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Editors

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This book is dedicated to the Institute of Vegetable Science of Zhejiang University for advancing vegetable research and industry in China.

Preface

In nature, plants are often exposed to different environmental adversities. Although success in crop cultivation involves good care and management, some environmental factors at extreme states pose serious challenges that ultimately limit crop production. Abiotic stress is one of the primary causes of crop losses worldwide. The stress caused by abiotic factors alters normal plant metabolism leading to various negative effects on plant growth, development, and productivity. To survive, sessile plants have evolved a wide array of molecular programs to perceive environmental stimuli rapidly and adapt accordingly. In recent years, much progress has been achieved in unraveling the complex stress response mechanisms, particularly the involvement of different phytohormones in stress perception and signal transduction. Phytohormones are the most fascinating features of plant system that precisely regulate growth, development, and responses to stresses. In addition to normal regulatory functions, classical phytohormones such as auxins, cytokinins, gibberellins, abscisic acid, and ethylene could induce stress tolerance to various abiotic factors. New plant hormones such as jasmonates, salicylates, brassinosteroids, strigolactones, etc., have also been implicated in plant growth and stress adaptation. Although the in-depth mechanisms of phytohormone-mediated stress tolerance still remain largely unknown, plant growth regulators or hormone analogues are being used to manage different environmental adversities. Nonetheless, there is still a remarkable gap between theory and practice in terms of large-scale field application.

In this book, we tried to provide a unique compilation of the roles of phytohormones in the response of plants to abiotic stresses considering heat, cold, drought, salinity, flooding, soil acidity, heavy metals, light, and ozone as an individual environmental hazard in each chapter. The physiological and molecular mechanisms controlling phytohormone-mediated tolerance to a single abiotic stress and interactions among them are discussed for relevant cases. In the last chapter, genetic engineering aspects of phytohormone metabolism along with major challenges and future research directions are suggested. Much attention has been paid to adhere with the focus in each chapter that enabled the authors to avoid repetition of similar issues. It is worth mentioning that all authors of this book have recently contributed original research articles in the field of phytohormone research. The chapters are

written at the levels intended to be useful to students (senior undergraduate and postgraduate) and researchers in plant physiology, biochemistry, and biotechnology. Although minor editorial changes were adopted, author's justification was kept intact in each chapter. However, some errors may still exist in the book, and thus we would greatly appreciate reader's feedback for potential improvement in future edition. We wish to thank all the authors who joined this book project by contributing their valuable works. We extend our sincere thanks to Springer Science+Business Media, especially Mr. Zachary Romano (editor, biochemistry and molecular biology, Springer New York), Mr. Abbey (Xiaojin) Huang (assistant editor, medicine and biological sciences, Springer Beijing Office), and all the other staff members of Springer involved in this book project for their generous cooperation.

Hangzhou, Zhejiang, People's Republic of China

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Golam Jalal Ahammed is a Research Associate at the Department of Horticulture, Zhejiang University, Hangzhou, China. He obtained his B.Sc. (Hons.) in Agriculture in 2004 and M.S. in Horticulture in 2006 from Bangladesh Agricultural University, Mymensingh, Bangladesh. Dr. Ahammed received his Ph.D. in Olericulture with major focus on plant stress physiology and hormonal regulation in 2012 from Zhejiang University, China. His major research interests include plant stress physiology, phytohormones, climate change effect on plants, and environmental pollution. He has authored over 30 research papers in peer-reviewed journals. Dr. Ahammed is a regular reviewer for a number of prestigious journals. Moreover, he has edited special issues for *Current Protein & Peptide Science*. Dr. Ahammed has been awarded an International Young Scientist Fellowship in 2015 by the National Natural Science Foundation of China. He is also a recipient of China Postdoctoral Science Foundation Award (56th and 58th). Currently, Dr. Ahammed is actively engaged in unraveling hormonal involvement in iron homeostasis under elevated carbon dioxide and interaction with cadmium in tomato.

Chapter 1
Role of Hormones in Plant Adaptation to Heat Stress

Golam Jalal Ahammed, Xin Li, Jie Zhou, Yan-Hong Zhou, and Jing-Quan Yu

Abstract Heat stress is one of the devastating abiotic stresses that cause substantial crop loss around the world. The frequency and magnitude of heat stress are being intensified due to global climate change. Heat stress induces excessive production of reactive oxygen species that cause damage to lipids, proteins, and nucleic acids in plants. Plants have evolved various sophisticated mechanisms to sense heat stimuli and activate different defense responses rapidly to protect its vital cellular structures from heat-induced damage. Phytohormones are the endogenous messenger molecules that precisely mediate plant growth, development, and responses to various biotic and abiotic stresses including heat stress. With the advancement of molecular technologies, several hormones that were previously known only for their roles in plant growth and development have also been implicated in the heat stress response. To date, all major hormones such as abscisic acid, auxin, gibberellins, cytokinins, salicylic acid, jasmonic acid, ethylene, and brassinosteroids have been reported to play critical roles in response of plants to heat stress. In this chapter, we intend to review how various phytohormones are involved in plant adaptation to heat stress. Furthermore, we discuss the potential role of important plant hormones in the enhancement of heat tolerance. Hormone cross talk that mediates the response of plants to heat stress is also discussed.

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1.1 Introduction

Plant growth and development are precisely regulated by coordination of both exogenous (environmental) and endogenous signals (Peleg and Blumwald 2011; Santner et al. 2009). External environmental factors such as light, temperature, moisture, and atmospheric carbon dioxide at optimum levels are essential for normal metabolic processes in plants. Among those various environmental factors, temperature is of great significance in the regulation of plant phenological development (Bahuguna and Jagadish 2015). A temperature beyond the "physiological optimum" that disturbs normal growth and development of a plant is generally considered as "high temperature" for that plant. Extremely high temperatures have the potential to cause "heat stress" in plants. However, the physiological threshold for the highest temperature that causes irreversible damage varies significantly across the plant kingdom even within genotypes. Heat-induced damages include changes in stability of proteins, enzymes, nucleic acids, biomembranes, and cytoskeletal structures (Asthir 2015). In order to maintain appropriate balance in metabolic processes, plants thoroughly or partially reprogram its transcriptome, proteome, metabolome, and lipidome (Mittler et al. 2012).

The frequency and magnitude of temperature extremes are being increased due to global climate change. Extreme seasonal heat which is considered as an important attribute of climate change could have detrimental impacts on agricultural productivity and food security by directly affecting crop growth and yield. In the tropics and subtropics, growing season temperatures will exceed the recorded highest seasonal temperatures of the last century by the end of the twenty-first century. Empirical data show that every 1 °C increase in seasonal temperature will likely to cause 2.5 to 16 % direct yield losses (Battisti and Naylor 2009). A recent study that analyzed national disaster-caused crop production losses (during 1964–2007) across the globe shows that drought and extreme heat dramatically decreased national cereal production by 9–10 %, and reduction in cereal yields was mainly due to extreme heat (Lesk et al. 2016). The authors estimated that a 1 °C increase in seasonal mean weather associated with extreme heat disasters results in a yield sensitivity of 6–7 %. Thus, rising temperature represents severe risks of food insecurity. It is anticipated that increasing heat stress will cause complete extinction of many species (Field et al. 2014). Therefore, climate change will obviously intensify the deleterious effect of heat stress on crop yield in the future.

As plants cannot relocate, they have to experience brutality of environmental extremes *in situ*. Extreme high temperature severely affects different biochemical processes in plants that are sensitive to heat shock. Heat stress causes changes in respiration and photosynthesis leading to a decreased plant productivity. Heat stress-

induced injuries are associated with over production of reactive oxygen species (ROS) that cause cellular damage. Through evolutionary success, plants have developed a complex but effective heat stress response (HSR) pathway that precisely regulates both short-term and long-term heat responses in order to minimize or prevent damage from heat stress. A better understanding of this complex heat response mechanism is important for future genetic manipulation of crops to ensure food security in the face of climate change (Hasanuzzaman et al. 2013; Kazan 2015).

Phytohormones are the endogenous messenger molecules that play a critical role in plant growth, development, and responses to various stresses. A number of previous reports provided solid evidence that phytohormones are actively involved in the response of plants to heat stress (Ahammed et al. 2014; Peleg and Blumwald 2011; Xia et al. 2015). Moreover, majority of the phytohormones provide physiological protection against heat stress. In addition to the individual role of a plant hormone, cross talk between multiple hormones precisely coordinates plant defense response to heat stress. Although significant advancement has been achieved in the molecular mechanisms of heat perception, the mechanisms that control phytohormone-mediated responses to heat stress still remain largely unknown. In this chapter, we briefly highlight the key progress on plant hormone research relating to the mechanisms of temperature sensing and subsequent thermal responses that are mediated by complex phytohormone networks. Additionally, we discuss the potential role of important phytohormones in the enhancement of heat tolerance.

1.2 Sensing Thermal Stimuli by Plants

In plants, changes in temperature are simultaneously perceived by all plant cells that are exposed to high temperature. Thus, at the cellular level, thermal perception and signaling may involve similar components across the plants (Mittler et al. 2012; Saidi et al. 2011). Plants have developed highly sophisticated mechanism that can even sense small changes (even 1 °C) in temperature (Kumar and Wigge 2010). Multiple pathways, regulatory networks, and cellular compartments are involved in the heat stress response (HSR) in plants (Mittler et al. 2012). The HSR is a highly conserved environmental and developmental program in plants and is an important component of the acclimation response. Using a wide array of thermosensors, plants precisely sense the absolute and gradual changes in diurnal and seasonal temperature (Bahuguna and Jagadish 2015).

