

Special attention was paid to Ca^{2+}-sensitive Na^+-permeable VICCs, whose suppression by high external Ca^{2+} in some plant models (wheat, *Arabidopsis*, pepper *C. annum*, rice) resulted in a very substantial decrease of Na^+ influx, thus, probably, underlying a well-known effect of Ca^{2+} amelioration of the plant growth under salinity (Davenport and Tester 2000; Demidchik and Tester 2002; Rubio et al. 2003; Shabala et al. 2006; Wu and Wang 2012). The study of Na^+ influx

channels is strongly handicapped due to the absence of established links between electrophysiologically characterized NSCCs and encoding genes. Basing on the molecular biology data, likely candidates may be the members of the glutamate receptor (GLR) and cyclic nucleotide gated channels (CNGC) families, which alone in *Arabidopsis* comprise 20 members each, compared to 61 and 12 in poplar, and 13 and 10 in rice, respectively (Mäser et al. 2001; Ward et al. 2009).

When it comes to CNGC, a rapid increase of the intracellular cGMP levels under salt stress may serve as a clue for the role of these channels in stress response (Donaldson et al. 2004). Further, several CNGCs genes, in particular CNGC3, CNGC19, and CNGC20, rapidly (after few hours from the onset of stress) are up-regulated by salt (Maathuis et al. 2003; Kugler et al. 2009; Dietrich et al. 2010). CNGC3 is expressed in root cortex, epidermis, and shoots, but not in stele; *cngc3* null mutants show higher salt resistance (Gobert et al. 2006). Loss-off-function CNGC10 mutants show a lower Na^+ uptake (and less K^+ efflux by roots), but at the same time higher salt sensitivity at prolonged salt stress, attributed to Na^+ accumulation in leaves. Consequently, a role of CNGC10 in phloem loading/xylem retrieval was proposed (Guo et al. 2008). Using heterological expression system, some leak-like (nonselective currents with entirely linear current–voltage relation) were recorded for CNGC4 and CNGC10 in the presence of high, 0.1 or 0.5 mM, concentrations of cyclic nucleotides, but no direct evidence was presented for their activation by these compounds (Balagué et al. 2003; Christopher et al. 2007). Moreover, in planta studies demonstrated that cGMP or cAMP partly inhibit VICC activity and reduced Na^+ influx in *Arabidopsis* roots (Maathuis and Sanders 2001; Essah et al. 2003). Hua et al. (2003) reported that heterologously expressed AtCNGC2, in contrast to AtCNGC1, forms channels with a high K^+/Na^+ selectivity and further, attributed it to unique amino acid sequence in the selectivity filter of the AtCNGC2 (Hua et al. 2003). However, ionic currents, supposedly encoded by AtCNGC1 and AtCNGC2 were quite dissimilar to those, reported by other authors, displaying a time- and voltage-dependent KIR-like pattern. Clearly, more electrophysiological work needs to be done to yield more consistent results on the properties and regulation of currents, mediated by the members of CNGC gene family.

Even less is known on the properties of currents, encoded by GLR gene family. However, Demidchik et al. (2004) demonstrated glutamate-activated Na^+ and Ca^{2+} inward currents in *Arabidopsis* roots. Among multiple efforts to demonstrate agonist-activated currents, mediated by plant GLRs, pore domains of 17 *Arabidopsis* GLRs were transplanted into the rat GluR1 and GluR6 environments. This study demonstrated that at least those chimaeras, which contain AtGLR1.1 or AtGLR1.4, form functional Na^+-, K^+-, and Ca^{2+}-permeable pores; respective hybrid channels may be gated open by selected agonists (glutamate or kainate), and, once open, were sensitive to a variety of selective blockers of animal GluRs (Tapken and Hollmann 2008). These results imply that some members of plant GLR family contain a functional pore domain, but it remains unclear whether and how these NSCCs are gated in planta. Some clues for functional roles of plant GluRs come from expression studies. Plant *AtGluR2* is mainly expressed in vascular tissues; its overexpression resulted in high sensitivity to elevated external Na^+ and K^+, and in a

worse Ca^{2+} use efficiency, with symptoms of Ca^{2+} deficiency in shoots. The latter may be ameliorated by medium Ca^{2+} supplement (Kim et al. 2001). In a liverwort (*Conocephalum conicum*), sudden application of glutamate, glycine, NMDA, but not aspartate caused generation of action potentials. When plants were adapted to the presence of glutamate and glycine, the response to these substances was desensitized. Yet action potentials, caused by different stimuli, and their Ca^{2+} components were substantially modified under these conditions (Krol et al. 2007). Glutamate-induced spikes of intracellular Ca^{2+} were demonstrated also in higher plants (Dennison and Spalding 2000; Dubos et al. 2003; Stephens et al. 2008). In the latter study on *Arabidopsis* hypocotyls, it was demonstrated that six amino acids, alanine, asparagine, cysteine, glutamate, glycine, and serine are capable to induce cytosolic Ca^{2+} signal and respective depolarization. Complex, asymmetric pattern of the mutually caused desensitization was observed for the responses to sequential application of these compounds. In a recent paper, Michard et al. (2011) have shown that D-serine activates Ca^{2+} influx in pollen tubes, which modulates Ca^{2+} signaling and polarized growth. Thus, most of the studies made in planta addressed the participation of plant GLRs in Ca^{2+}-signaling, whereas their possible role in mediation of Na^+ transport was restricted to a single paper (Demidchik et al. 2004). It remains to be established, also, which of plant CNGCs and GLRs form functional cation channels, and whether these channels are truly ligand-gated ones and what are their natural ligands, or, alternatively, whether they may be constitutively active (Maathuis 2004; Dietrich et al. 2010).

Search of candidates for low-affinity Na^+ influx mediators in no case should be restricted to the members of GLR and CNG-regulated gene families. Recent studies demonstrated that some transporters from the HKT family can mediate channel-like currents. For instance, rice OsHKT2;4 under certain conditions forms a weakly selective cation channels, with some preference to K^+ over Na^+ (Lan et al. 2010; Sassi et al. 2012). In total, OsHKT2;4 may adopt two different modes of activity: as a K^+-selective uniporter at low Na^+ and as K^+-Na^+ symporter at high (>10 mM) external Na^+ and K^+ <3 mM, with K^+ and Na^+ sharing the same pore as evidenced by X-ray structural analysis (Cao et al. 2011; Sassi et al. 2012). On the contrary, TaHKT2;1 and OsHKT2;1 mediate high-affinity K^+-Na^+ symport at low Na^+ and K^+, but act as Na^+ uniporters at high Na^+ as under salt stress (Rubio et al. 1995; Jabnoune et al. 2009). OsHKT2;1-regulated Na^+ influx may be useful at a moderate salt stress, supporting plant growth, especially at low external K^+. Under strong salt stress, its activity, however, is rapidly post-translationally down-regulated, minimizing its role under sustained salinity (Horie et al. 2007). HKT transporters is not a multigene family, with an exception of rice, expressing nine different genes, four of Class 2 (described above) and five belonging to the Class 1. In genomes of other plants frequently two or just single HKT gene may be found, e.g. AtHKT1;1 in *Arabidopsis*. Class 1 HKT transporters are Na^+-selective and catalyze Na^+ influx across the plasma membrane (Horie et al. 2001, 2009, 2012). For example, rice OsHKT1;5 encodes Na^+-selective transporter when expressed in *Xenopus* oocytes (Ren et al. 2005). Recently, a direct electrophysiological evidence was obtained that AtHKT1;1 mediates passive Na^+ transport, with a reversal potential following

Nernst potential for this ion (Xue et al. 2011). Yet in the absence of single channel data, it is not possible to discriminate, whether it operates as a channel or uniporter. But how a Na^+-selective uniporter can in principle enhance salt tolerance? One possibility is its participation in phloem loading, thus, removing Na^+ from leaves (Horie et al. 2009). Another, more convincing, possibility, is based on the preferential expression of *Arabidopsis* AtHKT1;1 and rice OsHKT1;5 in xylem parenchyma, so that its role in re-absorption of Na^+ from xylem vessels was proposed (Sunarpi et al. 2005; Horie et al. 2007). In rice, OsHKT1;5-controlled Na^+ transport rate was larger in salt-tolerant as compared to salt-sensitive variety (Ren et al. 2005). And, more directly, the enhancer trap-mediated targeting expression of the AtHKT1;1 in root stele increased the efficiency of shoot Na^+ exclusion and underlies more salt-tolerant phenotype in *Arabidopsis* (Møller et al. 2009). Moreover, Na^+ uptake by xylem parenchyma may indirectly promote K^+ loading into xylem via membrane depolarization, so that xylem K^+ and Na^+ levels would be related reciprocally (Horie et al. 2012). In line with this hypothesis, *athkt1;1* loss-of-function mutants are characterized by increased Na^+ and decreased K^+ contents in xylem vessels (Sunarpi et al. 2005). A different role under salt stress was suggested for McHKT1 from the halophyte *M. crystallinum* or ice plant. Although this transporter is predominantly expressed in xylem parenchyma, its expression in different tissues, in particular in leaves and stems, is also significant. And, unlikely other HKT1 transporters, it conducts Na^+ and K^+ equally. Onset of the salt stress is followed by a transient increase of the McHKT1 expression, paralleled with the initial osmotic adjustment in leaves (Su et al. 2003). Thus, authors speculated that McHKT1 at early stages of stress can mediate Na^+ transport from roots to leaves, where it is safely deposited into vacuoles.

High-affinity KUP/HAK/KT transporters are undisputedly key players in K^+ uptake, especially under K^+-deficient conditions, and most of them display a very high affinity for K^+ over Na^+. Yet, Na^+ transport function is not yet ruled out for this class of transporters, so that they can mediate high-affinity K^+ and low-affinity Na^+ influx. However, down-regulation of some of these transporters by salt, absence of reduction of Na^+ influx in loss-of-function mutants, and lack of sensitivity of low-affinity Na^+ influx to NH_4^+, a competitive inhibitor of KUP/HAK/KT transporters, raise doubts on their possible involvement in Na^+ influx under salt stress (Kronzucker and Britto 2011). Another low-affinity Na^+ transporter LCT1 is found so far solely in wheat (Amtmann et al. 2001), so its functional importance may be restricted to this species. Finally, cation-Cl^- contransporters (CCCs), whose function is well known in animals, are described also in plants (Colmenero-Flores et al. 2007). Heterologically expressed AtCCC significantly increased Na^+ and Cl^- uptake, which was inhibited by diuretic bumetanide. Bumetanide also inhibited up to 50 % of Na^+ accumulation in planta, *Arabidopsis* or halophyte *Suaeda maritime*, which argued for its contribution to a low-affinity Na^+ uptake under saline conditions (Colmenero-Flores et al. 2007; Zhang et al. 2010).

Summarizing, there are good reasons to think that multiple pathways contribute to low-affinity Na^+ influx under salt stress (Fig. 12.1), that their relative contributions may depend on species and growth conditions as well as on the severity of the

stress, and that changes in expression and regulation during sustained salt stress may exert further effects on the implemented pathways. Already in early studies (Epstein and Rains 1965), a spectrum of transport states for K^+ and Na^+ was observed, which were partly overlapped and can not be easily separated. In *Arabidopsis* a low-affinity Na^+ influx is clearly multi-component and does not show any saturation on external Na^+ concentration up to 250 mM (Essah et al. 2003). Application of nonspecific inhibitors (and specific ones are not available), such as *N*-ethylmaleimide for high-affinity K transporters, tetraethylammonium (TEA^+) as a general potassium channel blocker, or lantanides as generic NSCC blockers may result in misinterpretations. For instance, high-affinity K^+ transporters (AtKUP1) are sensitive to TEA^+ and other nonspecific potassium channel blockers. And different NSCC possess a variable sensitivity to Gd^{3+}, whereas not only VICCs, but also LCT1 and HKT2 are sensitive to external Ca^{2+} (Amtmann et al. 2001; Demidchik and Maathuis 2007; Cuin et al. 2008; Yao et al. 2010). Use of voltage-dependent blockers like Ba^{2+} and Cs^+ may be even more misleading, when results of in vivo experiment (e.g. of ion flux or tracer measurements), with a free running membrane PD are compared with a data of patch- or voltage-clamp experiment under fully controlled membrane PD. Consequently, the extent of block, reported in these two types of experiments, may be quite different. Also, buffering properties of the cell wall and kinetics of the blocker delivery may affect the result. In general, comparison of in vivo ion flux data, especially unidirectional fluxes, with net currents in a typical patch-clamp experiment deserves a lot of caution.

4 Acute Salt Stress, Membrane Depolarization, and K^+ Efflux

Resting membrane potential difference (PD = −100 to −150 mV) in cells of the most plants only weakly reflects external (either K^+ or Na^+) salt concentration changes in submillimolar to low millimolar range (Etherton 1963; Higinbotham et al. 1964; Davis 1972; Roberts and Snowman 2000), although some authors report larger changes already for low millimolar range of K^+ (Dunlop and Bowling 1971; Hirsch et al. 1998). This is because the plasma membrane is normally encountered in so-called "pump-state," when its conductance is dominated by H^+ pumps (Spanswick 1981). Yet, changes of external Na^+ (or K^+) above 10 mM can only partly be counterbalanced by H^+-pump current and a significant (by 60–80 mV in response to 50–100 mM NaCl) depolarization results (Higinbotham et al. 1964; Shabala et al. 2006, 2007; Chen et al. 2007a; Hua et al. 2008).