All macromolecules in a plant cell such as protein complexes, membranes, and nucleic acids can simultaneously perceive heat stimuli from the surrounding atmosphere, and thus they serve as thermosensors through reversible physical changes such as increased membrane fluidity, partial melting of DNA and RNA strands, dissociation of proteins, etc. (Mittler et al. 2012; Richter et al. 2010). However, primary heat sensors or heat-responsive macromolecules must have the potential to perceive heat stimuli precisely followed by differential response to temperature change depending on the extent of variation. More importantly, they must possess

capability to differentially activate a unique signaling pathway that ultimately upregulates hundreds of HSR genes. As reviewed in detail elsewhere, at least four putative sensors are assumed to be involved in triggering the HSR (Mittler et al. 2012): such as (1) a plasma membrane channel for initiating an inward calcium flux, (2) a histone sensor localized in the nucleus (H2A.Z nucleosomes), (3) one unfolded protein sensor in the endoplasmic reticulum, and (4) one unfolded protein sensor in the cytosol.

Briefly, heat stress alters membrane stability and activates a plasma membrane (PM) calcium channel that causes an inward flux of calcium. In addition, lipid signaling is also stimulated by the alteration in membrane stability. Upon binding with the calmodulin CaM3, calcium activates various kinases and transcriptional regulators of the HSR, including heat shock transcription factors (HSFs), multiprotein-bridging factor 1c (MBF1c), WRKY, and dehydration-responsive element-binding transcription factors (DREB). PM-localized ROS-generating enzyme respiratory burst oxidase homolog D (RBOHD) is also activated by enhanced cytosolic calcium flow. ROS play a dual role; while NADPH oxidase-dependent ROS generation triggers redox signaling that activates MBF1c, HSFs, and mitogen-activated protein kinases (MAPKs), heat-induced excessive accumulation of ROS causes metabolic imbalance. The unfolded protein sensor in the cytosol functions through HSFs, whereas the unfolded protein sensor in the endoplasmic reticulum (ER) acts through the inositol-requiring enzyme 1 (IRE1) and transcription factors (TFs) such as basic leucine zipper bZIP17, bZIP28, and bZIP60. However, activation of the unfolded protein response (UPR) possibly requires specific calcium signals from the PM, indicating that UPR does not meet the characteristics of primary heat sensor in plants. The activation of the ER UPR pathway releases different bZIP TFs from the ER membrane, and then the released TFs enter the nuclei causing accumulation of ER chaperone transcripts and the activation of brassinosteroid (a steroidal plant hormone) signaling by inducing transcription of specific genes (Che et al. 2010; Mittler et al. 2012). The occupancy of the H2A.Z histone sensor localized in the nucleus is decreased following warming in *Arabidopsis*. H2A.Z-containing nucleosomes also coordinate the ambient temperature transcriptome, suggesting a temperature-dependent regulation of transcriptome by H2A.Z nucleosomes (Kumar and Wigge 2010). Perhaps, a complex signal transduction network integrates signals from all these different sensors involving calcium fluxes, calmodulin, calcium-dependent protein kinases (CDPKs), MAPKs, phosphatases, and transcriptional regulators such as HSFs, MBF1c, WRKY, DREB, and bZIP. Eventually, all these sensors can activate similar set of HSR genes that improve thermotolerance in plants (reviewed in Mittler et al. 2012). It is also becoming evident that phytohormones are involved in this signal transduction network. Nonetheless, the activation of the different pathway upon heat stress may vary depending on tissue type, more specifically in between vegetative and reproductive tissues. Although a significant progress has been achieved in the molecular mechanisms of heat perception, physiological relevance of plant thermal responses under a complex environmental condition is largely unknown (Bahuguna and Jagadish 2015).

1.3 The Concept of Basal and Acquired Thermotolerance

It is well conceived that gradual and abrupt fluctuations in temperature pose a serious threat to sustainable crop production and global food security. Short-term changes in temperature may trigger acclimation response, while gradual changes may activate adaption response in plants (Bahuguna and Jagadish 2015). The ability of a plant to tolerate high temperature without prior exposure to mild high temperature is generally referred as basal thermotolerance, whereas the adaptive capacity of a plant to survive under extremely high temperatures following preexposure to mild high temperature is considered as acquired thermotolerance. The ultimate survival of the plants depends on both basal and acquired thermotolerance (Ahuja et al. 2010). The requirement for certain regulatory and acclimation proteins may vary based on the kinds of tolerance such as basal and acquired thermotolerance (reviewed in Mittler et al. 2012). For instance, transcriptional regulator MBF1c or the ROS-detoxifying enzyme catalase is required for basal thermotolerance, while they are not required for acquired thermotolerance. However, some HSFs and the disaggregating chaperone heat shock protein 101 (HSP101) may be required for both basal and acquired thermotolerance. In *Arabidopsis*, pre-acclimation of seedlings (38 °C for 3 h) significantly increases thermotolerance at 45 °C, and accumulation of HSP101 is found positively associated with seedling survival and post-stress root growth. It is suggested that the modulation in HSP101 expression and associated thermotolerance appear to be interrelated and might be evolved adaptively in natural populations of *A. thaliana* (Zhang et al. 2015).

1.4 Hormones Involved in the Response of Plants to Heat Stress

The involvement of phytohormones in the plant response to heat stress can be investigated in many ways. Exposure approaches include short-term heat shock with extremely high-temperature, heat acclimation study by exposing plants to a mild heat stress before imposing extreme heat stress and also long-term high-temperature treatment. In most researches that are limited to the analysis of biochemical and physiological parameters, endogenous content of one or multiple hormones following heat stress is quantified together with the evaluation of different growth parameters such as biomass production and photosynthesis. This type of study is often accompanied with pre- or post-application of exogenous hormones predominantly on foliar portion. Thus, changes in certain phytohormone level and/or plant tolerance in response to high temperature are indicated as potential involvement of that hormone in the plant response to heat stress. Advanced studies that employ sophisticated molecular techniques coupled with functional analysis through generating impaired mutants or overexpression plants for a specific gene involved in hormone biosynthesis or signaling have greatly unraveled complex hormone signaling pathway under heat stress.

Nonetheless, the response remarkably varies with the stage (vegetative or reproductive) of plant when heat stress is imposed (Mittler et al. 2012).

Heat stress alters hormone homeostasis, stability, content, biosynthesis, and compartmentalization in plants (Maestri et al. 2002). With the advancement of molecular technologies, several hormones that were previously known only for their roles in plant growth and development have also been implicated in the response of plants to heat stress (Dobra et al. 2015). In addition, participation of multiple hormones that fine-tunes the plant response to heat stress has made hormonal cross talk much more complex. To date, all major hormones such as abscisic acid (ABA), auxin, gibberellins (GAs), cytokinins, salicylic acid (SA), jasmonic acid (JA), ethylene, and brassinosteroids (BRs) have been reported to play critical roles in the response of plants to heat stress (Mittler et al. 2012; Peleg and Blumwald 2011; Xia et al. 2015; Zhou et al. 2014). Moreover, requirement for certain hormone and/or its associated signaling varies depending on the kinds of thermotolerance. For instance, SA-dependent signaling improves basal thermotolerance; however, SA signaling is not required for acquired thermotolerance in *Arabidopsis thaliana* (Clarke et al. 2004). In the following section, involvement of different phytohormones in the response of plants to heat stress is discussed mentioning some specific circumstances that are unique to hormone-mediated response to heat stress. The mechanism of hormone perception and their roles in growth and development under normal temperature are not emphasized in this chapter.

1.4.1 Abscisic Acid

About one and a half decade ago, a study conducted by Larkindale and Knight (2002) showed that heat stress tolerance involves participation of multiple hormones such as ABA, ethylene, and SA in *Arabidopsis*. They noticed that the ethylene-insensitive mutant *etr-1*, the ABA-insensitive mutant *abi-1*, and the SA-deficient *NahG* (constitutively expressing the *Pseudomonas putida* SA hydroxylase transgene *NahG* that inhibits SA accumulation) transgenic plants are sensitive to heat stress. However, application of ABA, SA, and 1-aminocyclopropane-1-carboxylic acid (a precursor to ethylene) protected wild-type *Arabidopsis* plants from heat-induced oxidative damage. Using calcium channel blockers and calmodulin inhibitors, they found that calcium is required for protecting plants from heat-induced oxidative stress especially during/after recovery. In addition, *Arabidopsis* mutants, impaired with ABA biosynthesis and signaling, show decreased basal and acquired thermotolerance (Larkindale et al. 2005). In one of our earlier studies, we also noticed that ABA-deficient mutant notabilis (*not*) tomato genotype is sensitive to heat stress (42 °C for 24 h) as evidenced by decreased photochemical efficiency (Fv/Fm) and increased lipid peroxidation compared with wild-type Ailsa Craig (Li et al. 2015). ABA levels are rapidly and transiently increased following exposure of plants to heat stress as early as 10 min in *Pisum sativum*, which might indicate its involvement in heat sensing and acclimation (Liu et al. 2006).