Salt or osmotically equivalent mannitol treatment causes the acute activation of H^+ pumping in root and mesophyll cells. But salt stress provokes membrane depolarization and K^+ efflux, whereas hypertonic stress (mannitol) on the contrary, induces membrane hyperpolarization and K^+ influx (Shabala 2000; Cuin et al. 2003; Ober and Sharp 2003). In case of salt stress, the fast hyperpolarization caused by plasma membrane H^+ pump activation is simply masked by a massive

depolarization caused by Na^+ entry. Once salt-induced depolarization occurred, passive (channel-mediated) K^+ influx will be not possible unless the repolarization takes place. It may be rapid in some species (10–15 min in pea roots, Bose, Pottosin and Shabala, unpublished) or depolarization may last over several days (barley roots and leaves, Carden et al. 2003; Cuin et al. 2003). Depolarization over E_K provides a driving force for K^+ efflux via any available cation channel (activated by depolarization or simply active at these conditions). In *Arabidopsis* and barley roots and isolated steles, most of the Na^+-induced K^+ efflux is inhibited by nonspecific K^+ channel blocker TEA^+, which implies that it is mainly KOR-mediated (Shabala et al. 2006, 2010; Chen et al. 2007a). *Arabidopsis* loss of KIR function mutant *akt1* displays the same response to salt as wild type. In contrast, *gork1* mutant, lacking KOR currents, shows greatly reduced Na^+-induced K^+ efflux. Conversely, *akt1* mutants respond by K^+ efflux instead of influx to hypertonic (300 mM mannitol) treatment (Shabala and Cuin 2007). Thus, in roots of *Arabidopsis* Na^+-induced K^+ efflux is mainly mediated by KOR. However, in pea mesophyll, both Na^+ influx and Na^+-induced K^+ efflux are likely mediated by NSCC (Shabala et al. 2007). Salt-induced Na^+ influx in root and leaves is manifold (at least by order of magnitude) higher than concomitant K^+ efflux (Shabala 2000; Shabala et al. 2006), but it is the loss of intracellular K^+, which per se may cause severe penalties for plant performance under stress (see below).

Changes in the intracellular K^+ are quick. In root epidermis, for instance, K^+ activity is halved after 10 min of the onset of salt stress, although it is partly restored after half an hour, due to K^+ release from the vacuole (Shabala et al. 2006). Similarly, after 10 min there were transient decreases by 40–60 % of xylem K^+ (Wegner et al. 2011). Xylem K^+ is further restored, but tissue K^+ decreases. After less than half an hour of application of 50 mM KCl or NaCl to barley roots, correlated alkalinization of the leaf apoplast and increase of apoplastic K^+ (tenfold, from 3 to 30 mM) occurred. This happens more likely due to a direct travel of K^+ and Na^+ in the transpiration stream, because the closure of stomata retarded the response. Ionic basis of this response was demonstrated by the fact that no response to sorbitol treatment was observed (Felle et al. 2005).

5 Barley Case Study: K^+ Retention Under Salinity Is Crucial for Salt Tolerance

Barley is normally a salt-tolerant crop, but the existence of a large collection of barley genotypes, involving also salt-sensitive ones (Bothmer et al. 2003), makes barley an attractive model to study mechanisms of salt tolerance. Initial study by Chen et al. (2005), using seven barley varieties, contrasting in salt tolerance, revealed that traditional traits like growth rate or weight, CO_2 assimilation, chlorophyll fluorescence, and water and elemental (Na^+ and K^+ contents), measured in plants after 5 weeks under salt stress displayed none or relatively poor correlation with the salt tolerance, unless a very strong (320 mM NaCl) salt stress was applied. On the contrary, measuring of the magnitude of NaCl (80 mM)-induced K^+ efflux

from the mature zone roots of 3-day-old seedlings by noninvasive MIFE technique revealed a very substantial difference between salt-sensitive and salt-tolerant varieties. Na^+-induced K^+ efflux varied from 20 nmol m^{-2} s^{-1} (most tolerant) to 150–180 nmol m^{-2} s^{-1} (most sensitive). The extension of this work to a larger number of contrasting barley varieties revealed that for 62 out of 69 it was strong ($P<0.001$) inverse correlation between the magnitude of the Na^+-induced K^+ efflux from roots and relative grain yield after 4–5 months of growth under salinity (Chen et al. 2007b). This correlation seems striking, because a short-term response of the "first line of defense" somehow determined the final fate of the plant under long-term salt stress. Therefore, the mechanism of early response of barley to acute salt application was worth to be studied in detail. Chen et al. (2007a) first demonstrated that the large fraction of NaCl-induced K^+ efflux is inhibited by TEA^+. Keeping in mind the NaCl-induced depolarization, it strongly suggested the involvement of KOR channels. Larger KOR currents in salt-sensitive genotypes may be due to: (a) higher KOR functional expression, (b) altered regulation (e.g. by membrane voltage), or (c) larger depolarization. Further experiments have shown that hypotheses (a) and (b) were false, whereas (c) quantitatively fulfilled with the predictions. Larger depolarization (worse control of the membrane potential) was at least in part due to a lower activity of the plasma membrane H^+-ATPase. At the same time, H^+-ATPase expression levels were equal in salt-tolerant and salt-sensitive varieties, so observed differences in the H^+-ATPase activity was due to its posttranslational regulation. Depolarization in the root cortex maintained for several days under salt stress, yet more tolerant barley variety showed partial repolarization after 1 week under salinity (Carden et al. 2003). Summarizing the results of these and other studies (Ershov et al. 2005; Zepeda-Jazo et al. 2008a; Shabala et al. 2010) salt resistance in barley is relied on the following: (a) better control of membrane voltage, so retaining a more negative membrane PD in the root symplast and reducing depolarization-induced K^+ loss; (b) intrinsically higher H^+ pump activity in roots as a main cause of better membrane potential control; (c) better ability of Na^+ exclusion from roots to the external medium; (d) higher sensitivity of root plasma membrane Na^+ transporters to supplemental Ca^{2+}; (e) lower expression of nonselective NORC channels; (f) higher K^+ in xylem with approximately the same level of xylem Na^+, which may represent a fine balance between activities of SKOR, NORC, HKT1, and SOS1 at the xylem parenchyma–xylem boundary, and (g) higher transpiration rate paralleled with a better vacuolar Na^+ sequestration in mesophyll, due to a higher vacuolar Na^+/H^+ activity in salt-tolerant barley varieties.

6 Salt-Tolerant Plant Species Possess Higher Overall Na^+/K^+ Selectivity, Better K^+ Retention, and Control of Plasma Membrane Electric Potential Difference

Qualitatively similar results to those reported to barley were obtained by means of noninvasive flux measurements on roots of wheat (two bread and two durum wheat genotypes, contrasting in their sensitivity to salt, Cuin et al. 2008) and two poplar

species, salt-sensitive *P. popularis* and salt-tolerant *P. euphratica* (Sun et al. 2009). Importantly, bread wheat, in contrast to barley, which is "salt-includer" (sequestering Na^+ into vacuoles), relies more on the Na^+ exclusion. Quantitative trait loci (QTL) analysis shows co-localization of genes, controlling K^+ and Na^+ in barley, whereas in wheat they are located in different chromosomes (Nguyen et al. 2013). This is likely related to a special role of HKT transporters, restricting root-to-shoot Na^+ transport in wheat and their limited role in salt tolerance for barley. On the other hand, *P. euphratica* is woody plant, used for re-forestation of alkaline and salinized soils and it, like barley, relies on the vacuolar Na^+ sequestration (Chen et al. 2002). Thus, plants different otherwise in their stress-resistance strategies or taxonomically very different appear to use similar strategy for K^+ retention. In poplar Na^+-induced K^+ efflux was strongly potentiated due to the inhibition of plasma membrane H^+-ATPase by ortovanadate, partly inhibited by TEA^+, a K^+ channel blocker, and completely abolished in the presence of high external Ca^{2+} (Sun et al. 2009). These data imply important role of H^+-ATPase in preventing of Na^+-induced membrane depolarization and roles for KOR and NSC channels in salt-induced K^+ efflux. In case of poplar, the contribution of (Ca^{2+}-sensitive) NSCC in mediation of the Na^+-induced K^+ efflux was apparently larger as compared to barley. A very extreme case, little or no variation of the PD (−140 to −130 mV) in the root epidermis, subjected up to 600 mM NaCl, was reported for the halophyte plant *Atriplex hastate*. Consequently, there were no significant decrease in the K^+ content in salinized roots of this plant, although root Na^+ and Cl^- levels duplicated at 500 mM NaCl in the growing medium as compared to control (100 mM NaCl) conditions (Anderson et al. 1977).

Thellungiella halophila is a salt-tolerant relative of *A. thaliana*. It is unusual halophyte species, with a marked Na^+ exclusion strategy. *Thellungiella* and *Arabidopsis* generate unilateral Na^+ efflux of comparable magnitude, though *Thellungiella* shows a much lower unilateral Na^+ influx and accumulates less Na^+ than *Arabidopsis* (Wang et al. 2006). The patch-clamp study of Volkov and Amtmann (2006) revealed the presence of three major channels in the plasma membrane of *Thellungiella* and *Arabidopsis* root cells: highly K^+-selective inward rectifier (KIR), time-dependent outward current with $P_{Na}/P_K \sim 0.07$, and moderately ($P_{Na}/P_K \sim 0.15$) K^+-selective weakly voltage-dependent instantaneous current. The latter was ~5 times more selective than respective current in *Arabidopsis* ($P_{Na}/P_K \sim 0.72$). This difference, therefore, may account for relatively high K^+/Na^+ selectivity for the cation absorption by *Thellungiella* roots as compared to *Arabidopsis*. In addition, respective current density in *Thellungiella* was two times lower. This current was insensitive to Cs^+ or TEA^+, but was sensitive to external Ca^{2+}. The sensitivity to external Ca^{2+} was similar to those for Na^+ influx and root Na^+ accumulation, which suggests a large contribution of this instantaneous current to Na^+ influx. On the contrary, partial sensitivity of both KIR and K^+ content in roots to Cs^+ or TEA^+ suggests a role of KIR in K^+ uptake under both control conditions and salinity.

Thellungiella and *Arabidopsis* differed greatly in NaCl-induced depolarization: membrane PD upon acute application of 100 mM NaCl changed from −119 to −82 mV and from −105 to −42 mV, respectively. More strikingly, after several

successive depolarizations by NaCl, the PD in *Thellungiella* was hyperpolarized (−138 mV), whereas *Arabidopsis* displayed a rather depolarized value, −25 mV. Hyperpolarization in *Thellungiella*, below equilibrium potential for any present ion, indirectly indicated a strong contribution of the H^+ pump activity. Recent studies have shown that hyperpolarization induces increase in the transcription level of HAK5 and respective K^+ uptake (Amtmann 2009). When it comes to leaves of salt-grown *Thellungiella*, Na^+ accumulation and K^+ loss was higher in epidermis than in neighboring tissues (Volkov et al. 2003). As compared to *Arabidopsis*, *Thellungiella* deposited higher concentration of Na^+ in leaves. *Arabidopsis*, in contrast to *Thellungiella*, displayed an opposite tissue distribution of K^+, with a higher concentration in epidermis. The authors speculated that one of the winning strategies of *Thellungiella* was to use epidermis as a main store of Na^+ and, more importantly, as a source of K^+ for the rest of the leaf tissues with a higher metabolic activity, though for *Thellungiella* it is believed that Na^+ exclusion by roots is more crucial for salt tolerance. More specifically, higher K^+/Na^+ selective uptake by roots reduces Na^+ influx, thus reducing the need for the energy-costing Na^+ efflux. Reduced root-to-shoot Na^+ transport on the other hand is supported by the activity of SOS1-mediated Na^+ retrieval from the xylem (Amtmann 2009).

Preferential accumulation of Na^+ in leaf epidermis is typical for dicots (Conn and Gulliham 2010) but more pronounced in halophyte dicotyledonous species (e.g. in *Atriplex spongiosa*, Storey et al. 1983a). When it comes to roots, halophytes display unusually high degree of selectivity of uptake between Na^+ and K^+, $S_{K/Na}$ (ratio of K^+/Na^+ in plant to K^+/Na^+ in the growing medium) about 10 and 40, as an average, for dicots and monocots, respectively (Flowers and Colmer 2008; Shabala and MacKay 2011). Thus, halophytes maintain relatively high cellular K^+ on the saline background, but also absorb sufficient quantity of Na^+, required for osmotic adjustment and turgor maintenance. It should be noted again in this context that many halophytes utilize a strategy, which is different from that used by glycophytes: instead of limiting the root-to-shoot Na^+ transport, halophytes facilitate it, resulting in a high accumulation of Na^+ in the shoot vacuoles (Storey et al. 1983b).