An optimum balance between GA and ABA is required to control the seed dormancy and germination. However, both ABA and GA are regulated by H_2O_2 that mediates enhancement of ABA catabolism and biosynthesis of GA during seed germination. Analysis of ABA catabolism mutants revealed that endogenous ABA content is inversely correlated with GA biosynthesis. High concentration of ABA inhibits seed germination by repressing GA biosynthesis, while exogenously applied GA can only overcome inhibition of germination that is induced by low level of ABA (Liu et al. 2010). In *Arabidopsis*, high temperature inhibits germination by stimulating ABA levels, and ABA in turn represses both GA synthesis and signaling. Under high temperature, the increase in ABA level in imbibed seeds is achieved by upregulation of ABA biosynthetic genes such as *ABA1/ZEP*, *NCED2, NCED5*, and *NCED9*, while GA levels remain low due to suppression of GA 20-oxidase genes, *GA20ox1, GA20ox2*, and *GA20ox3*, and GA 3-oxidase genes, *GA3ox1* and *GA3ox2*. Furthermore, ABA-deficient *aba2-2* mutant seeds showed increased expression of GA synthesis genes, but suppressed expression of GA negative regulator gene *SPINDLY (SPY)* under high temperature, indicating that ABA levels are important for controlling GA levels in seeds under high temperature (Toh et al. 2008). In *Arabidopsis*, five *NCED* genes such as *NCED2, NCED3, NCED5, NCED6*, and *NCED9* are involved in ABA biosynthesis. Among them *NCED9* plays a major role, while *NCED5* and *NCED2* play relatively minor roles in high-temperature-induced ABA synthesis and subsequent inhibition of seed germination (Toh et al. 2008).

One of the most adverse effects of high temperature is noticed during grain filling stage as high temperature limits the rate of dry matter transport from vegetative organ to kernel. Changes in hormonal levels (imbalance) in grain were found to be associated with heat stress-induced disruption of grain development (Asthir 2015; Asthir and Bhatia 2014). In wheat, every 1 °C increase in temperature above 18 °C results in 3–5 % single-grain mass fall. High temperature disrupts starch biosynthesis resulting in increased free sugar accumulation and enhanced nitrogen metabolism in wheat. Exogenous application of GA_3 and ABA promotes starch accumulation under heat stress by increasing the activities of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase (Asthir and Bhatia 2014). Although exogenous ABA does not influence endogenous ABA concentration at normal temperatures, application of ABA to ears followed by imposition of heat stress (45 °C for 2 h) significantly doubled ABA concentration in grain, suggesting an active involvement of ABA in thermoprotection.

Regulation of stomatal aperture is an important adaptation strategy in response to heat stress. Upon heat stress, tobacco (*Nicotiana tabacum* L.) plants transiently increase stomatal conductance which is mediated by an enhanced catabolism of ABA. Thus, an elevation of temperature beyond physiological optimum initially stimulates transpiration that helps plant to decrease its leaf temperature; however, enhanced transpiration causes water deficit that eventually induces ABA level for stomatal closure (Mackova et al. 2013).

As mentioned earlier the physiological threshold for the highest temperature that causes irreversible damage to cellular macromolecules varies significantly across

the plant kingdom. For instance, *Portulaca oleracea* that is widely distributed in tropical region can survive above 35 °C and 90 % relative humidity for several days, while such condition is unbearable for survival of *Arabidopsis*. *P. oleracea* applies multiple strategies for survival that include ABA-mediated regulation of stomatal conductance and respiration (Yang et al. 2012). Interestingly, depleting ABA content due to increasing ABA-8'-hydroxylase results in greater stomatal conductance and respiration rates in *P. oleracea*. Moreover, plant tolerance to heat stress varies with the species, even though they are habituated in the same geographical location. For instance, Mediterranean plants such as rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), and lemon balm (*Melissa officinalis* L.) show different tolerance to heat stress recurrence, although they exhibit similar tolerance to single heat stress event (Asensi-Fabado et al. 2013). Compared to rosemary and sage that show some sort of acclimation response with decreased JA content in rosemary and reduced ABA level in sage following stress reiteration, lemon balm is found most sensitive showing decreased relative water content, but enhanced levels of α-tocopherol and SA. Even when heat stress was combined with water deficit, rosemary and sage were much more resistant than lemon balm; although the hormonal content was not changed in sage, ABA and SA levels in rosemary were increased and decreased, respectively following repeated stress exposure, indicating that changes in hormonal levels that impact plant tolerance following stress imprints are species specific.

1.4.2 Auxins

In addition to well-established role of auxin in cell division and elongation, its role as a key regulator of adaptive growth response to high temperature has emerged during the last decade. As an adaptive response to high temperature, seedlings may elevate the photosynthetic and meristematic tissues away from heat-adsorbing soil by elongating hypocotyls and thus providing better advantage of the cooling effect of moving air (Gray et al. 1998). In accordance with this principle, *Arabidopsis* seedling elongates its hypocotyl upon mild heat stress; however, inhibition of auxin biosynthesis and mutation relating to auxin response or transport all compromise high-temperature-induced hypocotyl elongation. Similarly, BR biosynthetic or signaling mutants such as *de-etiolated2-1 (det2-1)*, *brassinosteroid insensitive1-5 (bri1-5)*, and *dwarf7-1 (dwf7-1)* also fail to show increased hypocotyl elongation in response to high temperature (Gray et al. 1998; Maharjan and Choe 2011). On the other hand, exogenous application of auxin upregulates expression of the BR biosynthetic gene *DWARF4*, indicating some functional interactions between auxin and BR to regulate temperature-induced hypocotyl elongation (Gray et al. 1998). It is suggested that temperature-induced synthesis of free auxin may stimulate BR biosynthesis via upregulating *DWARF4* that eventually regulates hypocotyl growth under high temperature (Maharjan and Choe 2011).

Auxin accumulation coupled with induction of *YUCCA* (flavin-containing monooxygenase) genes is rapidly increased upon exposure of *Arabidopsis* to mild high temperature (28–29 °C). Although all parts of *Arabidopsis* are capable of synthesizing auxin, its regulatory action also depends on polar auxin transport. For instance, upon mild heat stress, transcripts of auxin biosynthetic genes *YUCCA8* and *YUCCA9* are induced more significantly in cotyledons compared to that in hypocotyls, indicating that cotyledons serve as main auxin source under high-temperature stress, which is then transported to hypocotyls. When auxin transport is blocked using the inhibitor of polar auxin transport 1-naphthylphthalamic acid, high-temperature-induced hypocotyl elongation response is abolished, suggesting that auxin transport is essential for the response to increased temperature (de Wit et al. 2014; Stavang et al. 2009). In addition, petioles of *Arabidopsis* become elongated following exposure to high temperature, even adult plants show hyponastic leaves with long petiole and small lamina. *Arabidopsis* seedlings that are grown in 28 °C have lower number of stomata, but possess better cooling ability, possibly due to less compact shoot architecture and leaf hyponasty that facilitate transpiration. Nonetheless, long-term exposure of plants to increased temperature induces early flowering as an escape strategy in *Arabidopsis* (reviewed in de Wit et al. 2014).

In addition to phytohormones, high-temperature (28 °C)-induced hypocotyl elongation response requires a basic helix-loop-helix transcription factor, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) (Koini et al. 2009). In *Arabidopsis*, PIF4-deficient mutants (*pif4* mutants) do not show elongation responses or leaf hyponasty when transferred to high temperature. Moreover, *pif4* mutants fail to induce auxin-responsive gene *IAA29* upon mild heat stress. PIF4 regulates expression of auxin biosynthetic genes as well as endogenous levels of auxin that might mediate stem elongation under high temperature (Franklin et al. 2011). A family of *SMALL AUXIN UP RNA (SAUR)* genes is expressed under high temperature in a *PIF4*-dependent manner to trigger high-temperature-induced elongation response. Box et al. (2015) have shown that in addition to phytohormone and PIF4, transcriptional regulator *EARLY FLOWERING3 (ELF3)* controls elongation growth in a temperature-dependent manner in *Arabidopsis*. As ELF3 disappears at 27 °C (high temperature for *Arabidopsis*), it is quite likely that the locus harboring ELF3 is involved in a gene-by-environment interaction (Box et al. 2015). The gating of growth at night is relieved by high temperature which indicates the significance of temperature-dependent repression of growth in *Arabidopsis*. Until the recent past, PIF4 was considered as the key molecular player that integrates environmental signal (such as heat stimuli) and endogenous signal (such as auxin). Very recently, Wang et al. (2016) showed that HSP90 which is considered as key molecular chaperone during heat stress is also involved in mild high-temperature increase-dependent and auxin-mediated growth response in *Arabidopsis*. The authors showed that auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE1) is a HSP90 client and HSP90-SGT1 (SUPPRESSOR OF G2 ALLELE SKP1, a protein required for auxin response) chaperone system is essential for ambient temperature increase response in plants. In addition to elongated hypocotyl response, HSP90 was also found to be involved in ambient temperature increase-induced primary root growth

and lateral root formation (Wang et al. 2016). Interestingly, inhibition of HSP90 by geldanamycin significantly prevented 29 °C-induced upregulation of auxin-responsive genes such as *GH3.17*, *IAA19*, and *IAA5*, but did not alter the expression of auxin biosynthetic gene *YUCCA8*, indicating that HSP90 regulates temperature growth response by stimulating auxin signaling rather than modulating auxin biosynthesis. They also found that HSP90 plays a critical role in stabilizing TIR1 (an unstable protein) which results in enhanced accumulation of the TIR1/AFBs (auxin co-receptors). Finally, they concluded that HSP90 and SGT1 integrate temperature and auxin signaling in order to regulate plant growth in a rising ambient temperature. As plants gradually adapt to temperature increase in the real world, this finding may have significant implication in understanding of acquired thermotolerance.

Compared with vegetative stage, reproductive development of plants is more prone to high temperature (Sobol et al. 2014). Due to climate warming, global minimum night temperatures are increasing much rapidly than maximum day temperatures, which has significant negative effect on global rice yield (Shi et al. 2013). The decreased yield caused by high night temperature is partly due to increased respiration rate and membrane damage, decreased pollen germination, poor translocation of photoassimilates to grain, low seed set, and reduced grain weight. In heat-tolerant rice variety (*Oryza sativa* L. genotype N22), a concomitant induction of HSPs, calcium signaling proteins, and efficient protein modification and repair mechanism mediate enhanced tolerance to high night temperature (28 °C) especially during early grain filling stage (Shi et al. 2013). During the early phase of anther development, the occurrence of high temperature results in proliferation arrest and premature degradation of anther wall cells. Moreover, high temperature inhibits DNA proliferation in mitochondria, chloroplast, and nuclei of developing panicle. Notably, endogenous auxin levels in the developing anther and panicles are remarkably decreased under high temperature due to repressed expression of auxin biosynthetic genes (*YUCCA2*, *YUCCA6*) in *Arabidopsis* and barley. Exogenous application of auxin under high temperature induces anther cell proliferation and can reverse high temperature-induced male sterility, indicating that heat-induced tissue-specific reduction of auxin is the major cause of heat-related damage during reproductive development (Sakata et al. 2010; Oshino et al. 2011).

1.4.3 Cytokinins

Cytokinins are one of the major plant hormones that regulate numerous aspects of growth and development. Although the role of cytokinin in various developmental processes has been well characterized, our knowledge on its effect on plant stress tolerance is still fragmentary. This is possibly because of complex cross talk between cytokinin and stress signaling especially response related to abiotic stress tolerance (Zwack and Rashotte 2015). In many developmental processes, cytokinin and GA act antagonistically. Although most of the studies profoundly suggest a GA-regulated cytokinin action, evidence relating to cytokinin regulating GA activity cannot be

ignored. In tomato, cytokinins inhibit GA-dependent hypocotyl elongation as well as leaf serration (Fleishon et al. 2011). One of the most important mechanisms that plants deploy to minimize heat injury is by cooling leaf, the most important organ that performs photosynthesis. Leaf cooling is actually achieved by increasing transpiration under heat stress. In this response cytokinins play a critical role by stimulating stomatal opening that facilitates transpiration. Cytokinins can induce a number of heat-responsive proteins including small heat shock proteins (sHSPs) and glycine-rich protein under heat stress. Overexpression of cytokinin biosynthetic gene *ISOPENTENYLTRANSFERASE (IPT)* increases endogenous cytokinin levels that eventually enhance heat stress tolerance in grass (Xing et al. 2009). In addition to upregulation of endogenous cytokinin level by overexpressing *IPT*, exogenous application of cytokinin can also improve tolerance to heat stress in bent grass (Xu and Huang 2009). Transgenic tobacco plants overexpressing the *CYTOKININ OXIDASE/DEHYDROGENASE 1 (CKX1)* gene of *Arabidopsis thaliana* L. show reduced and delayed stomatal response, but maintain a lower leaf temperature (Mackova et al. 2013). In addition, overexpression of *CKX1* in roots using the WRKY6 promoter results in enhanced drought and heat tolerance in tobacco.

As mentioned earlier, the most devastating effect of heat stress is observed when stress occurs during reproductive stage particularly during anthesis period. Stress at anthesis causes floral abortion, resulting in severe yield loss. For instance, exposure of flower primordia of passion fruit (*Passiflora edulis*) to hot ambient temperatures causes total floral abortion (Sobol et al. 2014). Two growth hormones such as GAs and cytokinins play different roles in this condition. Application of GA_3 triggers floral abortion, but application of cytokinins improves heat tolerance by substantially minimizing floral abortion. More importantly, heat-tolerant passion fruit genotypes have been found to contain high levels of cytokinins in their leaves. In *Arabidopsis*, transgenic plants with low cytokinin levels are prone to heat stress, while transgenic plants with high cytokinin levels show increased heat tolerance during flowering. Moreover, application of exogenous cytokinins on wild-type *Arabidopsis* plants also demonstrates a protective role of cytokinins against hot air temperature during flower development stage. Cytokinins might either reduce endogenous GAs levels to improve heat tolerance, or it may simply affect downstream branches of GA signaling pathway without altering active GAs levels (Fleishon et al. 2011; Sobol et al. 2014). However, such protective effect of cytokinin is quite contradictory compared with its role in other stresses such as drought or water deficit. *Arabidopsis* mutant with low level of cytokinins showed increased tolerance to water stress, in which low levels of cytokinins perhaps increase ABA sensitivity that eventually promotes drought tolerance in plants (Nishiyama et al. 2011).

Studies have revealed that rapid perception of heat stimuli and subsequent transduction of its signal are crucial for activating plant defense system timely. Nonetheless, these phenomena are mediated by a complex signaling network where phytohormones play an important role. For instance, heat stress activates cascade of HSFs where HSFA2 serves as a master regulator of the HSR (Liu and Charng 2013). Activation of HSFs rapidly upregulates expression of various genes including those involved in the synthesis of HSPs that serve as molecular chaperons by preventing

protein denaturation (Mittler et al. 2012). In *Arabidopsis*, heat stress imposed to specific tissue such as shoots and roots or whole plants remarkably influences plant physiological responses (Dobra et al. 2015). These responses are found to be closely associated with the alteration in endogenous phytohormone levels such as cytokinins. Upon exposure to heat stress, transcript levels of *HSFA2* and heat stress associated 32-kD protein (*HSA32*) genes are rapidly induced in the stressed organ; however, expression of those genes in nonstressed organ remains low at least up to 2 h. Upon exposure of shoot or whole plants to heat stress, ABA content is transiently decreased, but active cytokinin levels are increased in leaves. These changes are associated with the stimulation of transpiration that might help plants to cool down leaf temperature. Furthermore, heat stress imposed to a part of the plants quickly stimulates the expression of the components of cytokinin signaling pathways even in the nonexposed tissues, suggesting a hormone-mediated rapid interorgan communication under heat stress. Until the stress becomes severe, the plant responses to heat stress also include a transient increase in active cytokinin levels and upregulation of the genes involved in photosynthesis and carbohydrate metabolism (Dobra et al. 2015).

1.4.4 Gibberellins

Gibberellins (GAs) are a group of natural diterpenoids primarily involved in plant growth and development such as seed germination, vegetative growth, flowering induction, and fruit development (Sun and Gubler 2004). GA-mediated growth promotion is induced by the degradation of DELLA proteins. As mentioned earlier, GA has been implicated in the response of plants to heat stress too. In *Arabidopsis*, germination and seedling growth are severely affected when seeds are heat stressed for 3 h at 50 °C. Exogenous application of GA_3 (50 μM) alleviates heat stress-induced inhibition in the germination and seedling growth (Alonso-Ramirez et al. 2009). Moreover, overexpression of a member of the GA_3 stimulated in *Arabidopsis* family gene (*GASA4*) from beechnut (*Fagus sylvatica*) in *Arabidopsis* improves plant tolerance to heat stress. Interestingly, exogenous application of GA_3- or overexpression of *FsGASA*-mediated enhanced tolerance to heat is accompanied with upregulated expression of the *ISOCHORISMATE SYNTHASE1* and *NONEXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1)* as well as increased accumulation of SA in *Arabidopsis* under heat stress, suggesting that GA alleviates heat stress-affected seed germination and seedling growth by modulating SA biosynthesis and signaling.

It is well conceived that rapid elongation of the stem is an adaptive response of plants to high ambient temperature. However, suppressed biosynthesis of GAs compromises high-temperature-induced hypocotyl elongation in *Arabidopsis* (Stavang et al. 2009). This implies that, in addition to auxin, rapid modulation of GA pathway is essential for high-temperature-induced hypocotyl elongation. BR pathway is also involved in such response; however, it is required at later stage of growth.

Additionally, GA pathway also promotes activity of PIF4 at the posttranslational levels under high temperature (Stavang et al. 2009).

Flowering time is not only important for shaping life cycles of plants but also that of the pollinator species. In higher plants, warmer temperatures accelerate floral transition, while such acceleration of flowering is dependent on the florigen (Flowering LOCUS T, FT). Under high temperature, FT is activated by PIF4 that mediates floral transition. Phytohormone GAs stimulate degradation of DELLA protein that influences PIF4 activity, while PIF4 can directly activate FT, suggesting a possible mechanism by which GAs levels influence flowering (Kumar et al. 2012). Warmer temperature increases the accessibility of PIF4-binding site at the FT promoter, which facilitates the release of H2A.Z nucleosomes (Bahuguna and Jagadish 2015). Previous studies have also shown direct molecular link between auxin, GA, PIF4, and growth elongation response under high temperature, which all together establishes PIF4 as a key node that integrates various environmental signals and hormone signaling pathways. In addition to solid evidence in support of coincidence model that mainly based on the transcriptional level of the PIF4 and PIF4-target genes, recent proteomics study has also verified the external coincidence model focusing on PIF4 protein level that integrates temperature-adaptive photoperiodic control of plant growth in *Arabidopsis thaliana* (Yamashino et al. 2013). PIF4-tag proteins are significantly accumulated predominantly at the end of the night specifically at 28 °C in a long day, but not at 22 °C.