7 Remodeling of the Plasma Membrane Ion Conductance by Stress-Related Factors: Polyamines, ROS, and Compatible Solutes

Salt by itself hardly affects the plasma membrane ion channels activity. However, there was a report on salt inhibition of KOR by intracellular Na^+ in a halophyte *Aster* species (*A. tripolium*) and the absence of such effect in non-halophyte *A. amelus* (Véry et al. 1998). The effect, therefore, was indirect in nature (via some messenger). Just another report is a selective inhibition of KIR but not KOR by cytosolic Na^+ as low as 10 mM in *Arabidopsis* roots (Qi and Spalding 2004). This finding, however, was not confirmed in the future studies (Volkov and Amtmann 2006). On the other hand, metabolic changes, accompanying salt stress, caused

enhanced production of polyamines (PAs), ROS, and so-called "compatible solutes" (principally, glycine betaine and proline). All these compounds are proved to regulate ion transport across the plasma membrane.

Polyamines are unique polycation metabolites, products of the catabolization of ornithine or arginine; only the latter pathway is operated in a model plant *Arabidopsis thaliana* (Hanfrey et al. 2001). Thus, in three enzymatic steps, with a crucial one catalyzed by arginine decarboxylase (ADC), diamine putrescine (Put^{2+}) is formed. Sequential addition of aminopropyl groups by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively, leads to a formation of higher polyamines, spermidine (Spd^{3+}) and spermine (Spm^{4+}). Expression of ADC and SPMS is up-regulated by salt stress (Alcázar et al. 2006, 2010; Gill and Tuteja 2010). Obviously, Put^{2+} and Spm^{4+} levels are important for salt stress tolerance, as loss of function for the enzymes of PAs biosynthesis mutants of *Arabidopsis* are oversensitive to salt stress (Kusano et al. 2008), whereas exogenous supply of Put^{2+} or Spm^{4+} reversed stress-oversensitive phenotype (Urano et al. 2004; Yamaguchi et al. 2006; Kusano et al. 2007a, b). Conversely, a variety of plants (rice, *Arabidopsis*, eggplant, apple, pear, tobacco) overexpressing enzymes for polyamine biosynthesis and displaying an overproduction of Put^{2+} and/or Spm^{4+}, also show an increased salt tolerance (Alcázar et al. 2010; Gill and Tuteja 2010; Hussain et al. 2011). But how PAs exert their protective function under salt stress remains elusive.

In animal cells, the principle targets for PAs are K^+-selective and NSCCs (Drouin and Hermann 1994; Ficker et al. 1994; Lopatin et al. 1994; Bähring et al. 1997; Williams 1997; Lu and Ding 1999). There are, however, only few reports of PAs effects on the plasma membrane ion channels in plants. All PAs inhibit KIR (KAT1) channels in *Vicia faba* guard cells and induced stomata closure (Liu et al. 2000). Of PAs, Spd^{3+} level greatly increased upon water stress, so the authors speculated that its contribution to stomata regulation may be important for the plant response to drought. It should be noted that the effect of Spd^{3+} was from the cytosolic side and indirect in nature, so that the activity of single KIR channels in a small isolated inside-out patch was unaffected by the application of Spd^{3+}, indicating that some important mediator is missing upon membrane patch isolation. In barley roots, salt-induced increases of PAs or their exogenous applications were paralleled with a decrease of root to shoot Na^+ transport and higher shoot Na^+/K^+ ratio (Zhao et al. 2003; Zhu et al. 2006). This appears to be a consequence of the following effects of PAs on ion channels in plasma membrane of root epidermal and stellar cells: inhibition of KIR and instantaneous Na^+-permeable NSC currents in epidermis and stele and increase of activity of KOR currents in stele (Zhao et al. 2007). It should be noted that only external but not intracellular PAs application was efficient in this case. Thus, rather than blockage, the effects of PAs on the plasma membrane ion channels in barley roots likely were indirect. In line with this proposal, we found a great variability of inhibition of whole cell KOR and KIR currents expressed in barley root epidermis, from almost complete suppression to the absence of any effect. Yet, as an average of all observed cases 1 mM of Put^{2+} or Spm^{4+} caused a reduction of KOR and KIR currents by 50 % and 60 %, respectively (Zepeda-Jazo and Pottosin, unpublished). In pea mesophyll, PAs acted on (inhibited)

constitutively expressed Na^+-permeable NSCC with a delay of 10 min, also suggesting an indirect mechanism (Shabala et al. 2007). In the latter study, inhibition of NCCC by PAs was associated with a decrease of NaCl-induced membrane depolarization and intracellular K^+ loss. PA-mediated decrease of the plasma membrane cation conductance, and especially, NaCl-induced K^+ loss, led to a hypothesis that this effect may be part of the salt-resistant strategy, with a potential impact on the improvement of cytosolic K^+/Na^+ relation (Kusano et al. 2007b; Zepeda-Jazo et al. 2008a, b). Yet a direct test revealed that such a scenario not necessarily dominates plant ionic response under salt stress. Although pretreatment by PAs indeed reduced NaCl-induced K^+ leak from roots in some cases, depending on plant species and growth conditions, amelioration of the K^+ loss by PAs may be cancelled or even, transformed to an increase of K^+ efflux (Pandolfi et al. 2010). Stimulation of the NaCl-induced K^+ efflux by PAs was especially intriguing and forced us to search for alternative effects of PAs, which are different from inhibition or block of ion channels.

Abiotic stresses, in particular drought and salinity, also cause increases in ROS production and in ROS-dependent signaling (Garg and Manchanda 2009; Miller et al. 2010). Therefore, increases in ROS and PAs levels under stress come hand-to-hand. Recent studies revealed important roles of PAs in balance of the ROS species. Although PAs may directly act as ROS scavengers (Das and Misra 2004), inhibit the activity of ROS-generating enzymes (Papadakis and Roubelakis-Angelakis 2005), or activate antioxidant system (Gill and Tuteja 2010), PAs catabolization by itself produces H_2O_2, a renowned signaling molecule. Apoplast is important compartment for the PAs catabolization, but they need to be exported there via yet unknown active mechanism, working against large electrical PD across the plasma membrane. Further, availability of either diamine oxidase (DAO) or polyamine oxidase (PAO), abundant in legumes or cereals families, respectively, may underlie some specificity of responses, involving diamine (Put^{2+}) or polyamines (Moschou et al. 2008a, b; Angelini et al. 2010). Moreover, due to the presence of transient metal ions, copper in the active center of DAO itself and iron in peroxidases associated with the cell walls, hydroxyl radicals ($OH^{\bullet}$), the most powerful ROS species, are generated via Fenton reaction and have direct effects on the cell wall loosening and organ growth (Schopfer 2001; Liszkay et al. 2004; Kukavica et al. 2009). There are two well-established examples of stress signaling, related to the PAs catabolization and ionic transport across the plasma membrane. First is the induction of stomatal closure by H_2O_2, generated by plasma membrane NADPH-oxidase and/or DAO. These are downstream elements in ABA-signaling cascade. H_2O_2 inhibits KIR and activates Ca^{2+} influx channels; high cytosolic Ca^{2+} activates anion channels, mediating anion influx coupled to K^+ efflux via GORK, thus releasing solute and water and causing, therefore, stomatal closure (Pei et al. 2000; An et al. 2008; Wang and Song 2008). Another example is the production of H_2O_2 by spermine export to apoplast and its oxidation by PAO, reduction of H_2O_2 to $OH^{\bullet}$, which in turn activates Ca^{2+} influx channel in plasma membrane. In leaves of salinized plants, plasma membrane NADPH-oxidase activity is suppressed, which may cause a reduction of leaf growth. Therefore, export of Spm^{4+} to the apoplast and its oxidation there becomes the major

source of ROS, necessary for the induction of Ca^{2+} influx and Ca^{2+}-dependent leaf blade growth (Rodríguez et al. 2009). ROS production and ROS-activated cation (Ca^{2+}) current is essential for the growth of roots, root hairs, and polarized growth in general (Demidchik et al. 2003; Foreman et al. 2003; Cárdenas 2009; Swanson and Gilroy 2010). This current was poorly selective and conducted a variety of mono- and divalent cations up to TEA^+ size. It appears that the populations of ROS-activated Ca^{2+}-permeable channels differ along the root: in the elongation zone both external H_2O_2 and $OH^\bullet$ activate Ca^{2+}-permeable currents, whereas cells in the mature zone sense only $OH^\bullet$ (Demidchik et al. 2007). OH, due to its very short half-life operates only within 1 nm distance from the point of its generation. Thus, $OH^\bullet$, externally generated via Fenton reaction, catalyzed by Cu^+ or Fe^{2+}, will affect only external face of the membrane. Increase of copper transporter expression lowers by 1–2 orders of magnitude the external Cu^+ concentration, which is required to induce significant cation (K^+ and Ca^{2+}) conductance in the plasma membrane of *Arabidopsis* root elongation zone (Rodrigo-Moreno et al. 2013). This result implies that respective conductance is more sensitive to cytosolic as compared to externally generated $OH^\bullet$. In guard cells, H_2O_2 at concentration as low as 10 μM inhibits KIR and GORK channels (Köhler et al. 2003), whereas $OH^\bullet$ activates GORK channels in roots, which mediate K^+ loss, eventually leading to a programmed cell death (Demidchik et al. 2010).

When it comes to the effects of PAs on the ROS-induced currents, two scenarios were conceivable in accord with the previous knowledge: (a) PAs catabolization may cause increase in ROS levels, which just sum up to ROS, applied experimentally or generated naturally via alternative pathways, promoting further activation of ROS-induced currents; (b) PAs may inhibit ROS-induced channels. In reality, however, PAs catabolization may have a limited effect, whereas PAs by themselves, instead of blocking the ROS-induced conductance, unexpectedly, acted as cofactors, sensitizing ROS-induced passive currents to $OH^\bullet$. This effect was restricted to the mature root zone (PAs did not potentiated ROS-induced current in the elongation zone) and was observed only in response to $OH^\bullet$. H_2O_2 up to 5 mM neither induced any ion current nor did it affect the transmembrane PD (Zepeda-Jazo et al. 2011; Pottosin et al. 2012). Similar studies on the two barley varieties, contrasting in their salt tolerance, revealed that the stimulation of the ROS-induced K^+ efflux by PAs (equally by Spm^{4+} or Put^{2+}) was observed mainly in salt-sensitive variety; this variety also displayed intrinsically higher PAs (mainly, Put^{2+}) levels (Velarde-Buendía et al. 2012b). In barley, salt-tolerance and K^+ retention were correlated with a tolerance to oxidative stress, but the activity of antioxidant enzymes was lower in salt-tolerant variety (Dragišić Maksimović et al. 2013). Thus, rather than due to better antioxidant function or reduced ROS levels, a weaker synergism between $OH^\bullet$ and PAs in the induction of K^+ efflux from roots may be responsible for a salt tolerance in this case. In addition, both PAs and $OH^\bullet$ activated plasma membrane Ca^{2+} pumps and this response displayed a lower threshold as compared to the activation of passive conductance and can be selectively inhibited by eosine yellow (Zepeda-Jazo et al. 2011; Bose et al. 2011; Velarde-Buendía et al. 2012b). Obviously, PAs in combination with $OH^\bullet$ can induce both Ca^{2+} efflux and influx;

the net effect, among all, was dependent on the PAs species and ROS level (Zepeda-Jazo et al. 2011). Thus, increase in PAs and ROS, both being stress-related factors, caused a substantial remodeling of plasma membrane cation conductance, with a potentially important impact on Ca^{2+} signaling and K^+ homeostasis.

Finally, increased biosynthesis of so-called compatible solutes under salt stress exerts protection functions beyond mere osmotic adjustment in cytosol of salinized plant cells. External addition of proline, betaine, and a variety of amino acids at low millimolar range efficiently inhibited NaCl-induced K^+ efflux from roots (Cuin and Shabala 2005, 2007). Summarizing, metabolic changes under salt stress often cause increases of PAs, ROS, and compatible solutes. These changes, in turn, may cause suppression of some constitutively expressed K^+-selective and NSC channels, induce novel low-selective cation conductance, activate ionic pumps, and modulate K^+/Na^+ exchange (K^+ efflux and Na^+ influx) across the plasma membrane.

8 Price to Pay: Na^+ Expulsion or Vacuolar Sequestration?

Salt-tolerant plants, for their growth and development under saline conditions, need to maintain turgor. Therefore, osmotic adjustment is required, to confront the decrease of water potential in external medium. It is widely accepted that cytosolic osmotic adjustment is largely done by increase of so-called "compatible osmolytes," organic compounds (mainly, glycine betaine and proline) with relatively neutral effects on the metabolism, whereas NaCl accumulation in central vacuoles ensures vacuole-to-cytosol osmotic balance (Storey and Wyn Jones 1977; Flowers and Colmer 2008; Shabala and MacKay 2011). In fact, total vacuolar and cytosolic pools of glycine betaine and proline may be fairly comparable. Yet, taking into the account that vacuole, especially under salt stress, occupies >90 % of the cell volume, it implies that absolute concentration of compatible osmolytes in the cytosol is more than by one order of magnitude higher than in vacuole (Leigh et al. 1981). These data indirectly suggest low permeability of the tonoplast to betaine and proline, which makes sense, because equal distribution of betaine and proline between the two compartments implies severe energy penalties. Indeed, synthesis of one molecule of compatible solute consumes between 40 and 50 ATP molecules, whereas import of Na^+ into the vacuole by Na^+/H^+ antiport costs less than 1 ATP molecule (Raven 1997). Thus, to store organic compounds in vacuoles exclusively for osmotic adjustment is 100 times more expensive, than storage of equivalent concentration of Na^+.