1.4.5 Salicylic Acid

The role of SA in basal thermotolerance is well established. The SA-mediated pathway improves heat tolerance in a wide range of plant species including potato, mustard, tobacco, tomato, bean, and *Arabidopsis thaliana* (Horváth et al. 2007; Li et al. 2010). In *Arabidopsis*, as compared to wild-type plants, the SA-deficient *NahG* transgenic plants show the lowest thermotolerance, while SA-accumulating *cpr5* (*constitutive expressor of PR genes*) mutants exhibit the highest thermotolerance, indicating the relevance of endogenous SA in basal thermotolerance. In addition to SA content, SA-dependent signaling plays an important role in basal thermotolerance. The SA-signaling mutants *npr1-1* and *npr1-5* are prone to heat stress (Ahammed et al. 2015). While the *npr1-1* mutant shows an increased electrolyte leakage and decreased thermotolerance, the *cpr5-1* mutants that are constitutively active in SA, JA, and ethylene signaling pathways show enhanced tolerance to heat stress (Clarke et al. 2009). However, NPR1-dependent pathway is only involved in the recovery period but not in the thermotolerance during heat stress (Clarke et al. 2004).

Exogenous application of SA induces certain *HSPs* gene expression and eventually improves heat tolerance in *Arabidopsis* (Clarke et al. 2009; Horváth et al. 2007). In wheat, SA pretreatment not only enhances the protein kinase activity but also inhibits the degradation of D1 protein under heat and high light stress (Zhao et al. 2011). Nonetheless, the recovery of D1 protein is accelerated by SA during recov-

ery period too. In grape vine (*Vitis vinifera* L.), pretreatment with SA alleviates heat stress-induced photosynthetic inhibition by maintaining a higher ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activation state and enhancing photosystem II efficiency (Wang et al. 2010). The mechanisms that control SA-mediated thermotolerance in grape vine also involve SA-induced accumulation of HSP21 in leaves especially in the recovery period. Studies using ABA and SA biosynthesis inhibitors indicate that possibly heat-induced increases in SA levels are not the result of a direct interaction, rather via an indirect interaction where heat stress increases endogenous ABA, which then functions to protect plant from heat injury (reviewed in Kurepin et al. 2013).

The WRKY transcription factor superfamily plays a crucial role in the response of plants to heat stress. Although a number of *WRKY* genes have been reported to be involved in heat stress response, a few of them play a critical role in plant hormone-mediated heat tolerance. For instance, *WRKY39*, a member of the group II WRKY protein, positively regulates collaboration between the SA- and JA-activated signaling pathways under heat stress in *Arabidopsis* (Li et al. 2010). Transcript of *WRKY39* gene can be induced by heat shock and exogenous SA or methyl jasmonate. Functional analysis revealed that the *WRKY39* knockdown mutants (*wrky39*) are sensitive to heat stress, while transgenic plants overexpressing *WRKY39* are relatively tolerant to heat stress in comparison to wild-type *Arabidopsis* plants. Interestingly, the expressions of SA-regulated *PATHOGENESIS-RELATED 1 (PR1)* and SA-related *MBF1c* genes are suppressed in *wrky39* mutants, but their transcript levels in *WRKY39* overexpressing plants remain upregulated. As transcript of *PR1* in *wrky39* mutants is decreased more significantly than that of *MBF1c*, it is speculated that *WRKY39* possibly acts upstream of *PR1* and downstream of *MBF1c*. MBF1c is a highly conserved transcriptional coactivator that precisely regulates thermotolerance in *Arabidopsis thaliana* (Suzuki et al. 2008). MBF1c functions upstream to SA, ethylene, and PR1 during heat stress, but not essential for induction of transcripts encoding HSFA2 and HSPs, indicating an existence of a tightly coordinated HSR network under the control of MBF1c.

1.4.6 Jasmonic Acid and Ethylene

Jasmonic acid (JA) is a long-distance signaling molecule that mediates plant response to biotic and abiotic stresses. In *Arabidopsis*, JA plays a positive regulatory role in basal thermotolerance, while ethylene shows the reverse effect (Clarke et al. 2009; Kazan 2015). As mentioned earlier, *Arabidopsis cpr5* mutants that are constitutively active in JA, SA, and ethylene pathways show enhanced heat tolerance, but such heat tolerance is compromised in *cpr5 opr3*, *cpr5 coi1*, and *cpr5 jar1* double mutants, indicating the essentiality of both JA biosynthesis and signaling in the process of thermotolerance. Nonetheless, exogenous application of JA remarkably improves heat tolerance in wild-type *Arabidopsis*. Although ethylene production is increased under heat stress, exogenous ethylene application cannot confer heat

tolerance in *Arabidopsis*. Accordingly, the ethylene-insensitive *ein2* mutant displays enhanced heat tolerance, implying that the EIN2-mediated pathway might suppress heat tolerance in *Arabidopsis* (Clarke et al. 2009).

Apart from the research on typical model plant *Arabidopsis*, the study using suspension culture of medicinal plant *Aquilaria sinensis* shows that heat shock (50 °C for 30 min) rapidly and significantly upregulates the expression of JA pathway genes and induces production of JA that is essentially important for the enhanced production of agarwood sesquiterpene (desired secondary metabolites) (Xu et al. 2016). Nordihydroguaiaretic acid, a specific inhibitor of JA, can block heat-induced upregulation of JA signaling pathway genes and can reduce accumulation of sesquiterpene compounds, indicating that JA mediates heat shock-induced enhancement in secondary metabolism in *A. sinensis*, which is possibly an adaptive response to heat stress.

In pepper (*Capsicum annuum*), *CaWRKY6* (subgroup IIb WRKY family gene) also plays a critical role in high-temperature response (Cai et al. 2015). Expression of *CaWRKY6* can be induced by exogenous application of JA, ABA, and ethylene. Suppression of *CaWRKY6* by virus-induced gene silencing (VIGS) increases susceptibility to heat stress which is associated with the downregulation of JA-, ethylene-, and ABA-induced marker gene expression. However, overexpression of *CaWRKY6* improves tolerance to heat stress, suggesting that CaWRKY6 is a positive regulator of heat stress tolerance and might mediate phytohormone-induced stress tolerance (Cai et al. 2015).

The key element of ethylene signaling and response pathway is the ethylene response factors (ERFs), which are under the APETALA2 (AP2)/ERF superfamily transcription factors. *ERF* expression is enhanced following exposure of plants to heat stress. ERFs can bind with dehydration-responsive elements (DREs) to activate transcription of stress-responsive genes that enhance heat tolerance in plants. In addition, ERFs act as a key node that integrates other hormone signaling pathways under abiotic stresses. While ABA downregulates *ERF1* expression under abiotic stress, this phenomenon does not block ethylene/JA signaling, and thus ERF1 can upregulate expression of *HSF3*, *HSP101*, and *HSP70* to promote heat tolerance (Muller and Munne-Bosch 2015).

1.4.7 Brassinosteroids

Brassinosteroids (BRs) are a group of steroidal phytohormones that regulate plant growth, development, and responses to a variety of environmental stresses including heat stress (Divi and Krishna 2009). In *Brassica napus*, BR-promoted enhancement in the basal thermotolerance is associated with increased synthesis and accumulation of HSP (Dhaubhadel et al. 2002). Exogenous application of BR alleviates heat stress by substantially increasing net photosynthetic rate, stomatal conductance, maximum carboxylation rate of RuBisCO, and maximum potential rate of electron transport contributed to ribulose-1,5-bisphosphate (RuBP) in tomato leaves

(Ogweno et al. 2007). In addition, antioxidant enzyme activity is remarkably induced, but the level of lipid peroxidation is decreased by exogenous BR application under heat stress. Nonetheless, the mechanisms that control BR-induced stress tolerance still remain largely unknown. As a consequence of excessive production of ROS, oxidative stress occurs as a secondary stress during heat stress (Qu et al. 2013). On the one hand, ROS at high level is harmful for normal metabolism, while on the other hand, low levels of ROS serve as a signal and thus have a regulatory role in plant stress response. ROS have been found to be involved in BR-induced oxidative stress tolerance in cucumber (Xia et al. 2009). BR-induced transient accumulation of NADPH oxidase-derived H_2O_2 mediates the transcriptional induction of antioxidant genes. Eventually, BR-induced increased activity of antioxidant enzymes improves ROS-scavenging capacity and thus minimizing oxidative stress. Silencing of *RESPIRATORY BURST OXIDASE HOMOLOG 1 (RBOH1)* compromises BR-induced tolerance to heat stress (Nie et al. 2013). Additionally, BR-induced heat tolerance is associated with upregulation of transcript levels of *RBOH1*, *MPK1*, and *MPK2*; however, MPK2 plays a more significant role than MPK1 in BR-induced H_2O_2 accumulation and subsequent stress tolerance. Heat stress-induced reduction in photochemical efficiency in BR-deficient mutant tomato (*d*^{im}) can be alleviated by exogenous application of BR or ABA; however, such inhibition in ABA-deficient tomato mutant (*not*) can be alleviated by ABA, but not by BR, indicating that stress ameliorative effect of BR largely depends on the endogenous level of ABA. H_2O_2 is not only required for BR- but also for ABA-induced heat tolerance in tomato. However, in addition to apoplastic H_2O_2, ABA induces chloroplastic H_2O_2 that might function in the induction of stress tolerance (Zhou et al. 2014).

A recent study conducted in *Arabidopsis thaliana* describes BR-induced transcriptome under heat stress conditions (Divi et al. 2016). It shows that HSF21 and HSF4 are putative BRASSINAZOLE-RESISTANT1 (BZR1) targets. In addition, BR-induced induction of *WRKY33*, *ACID PHOSPHATASE5 (ACP5)*, *BR-RESPONSIVE-RECEPTOR-LIKE KINASE (BRRLK)*, and *JACALIN-RELATED LECTIN1-3* (*JAC-LEC)* following heat stress might have a role in thermotolerance. BR transcriptomes carry distinct molecular signatures of ABA and JA. It is concluded that BR-induced stress tolerance mechanisms potentially involve posttranslational modification and protein turnover.

It is well recognized that BR signaling is mediated by the involvement of key transcription factor BZR1. PIF4 and BZR1 interact with each other to regulate hypocotyl elongation in *Arabidopsis* (Oh et al. 2012). A recent study shows that transcriptional regulator JUNGBRUNNEN1 (JUB1) directly represses *PIF4*, while BZR1 and PIF4 can also repress transcription of *JUB1* (Shahnejat-Bushehri et al. 2016). Furthermore, JUB1 induces DELLA accumulation but inhibits synthesis of bioactive GA and BR. Thus, overexpression of *JUB1* results in GA- and BR-deficient/signaling mutant phenotypes due to direct suppression of *GA3ox1* and *DWARF4* expression, respectively. On the other hand, *jub1-1* knockdown mutant shows enhanced hypocotyl elongation in the dark, indicating that JUB1 plays a negative role in mediating PIF4- and BZR1-promoted hypocotyl growth in the dark. It is

speculated that JUB1 forms a robust regulatory module involving GA, BR, and PIF4, which eventually enhances stress tolerance in *Arabidopsis*.

1.5 Conclusions and Perspectives

As a consequence of global climate change, the deleterious effects of heat stress on crop yield are expected to be increased in the future. Thus, a better understanding of the mechanisms involved in plant tolerance to heat stress is of vital significance. The deleterious effects of heat stress can be ameliorated through developing new varieties with improved thermotolerance. Manipulation of phytohormones through breeding or exogenous application of various phytohormones at optimal dose may help to mange heat stress and thereby sustaining crop production in the face of climate change. It is worth mentioning that activation of hormone-responsive genes in a specific tissue is crucial for the induction of stress tolerance and thus targeted manipulation of the plant hormone pool offers better efficacy for modulating the response of plants to heat stress (Mackova et al. 2013).

The general approach to study plant response to high temperature is by exposing plants to either short-term heat stress or extended high-temperature stress or heat acclimation and heat stress recurrence (Ahuja et al. 2010; Mittler et al. 2012). Although the purpose of studying high-temperature stress is to understand plant response, it often does not mimic the real-world situation even considering a single stress. For example, on a hot day temperature may vary a lot during hours of the day. Certainly, it declines at night providing the plants an opportunity to recover. However, the next day the plant may have to face another episode of abnormal high-temperature regimes. Many heat acclimation studies could explain plant response to heat stress; however, to a various extent, those conditions often cannot mimic the real-world temperature regimes. Nonetheless, multiple abiotic and/or biotic hazards may coexist in the natural ecosystem. Therefore, it is of great significance to study plant response to high temperature getting close to the real-world temperature hazard which may provide a better understanding of the mechanisms of plant tolerance to various high-temperature stresses. As phytohormones play a crucial role in imparting thermotolerance, establishing a role for a hormone in protection of crop yield from heat stress may have significant implications in breeding programs as well as field application of plant hormones.

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Chapter 2
Involvement of Plant Hormones in Cold Stress Tolerance

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Abstract The adaptation and survival of plants in challenging environments involve changes at the cellular and molecular levels. In particular, cold acclimation that is due to gradual exposure to low nonfreezing temperatures includes structural and morphological modifications, changes in cell membrane composition, and an accumulation of compatible solutes among other cryoprotective compounds. These processes are governed by plant hormones that are also involved in the adaptation to other abiotic and biotic stresses. Also, other hormones not usually associated with the response to stress have been shown to be participated in the plant cold response. Here, we review the latest information regarding the involvement of plant hormones and its cross talk during cold tolerance.

Keywords Abscisic acid • Chilling stress • Ethylene • Gibberellins • Jasmonic acid • Salicylic acid

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2.1 Introduction

Plants have developed a striking set of different mechanisms to adapt and survive in a challenging environment facing biotic and abiotic stresses. Temperature and water availability are the major environmental factors that significantly influence geographical distribution of plants and sustainability of agriculture worldwide (Chinnusamy et al. 2007). Since only 12 % of the Earth's ice-free surface is used for agriculture, most plants are exposed to low temperatures (Ramankutty et al. 2008). The temperature gradient also determines agricultural yield and crop productivity. In order to survive in such nonoptimal conditions, plants have developed complex mechanisms to perceive external signals and respond accordingly. Among abiotic stresses, cold stress is one of the most studied due to its key relevance in different developmental processes and impact on crop productivity (Rahman 2013).

Cold stress is associated with low chilling (nonfreezing/below 10 °C, depending on the plant species) and differs from freezing which usually occurs when temperatures drop below 0 °C (Gusta et al. 2005; Zhou et al. 2011). Cold stress directly affects plant growth and development, inducing several physiological, metabolic, and genetic mechanisms (Ruelland et al. 2009). First structural changes associated with cold response involve the alteration of membrane conformation, leading to a decrease of membrane fluidity (Sevillano et al. 2009). Furthermore, there is a rapid release of reactive oxygen species (ROS) that could cause oxidative stress if plants are not able to detoxify them (Ruelland et al. 2009; Sevillano et al. 2009). However, plants have various cellular enzymatic and nonenzymatic mechanisms to cope with this condition and, therefore, to minimize the low-temperature-induced damage (Ruelland et al. 2009).

In nature, plants may exhibit cold tolerance due to gradual exposure to low nonfreezing temperatures, a process known as cold acclimation (Thomashow 1999). Cold acclimation includes biochemical mechanisms (Thomashow 1999; Miura and Furumoto 2013; Wisniewski and Gusta 2014) such as changes in cell membrane composition and an increase in compatible solutes and other cryoprotective compounds (Wisniewski and Gusta 2014), as well as structural and morphological modifications (Miura and Furumoto 2013).

Different experimental evidences indicate that all these processes involve a fine tune of different signaling networks governed by phytohormones, responsible for the adaptation and responses against biotic and abiotic stress (Peleg and Blumwald 2011; Shi et al. 2015). These processes are usually mediated by a cross-regulation among the major "stress-related" phytohormones: salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET). In addition, cold responses also involve other phytohormones and signaling molecules such as brassinosteroids (BRs), strigolactones (SLs), nitric oxide (NO), cytokinins (CKs), auxins (AUXs), and gibberellins (GAs) (Peleg and Blumwald 2011; Shi et al. 2015). This chapter reviews the most relevant and latest information regarding cold stress sensing and signaling as well as the involvement of plant hormones and cross talk in the regulation of cold tolerance.

2.2 Cold Stress Sensing and Signaling Pathways

The identity of the cold stress sensor(s) in plants remains unknown. However, there are certain cellular components and processes that are firstly affected by cold and, therefore, may be the main candidates to play a role in stress sensing. Low temperatures trigger the production of second messengers which can be perceived by sensors to activate plant responses to cold stress.

The fluid mosaic physical state of the plasma membrane is vital for the structure and function of cells, as well as to sense temperature variations. These membranes undergo phase transitions, from a liquid crystalline to a rigid gel phase at low temperature (Chinnusamy et al. 2010). Currently, a hypothesis that is widely accepted is that the reduction in membrane fluidity caused by cold stress appears to be a primary event of cold perception to activate the Ca^{2+} channel in both prokaryotes and higher plants (Shi et al. 2014). In higher plants, cellular Ca^{2+} dynamics is detected as soon as 40 s after cold exposure through a novel aequorin-based Ca^{2+} signaling mechanism (Zhu et al. 2013). In addition to the plasma membrane, chloroplast may also play a role in sensing ambient temperature. Under low temperature, an imbalance between the capacity to harvest light energy and the ability to dissipate this energy through metabolic activity causes an excess of photosystem II (PSII) excitation pressure. The overreduction of PSII leads to the generation of ROS. Further, protein folding is influenced by temperature changes. Therefore, plant cells can sense cold stress through membrane rigidification, protein/nucleic acid conformation, and/or metabolite concentration, either a specific metabolite or the redox status (Chinnusamy et al. 2010).

Diverse plant species tolerate cold stress to different thresholds, which depends on the reprogramming gene expression to modify their physiology, metabolism, and growth (Chinnusamy et al. 2010). In higher plants, the major cold signaling pathway is the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE)-binding factor (CBF/DREB)-mediated transcriptional regulatory cascade (Fig. 2.1). This system is essential for the induction of a set of *cold-responsive* (*COR*) genes, which encodes cryoprotective proteins that protect plant cells against cold-induced damage (Thomashow 1999). There are three *CBF* genes (*CBF1/DREB1b*, *CBF2/DREB1c*, and *CBF3/DREB1a*) in the *Arabidopsis thaliana* (*Arabidopsis*) genome. Overexpression of these genes results in enhanced freezing tolerance (Thomashow 2001), whereas knockdown of *CBF1* and/or *CBF3* increases plant sensitivity to freezing stress after cold acclimation (Novillo et al. 2007). However, the *cbf2* mutant shows a freezing tolerance phenotype with or without cold acclimation. Gene expression analysis indicated that CBF2 plays a negative role in the expression of *CBF1* and *CBF3*, suggesting the existence of a negative feedback regulatory network in the cold stress response (Novillo et al. 2004).