In this context, there is no alternative for Na^+ sequestration in the vacuole under prolonged salt stress. Moreover, its extrusion to the external medium by plasma membrane Na^+/H^+ exchanger SOS1 would almost certainly result in a futile and energy-consuming Na^+ cycling across the plasma membrane as well as in cytosol acidification. Vacuolar Na^+ sequestration against large electrochemical gradient for Na^+ requires an active transport process. This utilizes electrochemical gradient for H^+, generated by two tonoplast H^+ pumps, pyrophosphatase (PPase) and H^+-ATPase (Apse and Blumwald 2007). Although, in terms of the H^+ gradient

there is no difference, which H^+ pump is involved, and genetically engineered plants, overexpressing PPase, show higher drought and salt tolerance (Gaxiola et al. 2001), naturally under salt stress conditions the vacuolar H^+-ATPase is up-regulated, whereas PPase is down-regulated (Nakamura et al. 1992). Applying inhibitors of the plasma membrane and vacuolar H^+ pump, respectively, Kader and Lindberg (2005) have demonstrated that in a salt-tolerant rice variety, in contrary to a salt-sensitive one, cytosolic Na^+ concentration under salt stress seems to be controlled by vacuolar Na^+ sequestration rather than by Na^+ exclusion to the apoplast. Data by the same group confirmed that Na^+ uptake into the cytosol of a salt-tolerant plant quince (*Cydonia oblonga*) is only transient and independent on external pH, which implies a more important role of the intracellular (vacuolar) Na^+ sequestration vs. its extrusion to the exterior (D'Onofrio et al. 2005). In bread wheat, salt tolerance was not correlated with Na^+ expulsion (Genc et al. 2007). Importance of vacuolar Na^+ sequestration strategy may be illustrated by interesting case of *Mesembryanthemum*, unique halophyte with CAM metabolism, which dynamically stores malate in vacuoles, releasing it into cytosol in the daytime. The latter occurs in parallel with the vacuole deacidification, which requires a down-regulation of the H^+ pump, to avoid futile malate cycling. But the H^+-pump down-regulation implies that Na^+ leak into the cytosol may not be reverted. To resolve this dilemma, ice plant developed two distinct types of vacuoles in the same mesophyll cells: one acidic, for a dynamic storage of malate, and a neutral one, used for NaCl storage and osmotic adjustment. Differential activity of malate transporters in the two vacuoles is likely controlled by high redox potential in the malate-storing vacuole (Epimashko et al. 2004).

Although tonoplast Na^+/H^+ antiporter can operate as K^+/H^+ antiporter, displaying a relatively poor Na^+/K^+ selectivity, it is currently considered as a key element in vacuolar Na^+ sequestration under salinity (Apse and Blumwald 2007). Timing is important for the tonoplast Na^+/H^+ exchange activity: halophyte plants normally display high constitutive Na^+/H^+ antiport (Shabala and MacKay 2011); in glycophytes it is stress-inducible (Shi and Zhu 2002; Fukuda et al. 2004).

9 Vacuolar Cation and K^+ Channels and Their Roles Under Salt Stress

Salt-resistant plants, and at the extreme end, halophytes, under salinity display opposite concentration gradients for K^+ and Na^+ across the tonoplast, with up to one order of magnitude higher Na^+ and up to fivefold lower K^+ in the vacuole as compared to the cytosol (Flowers and Colmer 2008; Shabala and MacKay 2011). In roots of salt-grown barley, vacuole to cytosol Na^+ concentration gradient was about fourfold and less than twofold in salt-tolerant and salt-sensitive varieties, respectively. Although, cytosolic K^+ decreased in both varieties (down to ~50 %), salt-tolerant variety on average was more capable to maintain high cytosolic K^+ on the NaCl background (Carden et al. 2003). In leaves of salinized barley, K^+ levels change in a contrasting manner. In epidermis, both vacuolar and cytosolic K^+ decreased approximately five times. In mesophyll, however, cytosolic K^+ level was

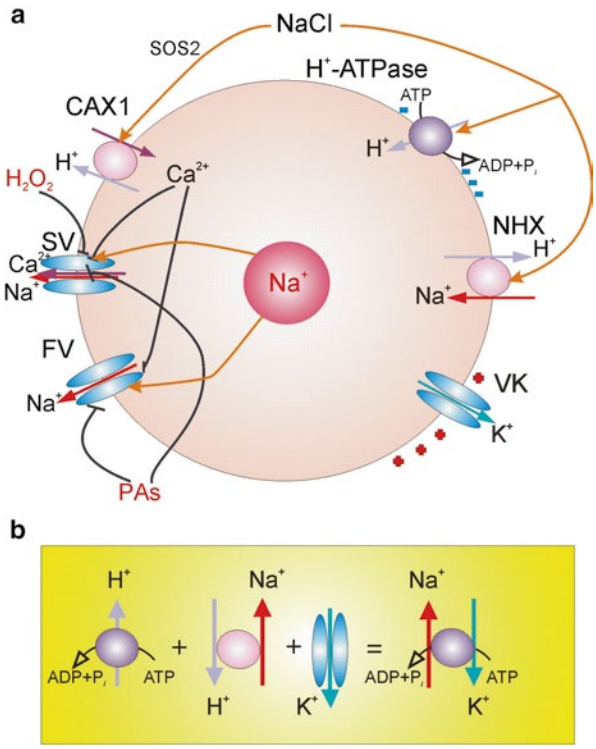

Fig. 12.2 K^+ and Na^+ transport systems of the vacuolar membrane and possible ways of their regulation under salt stress. (**a**) At the left, nonselective fast (FV) and slow (SV) vacuolar channels are presented. Under salt stress, to limit passive Na^+ leak from the vacuole, the activity of these channels has to be down-regulated, due to a decrease in their expression, inhibition by polyamines (PAs) or ROS (H_2O_2), or inhibition by increased vacuolar Ca^{2+} levels. At the right, the system involved in vacuolar Na^+ accumulation and Na^+/K^+ exchange across the tonoplast is presented. Vacuolar K^+-selective channel acts as a shunt conductance for electrogenic H^+-ATPase, to avoid the electrical overcharging of the tonoplast; at the same time it mediates K^+ leak to the cytosol, to compensate NaCl-induced K^+ loss to the external medium. (**b**) Altogether, tonoplast H^+-ATPase, Na^+/H^+ exchanger, and K^+-selective channel are mathematically equivalent to Na^+-K^+ ATPase, key enzyme in animal cells, which is absent in the plant kingdom

almost invariant, whereas vacuolar K^+ decreased by about 40 % (Cuin et al. 2003). These results suggest that cytosolic K^+ concentration in metabolically active compartment, cytosol of mesophyll, is maintained at the expenses of its own vacuole and epidermal cells. Conversely, at saline conditions barley leaf epidermis shows a higher Na^+ content as compared to mesophyll, although in both tissues substantial increase of vacuolar Na^+ was observed (Fricke et al. 1996). Therefore, vacuole may act as a source of K^+, but at the same time, as a safety store for Na^+.

It is obvious that under saline conditions tonoplast passive conductance for Na^+ has to be kept at minimum, whereas passive K^+ conductance may be allowed (until K^+ influx to cytosol becomes thermodynamically unfavorable). In the tonoplast, there are two major currents (Fig. 12.2), only weakly selective between K^+ and Na^+:

slow vacuolar (SV) and fast vacuolar (FV) ones (Brüggemann et al. 1999; Pottosin and Schönknecht 2007), as well as a strictly K^+-selective current, VK (Ward and Schroeder 1994; Pottosin et al. 2003). Both SV and FV channels display a complex gating by voltage and divalent cations (Pottosin and Muñiz 2002; Pottosin and Schönknecht 2007).

Increase of vacuolar monovalent cation content affects channels' gating: it increases the FV channels activity at physiological potentials (Pottosin and Martínez-Estévez 2003) and ameliorates the inhibitory effect of vacuolar Ca^{2+} on the SV channel, shifting the threshold of the SV activation to more negative (physiologically attainable) potentials (Pottosin et al. 2005). Thus, Na^+ leak through non-selective SV and FV channels under conditions of salt stress should increase due to the accumulation of salt in the vacuole, because of its effect on gating and on the ion permeation (increase of driving force), unless this increase is compensated by other factors (see Fig. 12.2 for possible mechanisms). The simplest way is a down-regulation of the channel expression. Yet, we are aware of a single study published to date, where salt-induced decrease of the activity of SV channels in *Plantago* roots was reported (Maathuis and Prins 1990). It was found also that more salt-resistant quinoa variety shows constitutively lower activity of FV and SV channels in mesophyll vacuoles as compared to a more salt-sensitive one; salt stress caused a decrease in the FV channels activity and an increase in the threshold for the SV channel activation in both varieties (Bonales-Alatorre et al. 2013). Another option should be a common factor, which turns off both SV and FV channels. As a candidate may be luminal Ca^{2+}, which likely accumulates at higher levels due to a salt-induced activation of CAX1, Ca^{2+}/H^+ exchanger of the tonoplast (Cheng et al. 2004). In *Arabidopsis* CAX1 is mainly expressed in leaves; it has a 400-fold higher expression in mesophyll as compared to leaf epidermis. Even more strikingly, TPC1 (encoding SV channels) expression is 1,000-fold higher in epidermis than in mesophyll. Thus, Ca^{2+} uptake in mesophyll vacuoles is enhanced, whereas its leak through SV channels is minimized. In line with this, there is a 100-fold higher accumulation of Ca^{2+} in mesophyll as compared to epidermis. At the same *tpc1-2* loss of SV function mutant shows only a threefold higher accumulation (Gilliham et al. 2011). Thus, in *Arabidopsis* the SV activity in mesophyll tonoplast may be efficiently suppressed, not only due to its lower expression, but due to higher Ca^{2+} uptake via CAX1, and a feedback down-regulation of SV (and also, expected down-regulation of the FV) by accumulated luminal Ca^{2+}. Yet this mechanism may not be generally applicable, because in monocot species, barley and wheat, Ca^{2+} accumulation in the mesophyll vacuoles was much lower than in epidermis (Conn and Gulliham 2010). In barley epidermis at saline or control growth conditions, there was clear parallelism for vacuolar Na^+ and Ca^{2+} accumulation for different cell types, with the lowest content of both ions was observed in vacuoles from lower epidermis. Yet, increase of vacuolar Ca^{2+} accumulation in epidermis was paralleled with increase of vacuolar Na^+ only at moderate (50 mM NaCl) salinity; at higher (150 mM NaCl) salinity, vacuolar Ca^{2+} dropped to a control value, whereas vacuolar Na^+ has shown a further increase (Fricke et al. 1996). It is not clear, whether such Ca^{2+}-dependent mechanism of the down-regulation of cation leak from vacuole may

be operative in roots, which accumulate much less Ca^{2+} in vacuoles than highly transpiring organs, like leaves. SV channels in all organs are activated under reducing conditions (Scholz-Starke et al. 2005) and are inhibited by H_2O_2 (Pottosin et al. 2009). Thus, increase of intracellular ROS production, which is especially large in leaves of stressed plants, may cause the reduction of the SV activity (Fig. 12.2).

Less is known on possible ways of the FV down-regulation under salt stress, which largely reflects the overall lack of studies on this vacuolar channel. However, an important result was obtained by Brüggemann et al. (1998), who demonstrated that PAs, $Spm^{4+} > Spd^{3+} \gg Put^{2+}$, produced a high-affinity inhibition of this current in barley mesophyll. This result was confirmed for different tissues and species (Dobrovinskaya et al. 1999a; Pottosin and Muñiz 2002). Moreover, Dobrovinskaya et al. (1999a, b) have shown that PAs, $Spm^{4+} > Spd^{3+} > Put^{2+}$ also blocked cation currents via SV channels, albeit at somewhat higher concentration as compared to their effects on the FV channels. PAs effects on the SV and FV channels were direct, fully reversible, and occurred at concentrations of few to hundred micromolar as compared to PAs effects on the plasma membrane cation and K^+ channels (apparent K_D about 1 mM). Contrary to nonselective FV and SV channels, VK channels are relatively insensitive to PAs (Hamamoto et al. 2008). Therefore, we hypothesized that under saline conditions major FV and SV channels are turned off by PAs and other stress-related factors, whereas the activity of K^+-selective VK channels sustains. Remaining ion transport activity includes vacuolar H^+-ATPase, electroneutral Na^+/H^+ exchanger, and shunt K^+ leak via VK channels (Fig. 12.2a). Algebraically, the sum of the activity of these three ion transporters is equivalent to Na^+/K^+ ATPase (Fig. 12.2b), the major enzyme in the plasma membrane of animal cells, lacking in plants and fungi, which controls high cytosolic K^+/Na^+ ratio. In contrast to terrestrial plants and fungi, multi-cellular animals may be considered under permanent "salt stress," due to a high NaCl concentration present in their body fluids. So, in animals Na^+/K^+ ATPase is constitutively highly active. In plants we speculate that respective activity may be implemented on demand under salt stress on a completely different molecular basis, involving the up-regulation of vacuolar H^+-ATPase and Na^+/H^+ antiporter and increase of the overall K^+/Na^+ selectivity of the tonoplast cation transport, by selective inhibition of FV and SV channels vs. sustained activity of the VK ones (Zepeda-Jazo et al. 2008b).