In *Arabidopsis*, the CBF/DREB1 pathway is controlled by an MYC-type factor ICE1 (inducer of CBF expression 1) (Fig. 2.1). The *ice1* mutant exhibits both chilling and freezing sensitivity, and the overexpression of *ICE1* confers increased freezing tolerance (Chinnusamy et al. 2003). Approximately, 40 % of *COR* genes and

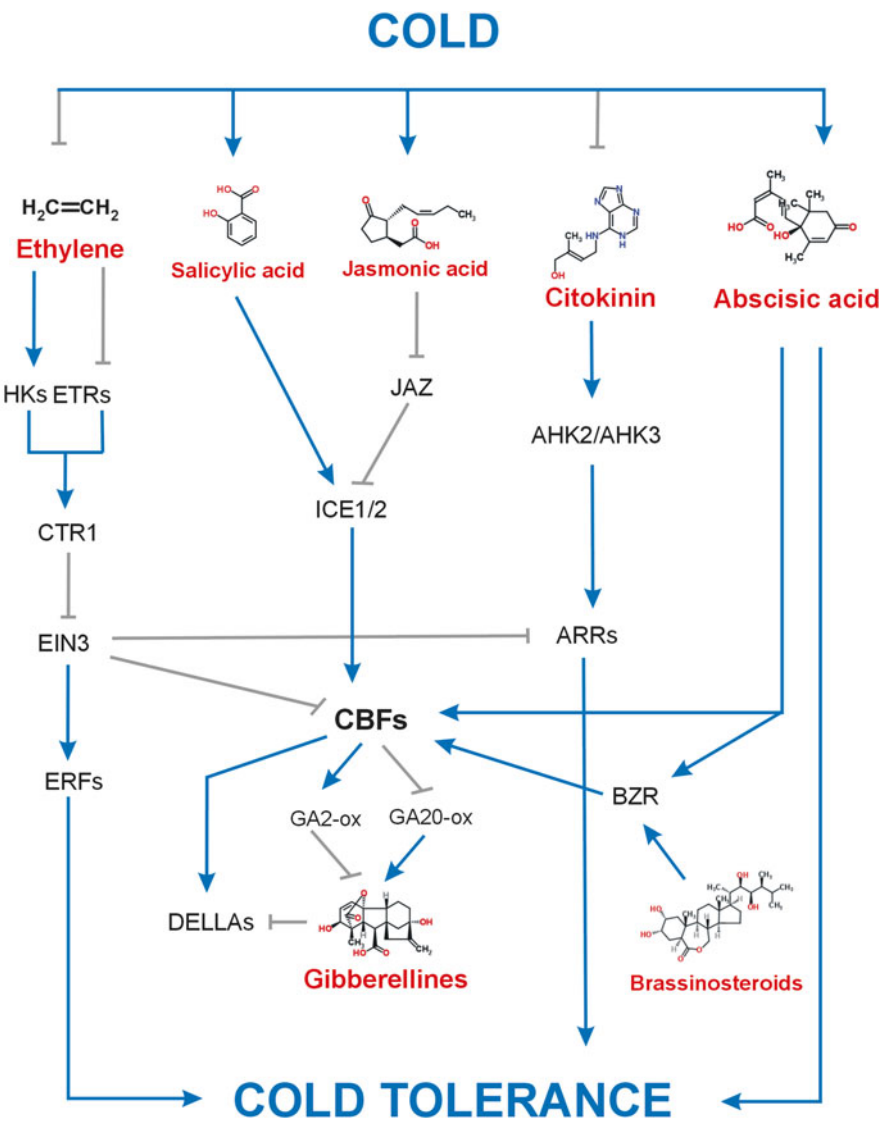

Fig. 2.1 Schematic representation of hormone signaling and perception in response to cold stress. AHK, membrane-located CK receptor; ARRs, *Arabidopsis* response regulators; BZR, BRASSINAZOLE RESISTANT; CBFs, cold-binding factors; CTR1, constitutive triple response1, EIN, ethylene insensitive; ERFs, ethylene response factors; ETRs, ethylene receptors; ICE, inducer of CBF expression; GA20-ox, GA 20-oxidase; GA2-ox, GA 2-oxidase; HKs, histidine kinases; JAZ, JASMONATE ZIM-DOMAIN

46 % of cold-regulated transcription factor genes are regulated by ICE1. This fact suggests that ICE1 functions as a master regulator controlling *CBF3/DREB1A* and many other *COR* genes (Lee et al. 2005). It is noteworthy that *CBF* genes are rapidly and transiently induced by cold, while induction of *COR* genes by cold is much slower. It is possible that under cold stress, CBF proteins accumulate to a certain extent to induce *COR* gene expression. It is unknown whether CBF proteins are alternatively modified or have partners that are slowly activated by cold (Shi et al. 2014). Key components of the ET and JA signaling pathways have been shown to modulate CBFs. It is thought that ABA, GA, and CK signaling regulates cold responses mainly via CBF-independent pathways (Kosová et al. 2012; Shi et al. 2014). However, certain *CBF* genes are induced by exogenous application of ABA, contributing to sustain cold tolerance (Knight et al. 2004). Therefore, CBF regulation constitutes a key point of phytohormones and cold stress interaction that would be further discussed.

In addition to the abovementioned CBF-dependent pathway, some CBF-independent components have a function in cold signaling (Fig. 2.1). In fact, Fowler and Thomashow (2002), through a transcriptome analysis, indicated that only ~12 % of the *COR* genes are controlled by CBFs. The *Arabidopsis esk1* mutant shows constitutive freezing tolerance that is independent of the CBF regulon (Xin and Browse 1998). Loss of *HOS9*, a homeobox transcription factor, causes reduced freezing tolerance without affecting the expression of CBFs and their target genes (Zhu et al. 2004). Also, *GIGANTEA* (*GI*), which encodes a nuclear-localized protein involved in flowering and the circadian clock, is induced by low temperature. The *gi-3* mutant shows both decreased constitutive cold tolerance and impaired cold acclimation ability without affecting *CBF* expression (Cao et al. 2005).

As it is well known, transcription factors (TFs) play central roles in gene expression by regulating transcription of downstream genes. The APETALA 2/ethylene-responsive element-binding factor (AP2/ERF) family is a large group of plant-specific transcription factors that includes the AP2, RAV, ERF, and DREB subfamilies (Sakuma et al. 2002). Many stress-inducible DREB subfamily members have been isolated and characterized, and it has been established that those are the major factors involved in plant abiotic stress responses for their involvement in regulation of gene expression via the DRE/CRT pathway. The ERF subfamily members, which bind to ET-responsive elements, are also involved in abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki 2006; Mizoi et al. 2012).

Histidine kinases (HKs) are important class of hormone receptors in plants. There are ten putative HKs in *Arabidopsis*, which are known as ET receptors and non-ET receptors. The ET receptors, localized to the endoplasmic reticulum (ER) membrane (Chen et al. 2002), act in concert with physically associated Raf-like kinase CTR1 (constitutive triple response 1) to block ET signal transduction pathway (Clark et al. 1998). Similarly, ER membrane-located CK receptors HK2 (AHK2), AHK3, and AHK4/CRE1 (CK receptor 1) belong to the non-ET receptors. In *Arabidopsis*, CK signaling pathway is also a two-component system, which consists of AHKs, *Arabidopsis* histidine phosphotransfer proteins (AHPs), and *Arabidopsis* response regulators (ARRs). Upon activation, AHKs transmit the sig-

nals via AHPs to ARRs through the phosphorelay cascade (Kakimoto 2003), determining the downstream response to CKs.

2.3 Hormone Functions and Cross Talk Toward Cold Tolerance

2.3.1 Stress-Responsive Phytohormones and Cold Tolerance

Plant hormones are involved in the regulation of plant interactions with the environment. Studies using hormone-biosynthetic pathway mutants have been instrumental to gain insight into the plant responses to changing environments (Kosová et al. 2012; Shi et al. 2014). Hormones do not act in isolation but are interrelated by synergistic or antagonistic cross talk so that they modulate each other's biosynthesis or responses (Peleg and Blumwald 2011). Therefore, each process could be affected by multiple hormones (Kosová et al. 2012).

ABA is the key plant hormone involved in the response to abiotic stresses associated with dehydration, which include not only drought but also salinity and cold stress (Gusta et al. 2005). Under low temperatures, ABA levels are rapidly increased to assure an adjustment of water relationships (Vanková et al. 2014). It is important to highlight that although ABA induces cold/freezing tolerance, it appears not to be the unique mediator in this process (Minami et al. 2003, 2005). For instance, Huang and coworkers (2015) proposed the ratio ABA/GA_3 to estimate cold tolerance in sugarcane.

In ABA-mediated cold tolerance, two major cis-acting elements known as ABRE (ABA dependent) and CRT/DRE (ABA independent) have been identified. Both can be activated by binding to different TFs known as ABRE-binding proteins (AREB) or ABRE-binding factors (ABFs). This interaction activates ABA-dependent gene expression (reviewed in Xue-Xuan et al. 2010). Although CBF signaling pathways have been traditionally shown to be ABA independent, *CBF1–3* could be part of this pathway as studies demonstrated that the *CBF1–3* responded to elevated ABA (Knight et al. 2004). Evidence shows that ABA can directly cause an increase in the generation of ROS, inducing an early expression of diverse antioxidant genes, contributing to cold stress tolerance (Xue-Xuan et al. 2010). The general mechanism of ABA-mediated cold tolerance has been mainly studied in herbaceous plants and confirmed in woody plants (Xue-Xuan et al. 2010).