10 Importance of the Cytosolic K^+/Na^+ Ratio

In this chapter, we have discussed the pathways for Na^+ and K^+ transport across the plasma membrane, tissue distribution of these ions, and their intracellular compartmentalization. As Na^+ (and Cl^-) uptake under strong salt stress is inevitable for the osmotic adjustment, and Na^+ and K^+ often share the same transport routes, intracellular Na^+ grows up and intracellular K^+ drops. Clearly, *total* K^+/Na^+ ratio will decrease then, but it may be misleading as a criterion for salt tolerance, keeping in mind that Na^+ may be preferentially concentrated in vacuoles. But what about the

cytosolic K^+/Na^+ ratio? Whether this parameter has fundamental physical meaning or it is merely mechanistic reflection of reduced intracellular K^+ and increased Na^+ levels?

A frequent lemma to hear is that Na^+ and K^+ are very similar in their physico-chemical properties (radius, hydration energy) so that they compete for the same binding sites in proteins. As K^+ required for the activity of 50 intracellular enzymes (Marschner 1995) such competition is believed to disrupt cell metabolism. In fact, however, hydration energy and radius for Na^+ and K^+ differed very significantly, by 25 % and 40 %, respectively (Hille 2001). Thus, the difference between Na^+ and K^+, rather than caused by their competition for a specific binding site, is likely due to their general effects (as the general effect of ionic strength, with some degree of ion specificity). For instance, for K^+ itself, an optimal range for protein synthesis by polyribosomes is normally between 100 and 200 mM, whereas inhibition is observed at higher K^+ concentration. When K^+ concentration is suboptimal, protein synthesis in ribosomal complexes, isolated from halophytes, may be stimulated by additions of Na^+ (Flowers and Dalmond 1992). Thus, K^+ and Na^+ may be mutually exchangeable to some extent. Phosphoenolpyruvate carboxylase (PEPC), key enzyme in CO_2 fixation, is another classical target for Na^+, which exerts a chaotropic effect on its activity (Osmond and Greenway 1972). Yet, under conditions of salt stress, overexpression of this enzyme, resulting in a higher protein aggregation, along with increased levels of compatible solutes in cytosol, acting as chaperons, may fully protect PEPC against high Na^+ (Manetas 1989). Thus, effects of Na^+ on intracellular enzymes depend on other factors and not necessarily are opposite to those of K^+. These considerations does not cancel the fact that at high concentrations, observed under salt stress, Na^+ may be toxic for cellular metabolism (so will be also K^+ at very high, naturally not occurring, concentration). However, toxic level of cytosolic Na^+ requires a re-evaluation, basing on the whole pool of experimental evidence. An "upper limit" of 30 mM set for cytosolic Na^+ (Munns and Tester 2008) is almost certainly an exaggeration. Besides, there are contradictions between cytosolic Na^+ levels, reported by different techniques. For instance, triple-barreled microelectrode measurements report cytosolic Na^+ activities up to 30 mM in salinized barley roots (Carden et al. 2003). On the other hand, usage of the X-ray microanalysis revealed cytosolic Na^+ activities, which were almost tenfold higher with the same plant model (Flowers and Hajibagheri 2001). "Apocryphal" data for high (>100 mM) Na^+ in cytosol, including the values as high as 300–400 mM (in leaves of salinized barley and durum wheat, James et al. 2006) are summarized for different plant species by Kronzucker and Britto (2011).

K^+/Na^+ or just K^+? Many halophytes, in contrast to glycophytes, respond to salinity with increase of K^+ in roots (Shabala and MacKay 2011). Na^+ retrieval from xylem is considered as a pivotal strategy for the salt tolerance (Tester and Davenport 2003). But halophytes (with some exceptions, like *Thellungiella*) tended to accumulate Na^+ in shoots, supporting osmotic adjustment and growth under saline conditions (De Boer and Volkov 2003; Shabala and MacKay 2011). So do also transgenic plants, with overexpressed vacuolar Na^+/H^+ antiporters, which display salt tolerance in parallel with a high tissue Na^+ concentration (Apse and Blumwald 2007).

Specifically speaking about cytosolic K^+, not the total one, there is ample evidence for a crucial role for K^+ maintenance in cytosol of metabolically active cells, like mesophyll, at the expense of vacuolar K^+ pool and of surrounding less metabolically active cells (Cuin et al. 2003; Volkov et al. 2003). Besides, cytosolic K^+ drop below certain threshold level (about 50 % of its concentration under non-stressed conditions), led to a programmed cell death (Shabala 2009; Demidchik et al. 2010; Poór et al. 2012). Therefore, cytosolic K^+ concentration by itself, rather than K^+/Na^+ ratio, may serve as a useful criterion for salt tolerance. This criterion, however, only makes sense when it is applied in a tissue-specific manner, as some tissues are apparently more vulnerable to decreased K^+ as compared to others.

11 Conclusion and Future Perspective

Salt tolerance is a multiple trait, and ways plants take up Na^+, transport, and re-distribute it between different tissues are also multiple. Relative contributions of different routes for low-affinity Na^+ uptake likely depend on tissue, species/varietal difference, and on specific growth conditions. Current state of our knowledge on the mechanisms of low-affinity Na^+ uptake perhaps raises more questions or doubts than answers. Use of varieties, contrasting in a salt tolerance, for the search of QTL, responsible for the control of Na^+, K^+, and Cl^- tissue levels, may provide partial solutions for given species. These studies will also help to identify, which genes, including those encoding ion transporters, are de facto important for each particular species or group of species. Hopefully, on this basis some more general, suitable for different crops, conclusions may be drawn. NaCl-induced loss of intracellular K^+ in many cases is mediated by KOR (GORK) channels, which may be a plausible target for genetic manipulations. Another plausible target may be plasma membrane H^+-ATPase, whose activity controls resting potential in plant cells and counter-resists its depolarization, decreasing, therefore, salt-induced loss of K^+. Yet, in roots and leaves of some plants, NSCCs may play equally important roles, mediating both K^+ loss and Na^+ uptake. To manipulate them, we need at least to know their molecular identity. The latter remains cryptic, and a large gap still exists between functional and genetic characterization of this group of channels. Considering tissue specificity, of primary interest are ion channels and transporters, which are expressed in the plasma membrane of root epidermis and hairs, xylem parenchyma, mesophyll, and leaf epidermis, which are critical spots for transmembrane K^+/Na^+ exchange and re-distribution in planta. If we manage, for instance, to improve Na^+/K^+ redistribution between mesophyll and leaf epidermis, by increasing channel-mediated Na^+ uptake from the apoplast into epidermis and reducing K^+ loss from mesophyll cells, this may eventually improve plant salt tolerance. On the intracellular level, vacuolar sequestration of Na^+ is an important and relatively general mechanism to solve a dilemma of the toxicity of Na^+ and its use as a cheap osmoticum. In addition to overexpression of tonoplast PPase and Na^+/H^+ antiporters, negative regulation of vacuolar SV and FV channels may help to reduce the energy cost of Na^+

sequestration. It remains to be elucidated, whether the activity of VK/KCO channels can assist salt tolerance as it is hypothesized here. Finally, a substantial remodeling of the membrane ion conductance by stress-related metabolic changes, including effects of increased concentrations of different ROS species, polyamines, and compatible solutes need to be taken into the account and used for improving of plants performance under salt stress.

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Chapter 13
Jatropha curcas: An Overview

Nisha Singh Tomar, Mohammad Abass Ahanger, and R.M. Agarwal

1 Introduction

Jatropha curcas L. (physic nut) (Fig. 13.1a, b) is a multipurpose drought-resistant, perennial small tree belonging to the family Euphorbiaceae (Ghosh et al. 2007; Shabanimofrad et al. 2011; Mastan et al. 2012; Wang and Ding 2012). Genus named *Jatropha* has been derived from the Greek word "Jatros" meaning "Doctor" and tropha "food" which implies its medicinal uses. It is commonly known as physic nut and *curcas* is a common name for physic nut in Malabar, India (Linnaeus 1753). *Jatropha* grows in a number of climatic zones including areas of low rainfall and is a native of tropical America, later introduced to Africa and Asia and is presently cultivated worldwide (Openshaw 2000; Tan et al. 2002). *Jatropha curcas* is gaining attention as biofuel crop because of its ability to grow on marginal and eroded soils. It can grow without much water, fertilizers and pesticides resembling other biofuel crops such as corn, oil seed rape, soybean and sunflower in this respect (Ho 2007).

It generally attains a height of 3–5 m, but under favorable conditions it may reach up to 8–10 m of height. It has green leaves which are three to five lobed with a length and width of 5–6 cm arranged alternately and the plant contains whitish latex. *Jatropha curcas* has thick glorious branchlets and stem with thin grey-colored smooth bark. It is a deciduous plant shedding leaves in dry season and winters. Flowering occurs during the wet season and two flowering peaks are often seen, i.e., during summer and autumn. Nevertheless, flowering occurs throughout the year in permanently humid regions. It is a monoecious plant with unisexual flowers borne on the same terminal inflorescence arising on branches but occasionally hermaphrodite flowers are also found. In androecium, ten stamens are arranged in two distinct whorls of five each. Gynoecium shows three slender styles which are connate

N.S. Tomar • M.A. Ahanger • R.M. Agarwal (✉)
School of Studies in Botany, Jiwaji University, Gwalior 474 011, Madhya Pradesh, India
e-mail: agarwalrm@rediffmail.com

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Fig. 13.1 (**a**) *Jatropha curcas* plants growing in Tapovan, Gwalior (rainy season). (**b**) *Jatropha curcas* plants growing in Tapovan, Gwalior (summer season)

to about two-thirds of their length, with a massive bifurcate stigma (Dehgan and Webster 1979).

Jatropha curcas is monoecious, protandrous and self-compatible, however shows out crossing and a tendency to promote xenogamy and minimize geitonogamy. Male to female flowers ratio is 29:1 which open synchronously. Fruiting behavior indicates that the plant might selectively eliminate the growing offspring, especially the geitonogamous fruit, to allocate the resources available to plant, mostly for xenogamous fruit. The ability to self-pollinate through geitonogamy is considered to be adoptive for *Jatropha curcas* for colonization (Raju and Ezradanum 2002; Ye et al. 2009). Each inflorescence yields a bunch of approximately 10 or more ovoid fruits. After pollination trilocular, ellipsoidal fruit is formed and exocarp remains fleshy green until the seeds are mature and turns yellow when mature. Seeds are black and about 2 cm long and 1 cm thick having small caruncle. *Jatropha curcas* produces 4–5 kg seeds/tree from fifth year onwards up to 45–50 years from the day of plantation (Gupta 1985; Poonia and Jethoo 2012).

Jatropha can be used to prevent soil erosion, to reclaim land, grown as a live fence, and is also planted as a commercial crop (Heller 1996). Various parts of the plant are of medicinal value, its bark contains tannin and the flowers attract bees indicating its honey production potential. Seeds yield oil which can be used for lighting, producing a lubricant, soap and most importantly as biodiesel (Rivera-Lorca and Ku-Vera 1997). *Jatropha* also provides a meal which is highly nutritious and a protein supplement in animal feed, if detoxified (Becker and Makkar 1998). Because of relatively higher moisture contents in trunk and twigs *Jatropha* plants exhibit better tolerance to fire therefore also planted as fire barrier to prevent the spread of fire outbreak. As a hedge, it also prevents spread of diseases and insect infestation in afforested area (Li et al. 2006).

Jatropha curcas can grow well in regions of low rainfall withstanding extremely low humidity in air and long-term drought by shedding its leaves thereby bringing down the rate of transpiration. These features furnish enough reasons for many countries to promote *Jatropha* for growing on marginal lands for biodiesel production

(Abou Kheira and Atta 2009). However, before using any plant for commercial production on marginal lands investigating its growth and development under such stressful conditions and impact of stress on its productivity may be meaningful (Niu et al. 2012).

2 Plantation Aspects

2.1 General

Jatropha curcas is easy to establish and is well adapted to grow under arid and semiarid regions with annual rainfall of 300–1,000 mm. Well-drained soil with good aeration favors its growth. However, it also grows on sandy, marginal and saline soils with poor nutrient soils but not in water-logged land. Nevertheless, this documentation of the establishment and productivity of *Jatropha* under various climatic conditions is yet to be completed (Openshaw 2000). Plants also grow well on moderately saline, degraded and eroded soils, and can easily be raised from seeds or cuttings (Katwal and Soni 2003).

Several morphological, anatomical and physiological changes have been induced by saline environment possibly in order to cope up such conditions. Adverse effects of salinity on various growth parameters such as plant height, stem diameter, root length, number of leaves/plant, leaf area, fresh and dry weight of plants have also been reported and depressing effects were more prominent with increase in salinity level. Increase in sodium percentage and proline contents have also been observed with higher salinity stress (Mazhar et al. 2011). Salt stress adversely affects growth and development of *Jatropha* plants. Nevertheless, *Jatropha* plants can grow well on moderate saline soils (Niu et al. 2012). However, Al-Busaidi et al. (2012) reported that higher salt contents did not reduce growth of *Jatropha* plants if soil is amended with mulch. Salts accumulated in salinity treated pots perhaps help to retain more water, reduce the temperature and provide nutrients to plants.

Jatropha seems to grow well in calcareous soils with pH up to 8.5. However, growth is restricted on acidic soils with pH below 5.2 or in high saline soils. At every stage of *Jatropha curcas* cultivation proper guidance and efficient management can be useful. Analysis of the proposed site is required at the initial stage of *Jatropha curcas* plantation. The implantation stage needs preparing the land for *Jatropha curcas* cultivation for planting and sowing seeds, managing the irrigation and controlling nutritional requirements. Besides, there is attention required to handle external factors such as pest and diseases (Raina 2009).

2.2 Raising Jatropha Plants

Jatropha can readily be grown from seeds or cuttings however, plants raised through cuttings show a lower longevity and possess a lower drought and disease resistance

than those propagated by seeds may be, because these plants do not produce true taproots (therefore show less tolerance to drought), however, pseudo-taproots are produced which may penetrate only one-half to two-thirds of the depth of the soil in comparison to the taproots produced by *Jatropha* plants grown through seeds. Methods used to cultivate *Jatropha* show variation depending upon the region and climatic conditions. Plants can be raised by direct seeding, pre-cultivation of seedlings (nursery raising), transplanting of spontaneous wild plants and direct planting of cuttings. Wider spacing (3 m × 3 m) gives higher fruit yield (Heller 1996).

Survival and vegetative development of the plant is affected by the propagation methods used. Sowing time, depth of sowing and choice of cultivation method also has a bearing on the survival rate of seedlings. Age and the position of the cutting within the plant influences sprouting as rooting ability of many woody plants may decline with age. Distal portion of the stock plants are first to show this reduced rooting potential, whereas cuttings from the lower or juvenile regions of the plants generally maintain a higher rooting capacity as compared with the upper regions (Hartmann and Kester 1983).

Seeds are kept at 6 cm depth and plants are raised preferably in poly bags of half kg capacity in month of May and June. About 8-week-old seedlings (6–8 in. height) are used for plantation during July–September. Plants can also be raised during February–March using cuttings (Poonia and Jethoo 2012) and position of cutting on the mother branch is important in rooting as the middle portion of the mother branch exhibits better rooting. Survival percentage of cuttings is improved by treatment of IBA and NAA (100 mg L^{-1}) and IBA is more effective in this respect (Kochhar et al. 2005).

2.3 Growth of Plants on Marginal or Waste Lands

The word "waste land" indicates unoccupied area whereas "marginal land" indicates areas unsuitable for crop production because of soil and climatic constraints (Chaudharry et al. 2007; Patolia 2007). Most of the marginal lands are located in arid and semiarid regions in many parts of the world where water supply is extremely limited. These soils exhibit high soil salinity, low fertility levels and less availability of water. Salinity stress significantly decreased the growth and dry weight of *Jatropha* plants and colonization of arbuscular mycorrhiza (AM) in roots of plants. However, inoculation of *Jatropha* seedlings with AM fungi can promote the establishment of plants even under salinity stress by reducing the harmful effects. In saline soils root (%), AM colonization and concentration of glomalin were found to be negatively correlated (Kumar et al. 2010).

Jatropha curcas can be grown on wasteland for biodiesel production and several related issues such as food versus energy and environment need to be focused on (Gheewala and Prueksakorn 2006). It has been found growing well in wastelands producing greater quantity of fuel per hectare than soybean and corn (Fitzgerald 2007). Certain governments and corporations in the world consider *Jatropha* plant

as one of the most promising renewable substitute for fossil fuels. *Jatropha* plants subjected to water stress show decreased leaf area, biomass and relative growth rate but maintain leaf water content and transpiration efficiency. Seedlings from different accessions exposed to different levels of drought stress exhibit more or less similar effects. *Jatropha* plants maintain their growth for several weeks during period of drought using stem water reserves. *Jatropha* seedlings do not shed their leaves immediately after exposure to water stress but develop leaves with higher adaxial stomatal density with the commencement of stress followed by gradual shedding of leaves. Succulent stem plays important role in the maintenance of water status of *Jatropha* plants (Maes et al. 2009).

Jatropha curcas thrives well in a number of climatic zones with rainfall of 250–1,200 mm but most of the *Jatropha* plantation is found on land receiving 600 mm annual rainfall and temperature ranges between 20 and 27 °C (Tarek 2009). According to Grass (2009), *Jatropha* plantation may be successful in dry regions of the tropics with annual rainfall of 500–600 mm but rainfall 900–1,200 mm or irrigation is conducive for better production. *Jatropha* sheds its leaves and the entire plant becomes leafless in summer (May–June) to cope up with drought by reducing transpiration rate and (December and January) to resist cold during winter. *Jatropha curcas* needs little water during its lifespan (which is about 50 years) as compared with other cash crops. However, life saving irrigation is required during dry periods.

Flowering normally starts after a dry and dormant period and is induced and continued by prolonged periods of soil water availability, either by precipitation or irrigation. Strong correlation between reproduction and vegetative growth has been found. Female flowers are slightly larger and are produced during hot season. Greater numbers of female flowers are produced during favorable conditions (Aker 1997). Each inflorescence produces a bunch of about ten or more oval fruits which mature 3–4 months after flowering. Flowering forms a significant phenological stage of *Jatropha curcas* for oil production as the number of female flowers eventually determines the number of fruits and seeds. Female flowers will open for 2–4 days only whereas the male flowers open for a period of 8–10 days (Prakash et al. 2007).

Jatropha curcas shows two flowering peaks, one during summer and other during autumn. Nevertheless, in humid regions flowering may occur throughout the year. Plants bear fruits 3–4 years after plantation and reach stability fifth year onwards (Heller 1996). Nutrient limitations reportedly hasten the end of flowering and abortion of flowers may be 60 % or more depending upon the soil, water and nutrient availability. If carbohydrates are insufficiently produced, e.g., in the first period after dormancy, flower abortion is a common phenomenon (Kumari and Kumar 2007).

According to Henning (1996) and Heller (1996) seed production of *Jatropha curcas* varies between 2.5–3.5 and 0.1–8 t ha^{-1} $year^{-1}$ respectively. Francis et al. (2005) observed variation in seed yield of *Jatropha curcas* from 0.2 to 2.0 kg per tree within plantation stands and Ouwens et al. (2007) reported regional variation in seed production ranging from 0.1 to 15 tones ha^{-1} $year^{-1}$. This range may be ascribed to variation in rainfall and soil nutrient status. When cultivated on poor soil, with no

irrigation and planted in full sunlight the plant takes 4–5 years to yield. However, much less time is required under optimum conditions of rainfall and soil and plantation continue yielding up to 45 years after establishment.

A systematic study on the morphological traits and relationship between genetic variability and economic yield of plant is required. Seed size, number of seeds or fruits per tree must be counted for estimation of oil yield per hectare (Chaudharry et al. 2007). Germplasm screening has led to identification of genetic variants responding differently to stress. Early, mid-late and late genotypes have been identified on the basis of data recorded on flower initiation and male:female ratio. Further evaluation and utilization of these variants in breeding programs shall help develop varieties suitable for different agro-climates of semiarid India. Commercialization of *Jatropha curcas* is limited due to lack of uniform seed production and maturity cycle. Additional irrigation at flowering, sudden withdrawal of irrigation results in dehiscence and senescence can also alter the flowering behavior, seed maturity and yields (Raina 2009).

2.4 Pruning

Pruning improves yield and product quality of horticultural crops. *Jatropha curcas* is pruned to control plant size with greater number of branches and increased seed yield. Commercial plantation of *Jatropha curcas* is pruned to promote production of more branches and abundant and healthy inflorescences resulting in improved fruit setting and seed yield (Gour 2006). Annual pruning of the *Jatropha* plantation by two-thirds of terminal branches is suggested during dormant period when leaves are shed (Achten et al. 2008). Pruning levels between 70 and 90 cm with nitrogen (312.5 kg ha^{-1}) application show consistently high fruit yield in *Jatropha curcas*, whereas pruning at 50 cm and no supplementation of fertilizers gives poorest fruit yield (Suriharn et al. 2011).

2.5 Role of Nutrients and Mycorrhizal Innoculation

Jatropha curcas is considered as a hardy and low nutrient requirement crop grown on marginal land with low soil fertility (Heller 1996; Jongschaap et al. 2007). However, *Jatropha* plants respond well to fertilizer application and without proper management and fertilizer application it may not be productive and profitable. Growing plants on low or non-fertile soils imply the need to use fertilizers at least in the beginning, to boost crop growth and seed production. Supplementation of nitrogen (0–60 kg ha^{-1}) and phosphorus (0–30 kg ha^{-1}) to marginal lands where *Jatropha* was planted in 2 × 2 m pattern, i.e., 2,500 plants per hectare showed significant increase in plant height, leaf area index, total above ground dry matter and seed oil yield (Patolia et al. 2007). Nitrogen fertilizers significantly affect growth, development, kernel set and yield of *Jatropha curcas* (Yin et al. 2010).

Application of nitrogen fertilizer has led to increase in leaf area index, photosynthetic rate and radiation use efficiency (Novoa and Loomis 1981). In grown-up plants nutrient contents of leaves and nutrient uptake from soil were negatively correlated with plant density. However, such a situation is not there during initial phase of *Jatropha* plantation due to lack of competition for radiation, water and nutrients between plants (Chaudharry et al. 2007). Organic or inorganic fertilization significantly improves seed yield and 24 % increase in total above ground dry matter was recorded after 2 years of nitrogen application at 45 kg ha^{-1} (Patolia et al. 2007). *Jatropha* plants supplemented with nitrogen fertilizer 312.5 kg ha^{-1} produce longer branches, result in maximum fruit and seed yield. Higher doses of fertilizers depressed the yield; however, lowest yield was noticed without any fertilization (Suriharn et al. 2011).

Jatropha curcas is assumed as low input crop and major parts of the plant together with seed coat can be recycled to maintain the soil fertility, especially on non-fertile marginal lands. The seed kernel of the plant contains about 45 % oil and the left out seed cake is an excellent source of plant nutrients and can be used as fertilizer. Whole seeds of *Jatropha curcas* are rich in Mn (28.37 mg kg^{-1}), Zn (47.13 mg kg^{-1}), K (103.13 mg kg^{-1}), Mg (109.89 mg kg^{-1}), P (185.17 mg kg^{-1}), Ca (34.21 mg kg^{-1}) and Na (8.44 mg kg^{-1}), although level of sodium was 18.22 mg kg^{-1} in shell (Abou-Arab and Abu-Salem 2010). Use of *Jatropha curcas* seed cake enhances seed yield up to a significant level (Ghosh et al. 2007).

The biofertilizers containing beneficial microbes promoted growth of *Jatropha*. More often the biomass yield was slightly higher with vermi-compost than farmyard manure; however, in some cases improvement in stem length was found with farmyard manure (Kumar and Sharma 2005). Jamaluddin and Singh (2006) have reported association of AM fungi viz. *Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora* with *Jatropha* roots. Improvement in uptake of phosphorus and micro-elements such as aluminum, zinc, copper, iron and lead with mycorrhizal inoculation has also been reported (Sharma 2007).

Production of *Jatropha curcas* as a biodiesel feedstock on marginal lands is growing rapidly but biomass production on these lands is limited. However, inoculation of arbuscular mycorrhiza (AM) fungi is reported to promote plant growth. *Jatropha* plants inoculated with AM show improved dry weight, leaf water status, and less damage to membrane. Moreover, these parts also had increased leaf chlorophyll concentrations, proline and sugars as compared to non-AM-inoculated plants (Kumar et al. 2010). *Acaulospora* and *Glomus* are the most common AM fungi reported from the rhizosphere of *Jatropha* plants where *Glomus* being the dominant genus (Kamalvanshi et al. 2012).

2.6 Role of Growth Regulators

Foliar application of plant growth regulators (50, 100 and 150 ppm) such as etherel, indole acetic acid (IAA) and naphthalene acetic acid (NAA) influenced different morpho-physiological characters of *Jatropha curcas* such as plant height, collar

diameter, tree spread, flower initiation, number of inflorescence per plant and the ratio of male/female flowers per inflorescence. Chlorophyll contents, nitrate reductase activity and proline content were also affected by combined effect of auxin and ethylene. Nitrogen status and chlorophyll content of the *Jatropha curcas* improved with the spray of plant growth regulators. Increase in the fruit and seed yield per plant due to etherel application may be because of balanced water use during vegetative phase and increase in the number of fertile female flowers and amelioration of stress condition whereas, in auxin-treated plants this increase in yield might be due to development of more female flowers and better networking between source and sink relationship (Joshi et al. 2011).

Gibberellic acid (GA_3) alters sex ratio resulting in increased number of female flowers and better yield. Increase in female flowers and seed yield was proportionate to the concentration of hormone applied up to 100 ppm but greater concentrations caused decreased seed yield (Makwana et al. 2010). Treatment of plant growth regulators significantly increased photosynthetic rate, nitrate reductase activity, chlorophyll, crude protein and proline content of the *Jatropha* plants (Joshi et al. 2011).

Higher doses of etheral (100 and 150 ppm) restricted the plant growth. Koch and Moore (1990) suggested inhibition of stem elongation in whole-green plants by ethylene resulting either by inhibition of basipetal IAA translocation or by influencing IAA metabolism or by some other auxin-independent mechanism. Reduction in the height of plants during early season helped the plants use available water more efficiently thereby resulting in better performance under field conditions. The collar diameter increased by the application of both plant growth regulators (etherel and auxins). Interaction between etherel-derived ethylene and endogenous auxin may have caused the increased xylem production and cambial growth and also induction of enzymes involved in lignification by etherel-derived ethylene. The increase in the tree spread and tree volume of *Jatropha* plants by application of auxins (IAA and NAA), and enhanced cell division and cell enlargement of the plant cells may be responsible for this (Joshi et al. 2011).

Small burst of ethylene production in the meristem initiated flowering in pineapple (Trusove and Botella 2006). Foliar spray of auxins (IAA and NAA) shortened the vegetative period of *Jatropha* plants and induced early reproductive growth. Spraying growth regulator at flower bud initiation stage might have suppressed the male bud initiation and enhanced female flower bud initiation. NAA spray (50 ppm) decreased the number of days for first female flower appearance during summer and kharif season and resulted in more number of female flowers. The increase in the number of inflorescence, male and female flowers per plant may be because of the synergistic effect of ethylene and auxin and their impact on concentration of other hormones within the cell. A significant increase in fruit and seed yield of *Jatropha* plants was observed with the application of plant growth regulators. Favoring feminization by ethylene in *Jatropha* plants may be attributed to regulatory role of ethylene on expression of specific floral genes; nevertheless, this remains to be ascertained. The increase in femaleness after etherel applications has also been related to the effect of ethylene on auxins and gibberellins (GA) and their interactions (Joshi et al. 2011).

Application of plant growth regulators (100 and 150 ppm) enhanced the proline content of *Jatropha curcas* plants and prominent effect was observed with higher concentration. Proline seemed to have diverse roles under osmotic stress conditions, such as stabilization of proteins, membranes and subcellular structures, and protecting cellular functions by scavenging reactive oxygen species (Van Rensburg et al. 1993).

2.7 Tissue Culture and Crop Improvement

Propagation and storage of selected genotypes of tropical plants have been undertaken with the application of tissue culture (Engelmann 1991). These techniques provide higher multiplication minimizing the risk of infections in comparison to conventional breeding procedure. Reports of aseptic culture of various genotypes of *Jatropha* are available from India and Nicaragua which can be used for future genetic improvement of this species (Machado et al. 1997; Sujatha and Prabakaran 2003; Wei et al. 2004). Genetic variation in seed morphology and oil content of *Jatropha* can be exploited in tree improvement programs (Kaushik et al. 2007).

Use of molecular markers helps in assessing the molecular diversity of *Jatropha* germplasm and can be useful in breeding programs (Mohan et al. 1997; Kumar 1999). A new full-length cDNA of stearoyl-acyl carrier protein desaturase obtained using RTPCR and RACE techniques has been obtained from developing seeds of *Jatropha* and the gene functionally expressed in *E. coli* (Tong et al. 2006). The enzyme is important for fatty acid biosynthesis in higher plants playing an important role in determining the ratio of saturated fatty acid to unsaturated fatty acids (Lindqvist et al. 1996). A new full-length cDNA encoding aquaporin (JcPIP2) has been isolated from seedlings of *Jatropha curcas*, induced by heavy drought stress can help comprehending the molecular mechanism of salt and drought tolerance (Ying et al. 2007).

3 Application Aspects

Jatropha curcas is used for soil water conservation, soil reclamation, erosion control, live fence, fire wood, green manure and lighting fuel (Jongschaap et al. 2007). Primarily seeds are used to produce oil which is used as biofuel either independently or mixed with diesel (Ramchandra et al. 2006). Large-scale promotion and cultivation of *Jatropha curcas* is undertaken by State and Central Governments. Exploitation of biofuels assumes importance particularly because exhaust gases from petroleum fuels not only have detrimental effects on environment but petroleum reserves are fast depleting. A biofuel policy has been announced by the Indian Government proposing 20 % blending of biofuels with petrol and diesel by 2017

(Gahukar 2009). By-products of *Jatropha curcas* such as fruit coats, seed hulls and press-cake can be used for organic fertilization. Leaf residue and seed cake are also used as biofertilizer after suitable processing in bio-gas plant and use of earthworms providing an important source of income and employment to the rural youth (Vyas and Singh 2007; Poonia and Jethoo 2012).

3.1 Biofuel Products

3.1.1 Biodiesel

In the last few years, the potential of the drought-resistant *Jatropha curcas* for production of biofuel and industrial products has been evaluated by several groups (Jiang et al. 2012). Analysis shows that the biodiesel production from *Jatropha* is very profitable providing valuable products as well (Foidl and Eder 1997). Oil from *Jatropha curcas* can be used directly or after blending it with methanol (Gubitz et al. 1999). *Jatropha*-based biodiesel, a non-edible, renewable fuel suitable for diesel engines is better known for its potential to generate large-scale employment and relatively low environmental degradation (Pradeep and Sharma 2007). Environmental degradation and depleting oil reserves are matter of great concern around the globe (Kumar and Sharma 2008). Oil extracted from its seeds can be used directly or subjected to the process of trans-esterification. It can also be used as a fuel for steam turbines to generate electricity (Ramchandra et al. 2006).

3.1.2 Biogas

Seed cake is utilized as feedstock and seed cake along with fruit pulp can also be used for the production of biogas by anaerobic fermentation (Staubmann et al. 1997; Visser and Adriaans 2007). *Jatropha* may be instrumental in providing employment and meeting to some extent the domestic need of energy and thereby improving the environmental and quality of rural life (Poonia and Jethoo 2012).

3.1.3 Charcoal

Jatropha wood is a very light and is not popular as a fuel wood source because it burns too rapidly. Charcoal is still one of the few simple fuel options; nevertheless, converting *Jatropha* seed shells into charcoal would be economically feasible, only if we have a large source of seed shells from *Jatropha* plantations. Scientists believe *Jatropha* wood would not be of much value for either charcoal or firewood and the extraction of oil from *Jatropha* seeds is of much higher economic value than converting the wood to charcoal (Benge 2006).

3.2 Medicinal Uses

For a long time different parts (seeds, leaves and bark) of *Jatropha* have been used in traditional medicine (Mastan et al. 2012) and for veterinary purposes (Duke 1985, 1988). Tender twigs are used in toothache, gum inflammation, gum bleeding and pyorrhea. Seeds are used to treat arthritis, gout and jaundice (Gupta 1985). Curcacycline A has reportedly been shown to possess antitumor activities (Vanden Berg et al. 1995). Seed oil is applied to treat eczema, skin diseases and is also used in rheumatic pain (Heller 1996). It has a strong purgative action and is also good for skin diseases and for pain relief as well such as that caused by rheumatism. Plant extract is used for wound healing, allergies, burns, cuts, inflammation, leprosy, leucoderma and scabies. Water extract from branches is used to treat tumor and HIV. Plant sap is used in dermato-mucosal diseases and emulsion of sap with benzyl benzoate can be effectively used against scabies and dermatites. Decoction of leaves and roots is given to treat diarrhea. Roots show strong antihelminthic action and its bark is externally applied to cure sores. Roots are also reported to be antidote for snake bite (Gubitz et al. 1999).

Goonasekera et al. (1995) have reported various solvent extracts of *Jatropha* to have an abortive effect. The latex has been found to be a strong inhibitor to watermelon mosaic virus (Tewari and Shukla 1982). Curcain, a proteolytic enzyme, has been reported to have wound healing activity in mice (Nath and Dutta 1997; Villegas et al. 1997). Latex considerably reduces the clotting time of human blood, whereas diluted latex prolonged the clotting time and at higher dilutions the blood did not clot at all (Osoniyi and Onajobi 2003). The methanolic extract of *Jatropha* roots exhibited systemic and significant anti-inflammatory activity in acute carrageenan-induced rat paw edema (Mujumdar and Misar 2004).

3.3 Industrial Uses

Jatropha curcas has not only been identified as potential plant for the production of non-edible oil but is also used for manufacturing of candles, lubricants, varnishes and in cosmetic industry (Foidl and Kashyap 1999). *Jatropha* oil has very high saponification value and glycerin is a by-product of biodiesel and being extensively used for making soap. It is used for making varnish in China by boiling the oil with iron oxide and for wool spinning in England. Protein contents of *Jatropha* oil cake can be used as a raw material for plastics and synthetic fibers. Bark of *Jatropha curcas* yields a blue dye used for dying clothes in Philippines. The dye may be extracted from leaves and tender stems and is concentrated to yellowish syrup, dried to blackish brown mass that imparts different shades of tan and brown to cotton clothes (Gubitz et al. 1999). Experimentation on solid-state fermentation of *Jatropha* seed cake has shown it to be a good source of low cost production of industrial enzymes (Mahanta et al. 2008).

3.4 Jatropha *Meal*

The seeds of a non-toxic variety of *Jatropha curcas* which are found in some provenances of Mexico and Central America have been boiled, roasted and eaten. The young leaves may be steamed or stewed and safely eaten (Duke 1988; Delgado Montoya and Parado Tejeda 1989). Nevertheless, *Jatropha curcas* has been reported to contain many toxicants such as jatrophine, lectin and curcin (Naengchomnong et al. 1986; Wink et al. 1997). Phorbolesters (phorbol-12-myristate 13-acetate) are toxic components in *Jatropha* (Gubitz et al. 1999; Makkar and Becker 1997). Though *Jatropha* leaves are used as feed for tusser silk worm, accidental consumption of the seeds have resulted in *Jatropha* poisoning in humans causing symptoms of giddiness, vomiting and diarrhea and in certain cases even death has been recorded (Becker and Makkar 1998). Seeds of *Jatropha curcas* are rich in curcin and phorbol esters and press-cake contains saponins and phytate (Mujumdar and Misar 2004). Concentration of phorbol esters varies with the soil type and climatic conditions; however, Mexican varieties of *Jatropha* have been reported to possess negligible amount of phorbol esters (Martinez-Herrera et al. 2006).

Non-protein nitrogen formed only 7.8–9.0 % of the total nitrogen in the *Jatropha* meals indicating the presence of greater true protein (Makkar et al. 1998). The level of essential amino acids of the defatted, kernel meal of the non-toxic variety is more than that of FAO reference protein leaving aside lysine (Makkar and Becker 1999). The nutritional composition of the extracted seed meal from the non-toxic variety seems to be similar or even better than the toxic variety. Digestible organic matter and metabolizable energy (in vitro) of the non-toxic *Jatropha* seed meal were less in comparison to soybean meal; nevertheless, it was comparable with those of cotton seed, rape seed and sunflower meal (Makkar and Becker 1999). In *Jatropha* meal and soybean similar pattern was observed for the essential amino acids but lysine is lower and sulfur amino acids are greater in soybean (Makkar et al. 2007).

The pepsin soluble fraction of the total nitrogen has been reported to be 94–95 % (Aderibigbe et al. 1997) which indicates that seed meal of the non-toxic *Jatropha* has high potential as a feed supplement for fish and monogastrics. Mexican, non-toxic varieties have the least of phorbol esters; however, other antinutrients such as trypsin inhibitor, lectin, and phytate are present in significant amounts and their levels are comparable to those in the toxic varieties. Moist heating of seeds resulted in complete inactivation of trypsin inhibitor activity and decreased lectin activity (Makkar and Becker 1999).

Heat treatment reduces heat-labile, antinutritional factors such as trypsin inhibitors and lectins and also increases protein digestibility. Heat treatment followed by aqueous methanol extraction eliminates most of the antinutrients and toxins from the toxic variety and the meal treated in this manner can be fed to rats (Makkar et al. 1997). *Jatropha curcas* is used as a nutritious and economic protein supplement in animal feed if detoxified. Seeds of non-toxic varieties of *Jatropha curcas* are roasted and eaten (Makkar and Becker 1999). Nevertheless, the presence of high-level

antinutrients prevents their use in animal feeding. In Mexico, *Jatropha* varieties have been screened with minimal phorbol esters that indicate the feasibility of products from these plants in animal and fish diet (Martinez-Herrera et al. 2006).

3.5 Source of Nutrients for Plants

Biomass of *Jatropha curcas* when applied as green manure in rice fields improved the crop yield (Sherchan et al. 1989). Press cake of *Jatropha curcas* being rich in nitrogen serves as the source of nutrients to plants and is used as a fertilizer (Gubitz et al. 1999). *Jatropha curcas* supports healthy growth of natural vegetation (Sahoo et al. 2009). Senescent leaf litter of *Jatropha curcas* added to the soil improved the growth of mustard (*Brassica juncea* cv RH-30), taramira (*Eruca sativa* cv T-27), chickpea (*Cicer arietinum* cv HC-5) and barley (*Hordeum vulgare* cv BH-393) up to 20 quintal per hectare but 25 quintal per hectare reduced the growth and yield of test crops but still showing better growth than untreated plants (Singh et al. 2010).

3.6 Insect and Pest Control

The seed oil contains phorbol esters which is a family of compounds possessing insecticidal, fungicidal properties due to their toxic nature (Solsoloy and Solsoloy 1997). According to Gubitz et al. (1999), phorbol esters isolated from *Jatropha* have been demonstrated to possess molluscicidal, insecticidal and fungicidal properties in laboratory and field experiments which are responsible for toxicity of *Jatropha curcas* to animals and humans. Seeds are considered antihelemintic in Brazil and ground with palm oil to use as rat poison. Leaves are used to fumigate houses against bed bug in Ghana. Ether extract exhibits antibiotic properties against *Staphylococcus aureus* and *Escherichia coli*. Methanol extracts of *Jatropha* seed (which contains biodegradable toxins) are being tested in Germany for the control of water snails. It causes a number of biological effects such as tumor and inflammation (Hass and Mittelbach 2000). Oil and aqueous extract is used to control cotton bull worm (pest of cotton), pest of pulses, potato, and corn (Kaushik and Kumar 2004).

3.7 Water Conservation

Jatropha curcas is considered as a drought-tolerant species growing well in semiarid and tropical areas. However, water use efficiency of *Jatropha curcas* is not exactly known. Increased shading by *Jatropha* plantation and presence of mulch layer of

senescent leaves may reduce evaporation from soil. Availability of soil moisture and water-holding capacity of the soil is determined by texture of soil and contents of organic matter present in it and is influenced by evapo-transpiration. Actual evapo-transpiration is determined by the dimension of the root system of *Jatropha* plants (including rooting depth, lateral soil exploration and functional root surface) and the ability of roots to take up the available water (Jongschaap et al. 2007). *Jatropha* plantation can promote infiltration, vertical and lateral redistribution and evapo-transpiration by combination of several hydrological processes thereby overcoming the limitations posed by compaction of soil surface to water infiltration in the soil (Qiu et al. 2001). Plantation of *Jatropha* may be one of the alternative to conserve water and can contribute toward the economic stability of farmers (Poonia and Jethoo 2012).

3.8 Soil Conservation and Fertility

Land degradation is one of the serious environmental problems resulting in loss of soil fertility and soil biodiversity (Lal 2004), and prolonged dryness, loss of vegetation cover, inappropriate land use and poor soil management are the major causes responsible for resulting in decreased agricultural potential of soil. *Jatropha curcas* can easily be grown on marginal soils and can survive on poor stony soil therefore helping reclaim land (Munch and Kiefer 1989). *Jatropha* leaves produce large quantity of organic matter increasing microbial and earthworm activity in soil indicating ecological improvement (Gubitz et al. 1999). *Jatropha* can be grown on barren lands for the removal of carbon from the atmosphere and the building up of soil carbon thereby reclaiming and restoring eroded areas (Makkar et al. 2007).

Adequate root system of *Jatropha* plants helps in recycling of nutrients from deeper soil and reclaim marginal soils. After 18 months of plantation macro-aggregate stability increased up to 30 % and bulk density of soil was reduced by 20 %, thereby bringing about change in soil structure (Chaudharry et al. 2007). The ability of *Jatropha curcas* to resist drought and grow wild in low rainfall harsh climatic conditions may be beneficial for restoration of degraded ecosystem, alleviate soil degradation, desertification, and deforestation (Francis et al. 2005; Juwarkar et al. 2008). *Jatropha* is suitable for preventing soil erosion and shifting of sand dunes as it is a highly adaptable species and has the ability to grow on very poor and dry sites. The organic matter from shed leaves enhances earthworm activity in the soil around the root zone of the plants leading to the improvement of soil fertility (Kumar and Sharma 2008).

3.9 Hedge Plant

Jatropha curcas is being promoted as hedge plant (live fence) to protect field particularly because it is not eaten by cattles (Heller 1996). Because of relatively higher

moisture contents in trunk and twigs *Jatropha* plants exhibit better tolerance to fire and is also planted as fire barrier to prevent the spread of fire outbreak. As a hedge it also prevents spread of diseases and insect infestation in afforested area (Li et al. 2006). *Jatropha* plants can be cut at any desired height and is well adapted for hedges around agricultural fields (Gubitz et al. 1999). Therefore, in addition to seed yields it serves the purpose of bio-fence with cost-effectiveness as compared to wire fence (Kumar and Sharma 2008).

4 Phytochemical Constituents

Many biologically active substances have been isolated and characterized from different parts of *Jatropha* (Gubitz et al. 1999). Stem extract of *Jatropha curcas* is reported to contain saponins, tannins, glycosides, alkaloids and flavonoids of phenolic nature (Akinpelu et al. 2009; Igbinosa et al. 2009). Though plant parts of *Jatropha curcas* differ in their metabolites, leaves and ovary walls possess much greater quantity (Table 13.1). Apart from the oil, *Jatropha* species are a significant source of many phytochemicals with varying biological activities (Devappa et al. 2010).

Saponins are natural triterpene plant glycosides found in *Jatropha curcas* seeds possessing some physiological activities (Fenwick et al. 1991). *Jatropha curcas* is rich in phenolic compounds (Bandoniene et al. 2002; Tape et al. 2006) and flavonoids (Saxena et al. 2005). *Jatropha curcas* contains polyphenolic compounds such as flavanols, cinnamic acid, coumarins and caffeic acid which scavenge free radicals and inhibit peroxidation (Bahman et al. 2007). Phenolic compounds in *Jatropha curcas* are used as natural antioxidants for the protection of oils and corresponding biodiesel in order to prevent their oxidative deterioration (Diwani et al. 2009). Igbinosa et al. (2009) have reported saponins, steroids, tannins, glycosides, alkaloids and flavonoids in the stem bark extract of *Jatropha curcas*. Aerial parts of *Jatropha curcas* contain o and *p*-coumaric acid, *p*-OH benzoic acid, protocatechuic acid, resorsilic acid, saponins and tannins. HPLC analysis revealed the presence of gallic acid, benzoic acid, quercetin, coumaric acid, benzoic acid and salicylic acid, out of which gallic and benzoic acid were predominant (Diwani et al. 2009).

Table 13.1 Total phenols, tannins, phytic acid and free amino acids (mg g^{-1}dr wt.) in different parts of *Jatropha curcas* L.

Plant parts	Total phenols (mg g^{-1} dr wt.)	Tannins (mg g^{-1} dr wt.)	Phytic acid (mg g^{-1} dr wt.)	Free amino acids (mg g^{-1} dr wt.)
Leaf	4.23 ± 0.28	41.0 ± 1.73	34.50 ± 2.08	14.66 ± 0.33
Stem	0.60 ± 0.03	8.50 ± 0.13	21.33 ± 0.60	5.20 ± 0.11
Root	0.57 ± 0.02	9.70 ± 0.40	25.93 ± 1.82	8.60 ± 0.17
Ovary wall	4.59 ± 0.10	44.43 ± 1.29	29.49 ± 0.66	3.90 ± 0.05
Seed	0.84 ± 0.03	8.46 ± 0.17	17.50 ± 0.28	7.93 ± 0.23

5 Allelopathic Effects

Allelopathy refers to the process by which plants produce certain compounds that are released into environment, where they interfere with the growth of other plants. Allelopathy can play an important role in the environmental impact of commercial plantation such as degradation of soil, reduction of productivity, and biodiversity (Vesterdal et al. 2002). *Jatropha curcas* L. is an exotic species that shows invasive characters and has been shown to possess certain antinutritional factors. Plantation of *Jatropha* species is being undertaken at a large scale. *Jatropha curcas* exhibits autotoxicity as higher concentrations of fresh leaf extracts inhibit its seed germination and lower concentrations show stimulation. Nevertheless, inhibitory effects of higher concentrations were more noticeable than stimulatory effects of lower concentrations (Cheng-Zhong et al. 2009).

Application of *Jatropha curcas* leaf leachate to soil resulted in reduced shoot and root length of marigold (*Tagetes erecta* L) and increase in membrane permeability and proline contents in roots of marigold seedlings. Residue incorporated into soil also showed similar effects on the growth of marigold plants. This indicates that *Jatropha curcas* plants release some phytotoxic compounds that are responsible for allelopathic effect (Wang et al. 2009). Higher concentration of *Jatropha curcas* leaf and root extracts has been reported to strongly inhibit germination, radical and plumule length of some test species such as *Phaseolus vulgaris*, *Zea mays*, *Lycopersicon lycopersicum* and *Hibiscus esculentum*. These inhibitory effects suggest presence of allelochemicals in leaf and roots of *Jatropha curcas* (Abugre and Quashie-Sam 2010).

Aqueous extracts from leaves and roots of *Jatropha curcas* inhibit growth of corn (*Zea mays*) and tobacco (*Nicotiana tabacum*). Degree of inhibition increases with increasing concentration of extracts. Chlorophyll contents, rate of photosynthesis and stomatal conductance decline but rate of transpiration increases with increasing concentration of the extracts. The presence of azelaic acid has been detected in *Jatropha curcas*, using GC-MS possibly providing a competitive advantage to *Jatropha curcas* in defense mechanism by inhibiting growth of neighboring plants (Ma et al. 2011). Inhibitory effects are attributed to allelopathic substances present in plants. Treatments of *Jatropha curcas* leaf extracts cause gradual decrease in germination percentage and growth of *Capsicum annum*, whereas germination and shoot length increases with increasing concentration of extract of *Sesamum indicum*; nevertheless, inhibition in root growth has been noticed in all treatments (Rejila and Vijaya Kumar 2011).

6 Conclusion and Future Perspectives

Jatropha curcas is a plant with many attributes and considerable potential and its ability to grow on waste, marginal lands in dry and low nutrient soils and its oil-producing potential has attracted the attention of scientists, ecologists and several government and non-government agencies to promote its plantation as biodiesel crop.

Table 13.2 Sodium, potassium, calcium and chloride (%) in different part of *Jatropha curcas* L.

Plant parts	Sodium (%)	Potassium (%)	Calcium (%)	Chloride (%)
Leaf	1.136 ± 0.008	5.340 ± 0.059	3.476 ± 0.053	4.262 ± 0.085
Stem	1.342 ± 0.003	4.927 ± 0.047	2.848 ± 0.044	3.971 ± 0.080
Root	2.038 ± 0.025	3.095 ± 0.064	2.486 ± 0.061	1.344 ± 0.060
Ovary wall	0.628 ± 0.010	4.196 ± 0.011	0.824 ± 0.034	0.926 ± 0.035
Seed	0.190 ± 0.030	1.376 ± 0.011	1.103 ± 0.058	0.533 ± 0.026
Soil	0.322 ± 0.014	1.710 ± 0.032	0.246 ± 0.001	1.042 ± 0.048

Table 13.3 Analysis of sodium, potassium, calcium and chloride (%) in roots of *Jatropha curcas* and corresponding soil

Percentage	Roots	Corresponding soil
Sodium (Na)	1.988 ± 0.025	0.375 ± 0.005
Potassium (K)	3.315 ± 0.062	1.460 ± 0.032
Calcium (Ca)	2.396 ± 0.041	0.430 ± 0.030
Chloride (Cl)	1.346 ± 0.006	1.036 ± 0.046

Promotion of biodiesel will not only provide ecofriendly source of alternative energy to reduce import of petro-diesel but also add to generation of employment opportunities in rural areas and reduce green house gases. Some of the areas which need greater attention in contemporary research are identified below.

Major constraints to *Jatropha* plantation is the lack of knowledge of its yield under suboptimal conditions and on marginal or wastelands. For success of new agro-industrial crop, stability in annual profitable seed production should be established. Despite being a xerophyte the arid environment need not be ideal for commercial plantation because mechanisms which enable *Jatropha* to survive under stress are not always complementary for sustained production as commercial crop. Generally, soil moisture and fertility levels are important for good seed yield and oil contents.

Though *Jatropha curcas* is a hardy plant with higher quality of oil contents and can grow on marginal land, good growth requires proper fertilization and water supply. It sheds all its leaves during summer (May–June) to reduce transpiration rate and minimize water loss that probably imparts *Jatropha* sturdiness toward stress. *Jatropha* plants accumulate sodium, potassium and chlorides in greater quantity and are well adapted to grow in saline soils (Tables 13.2 and 13.3). These plants show greater tolerance to saline soils, therefore can be used to improve saline soils by reducing its salinity. Further work may be useful pertaining to the utility of *Jatropha* for improvement of saline soils as well as on its allelopathic effects.

Further, organic manure from *Jatropha curcas* may improve nutrient status of soil and growth of some crops however, for which experiments must be carried out before its application. If grown as hedge plant it is useful in protecting crop plants from grazing animals. Hedge from *Jatropha* plants can be useful in honey production and also as fire barrier. *Jatropha* meal may serve as important source of nutrients for fishes and poultry after detoxification. Nevertheless, phorbol esters in *Jatropha curcas* plants may also be utilized for biological control of insect and pests which need extensive investigation.

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