In addition to ABA, the function of other "stress-related hormones" such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in cold response has been explored in different plant species (Miura and Furumoto 2013; Vanková et al. 2014; Shi et al. 2015). Low temperatures promote the accumulation of endogenous free SA and glucosyl SA in *Arabidopsis* shoots, wheat, and grape berry (reviewed in Miura and Tada 2014), suggesting that SA is also involved in the regulation of cold responses (Fig. 2.1). SA-treated plants show a higher tolerance to cold, whereas

application of SA biosynthesis inhibitors leads to a higher chilling damage and repression of cold-responsive genes (Dong et al. 2014). Low temperatures (8 °C) in cucumber seedlings increase SA levels by stimulating the activity of phenylalanine ammonia lyase (PAL) and benzoic acid-2-hydroxylase enzymes, which directly contribute to de novo SA formation (Miura and Tada 2014). SA could induce cold tolerance through the induction of *COR* and the modulation of cellular H_2O_2 levels (Dong et al. 2014). Similarly, exogenous SA or acetylsalicylic acid increases the chilling tolerance and alleviates MDA production by reducing the accumulation of both H_2O_2 and superoxide radials (O_2^-) in maize roots and leaves (Wang et al. 2012). In addition, in the *ice1* mutant, SA-inducible genes are upregulated (Chinnusamy et al. 2003), indicating an essential role of *ICE1* as an integrator of SA-signaling and cold response pathway. It is worth noting that the effect of exogenous application of SA on cold tolerance may be tissue specific and dependent on the organism, concentration, and period of application (Miura and Tada 2014).

Different works have revealed a positive role of JA in inducing cold tolerance in several plant species (Dar et al. 2015; Kazan 2015). The work of Hu and coworkers (2013) and Du and coworkers (2013) showed that the exposure to low temperature rapidly stimulated the accumulation of JA by inducing its biosynthetic genes. Moreover, *Arabidopsis* mutants defective in JA biosynthesis or signaling display an increased sensitivity to cold stress (Hu et al. 2013). Recently, a role for *Arabidopsis* jasmonate zim-domain (JAZ) repressors as key regulators of cold tolerance has been described. Under unstressed conditions, JAZ1 and JAZ4 proteins physically interact and suppress the transcriptional activities of *ICE1* and *ICE2* (Fig. 2.1), preventing the activation of cold stress responses (Hu et al. 2013). Cold stress increases JA levels by triggering *COI1*-mediated degradation of JAZs in WT plants, releasing ICEs from repression. ICE1 and ICE2 then activate CBFs by binding to the DRE/CRT box promoter sequence element found in the ICE-regulon promoters (Hu et al. 2013; extensively reviewed in Kazan 2015; Fig. 2.1).

ET appears to negatively regulate freezing tolerance since in vitro grown *Arabidopsis* seedlings treated with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) show a reduced freezing tolerance with or without cold acclimation, whereas the application of aminoethoxyvinylglycine (AVG), an inhibitor of ACC biosynthesis, increases freezing tolerance in these plants, indicating a negative effect of ET on cold tolerance (Shi et al. 2012). Moreover, ET signaling pathway seems to affect cold tolerance in *Arabidopsis* by repressing the CBF pathway (Shi et al. 2012). The *Arabidopsis* ET-insensitive mutants *etr1-1*, *ein4-1*, *ein2-5*, and *ein3-1* show an enhanced freezing tolerance. Moreover, A-type ARR genes are repressed by EIN3 (Shi et al. 2012), finally repressing CBF-induced responses. Similarly, in the model legume *Medicago truncatula*, a negative correlation was found between ET levels and cold acclimation, since ET-insensitive *skl* mutants were more tolerant to cold than wild-type plants (Zhao et al. 2014).

By contrast, in a recent work in *Arabidopsis* seedlings, it was described that freezing tolerance is enhanced by the application of ACC (Catala et al. 2014) and that an overexpressing *eto-1* mutant (an ET overproducer) displays a higher expression of CBFs (Catalá and Salinas 2015), suggesting therefore that ET acts as a posi-

tive regulator of freezing tolerance and cold acclimation. In this sense, a direct linkage has been established between the ET and the RARE COLD INDUCIBLE1A (*RCI1A*) gene, a negative regulator of cold acclimation in *Arabidopsis*. This gene regulates the expression of cold-induced genes mainly through an ET-dependent pathway, involving physical interaction with ACC synthase (ACS) isoforms. Therefore, *RCI1A* restrains ET biosynthesis contributing to establish the required threshold of this hormone to cope with cold stress (Catala et al. 2014). Similarly, in tomato and tobacco plants, the overexpression of *ERF2*, a positive regulator of ET responses (Zhang et al. 2009), leads to a higher cold tolerance in both species, whereas ACC application in *ERF2* antisense transgenic lines can restore cold tolerance, suggesting a positive role of ET in cold tolerance in both species (Zhang and Huang 2010). In addition, the overexpression of this ETR in rice leads to a higher cold tolerance linked to accumulation of osmoprotectants and chlorophyll and to a reduction in ROS production and oxidative damage (Tian et al. 2011). Similar to other hormones, the role of ET in cold tolerance appears to be species dependent and also influenced by the stage of plant development when the stress is imposed. A possible requirement of different internal threshold levels of the hormone to trigger cold-associated responses in different species and even cultivars cannot be ruled out.

The possible role of BRs in response to cold stress tolerance has been explored by transgenic approaches and exogenous BR application. Seedlings overproducing BRs were more tolerant to cold stress and had higher levels of some *COR* genes. Exogenous application of BR also induces the expression of stress-related genes, leading to the maintenance of photosynthesis activity, activation of antioxidant enzymes, accumulation of osmoprotectants, and induction of other hormone responses. The former suggest a role for BRs in promoting cold tolerance in *Arabidopsis* seedlings (Divi and Krishna 2010). Moreover, recent studies described the existence of several BRASSINAZOLE-RESISTANT (BZR) transcription factors (positive regulators of BR signaling) which are upregulated in response to exogenous ABA treatment and showed differential expression in response to low-temperature stress in *Brassica rapa* plants. Moreover, these TFs were proposed to activate CBF-mediated cold response pathway in these species (Fig. 2.1), displaying a key regulatory function in cold tolerance (Saha et al. 2015). As more transcriptomic, metabolomic, and proteomic information becomes available, these data can be mined to identify novel components involved in the cold signaling, and additional nodes of cross talk between cold stress signaling and phytohormone metabolism will be identified (Shi et al. 2015).

2.3.2 Growth-Promoting Phytohormones and Cold Tolerance

Phytohormones such as GAs, AUXs, and CKs display a key function in the control of plant growth and development from embryogenesis to senescence and are also involved in the response to cold stress. In this sense, the decrease in the growth rate

at the beginning of the cold stress response is associated with the downregulation of GA, AUX, and CK metabolism (Rahman 2013; Vanková et al. 2014). Wheat plants exposed to low temperatures decrease GA_4 content in all organs (Vanková et al. 2014). In *Arabidopsis*, CBFs induced by cold stress upregulate the expression of *GA2ox* gene, encoding the GA-catabolizing enzyme, and repress the *GA20ox* gene, encoding a GA-biosynthesizing enzyme, determining a final reduction in GA levels (Kurepin et al. 2013; Fig. 2.1). Low temperature reduces the level of GA which is associated with a reduction in plant growth, as demonstrated in the GA-insensitive wheat genotype with reduced height (*Rht3*) or dwarf phenotype (Tonkinson et al. 1997). In the same direction, growth retardation was observed in *CBF1/DREB1B*-overexpressing plants, in part, by the accumulation of DELLA proteins, a phenotype that was rescued by exogenous GA application (Achard et al. 2008a). It has been established that *CBF1* enhances the accumulation of DELLA proteins by reducing GA content through the stimulation of *GA2ox* gene expression (Achard et al. 2008a). DELLA protein degradation is stimulated by GAs by enhancing the proteasome activity (Dill et al. 2004). Moreover, DELLA proteins have been associated with abiotic stress survival due to the drop of ROS levels and a delay in cell death through the induction of ROS detoxification enzymes (Achard et al. 2008b). These data indicate that CBF/DREB1-dependent signaling pathway regulates plant growth through modulation of DELLA protein accumulation (Fig. 2.1).

Current knowledge about the role of AUXs under cold stress signaling is limited. Cold and AUXs are potentially linked since it was described that cold stress inhibits the root gravity response in *Arabidopsis* by around 50 %, a process basically governed by AUXs (Shibasaki et al. 2009), suggesting that cold decreases internal indol-3-acetic acid (IAA) levels in plants. However, since AUX signaling mutants (*axr1* and *tir1*) showed a reduced gravity response but a normal cold tolerance, it was suggested that cold stress affects AUX transport rather than AUX signaling (Shibasaki et al. 2009). Indeed, an AUX transporter (PIN3), which has been suggested to play a role in earlier stages of root gravity response, is inhibited by cold stress (Shibasaki et al. 2009). Moreover, direct transport assays confirmed that cold inhibits AUX transportation, suggesting that the effect of cold stress on AUX is more linked to the inhibition of intracellular trafficking of specific efflux carriers (Shibasaki et al. 2009; Rahman 2013).

The reduction in GA and AUX levels during cold stress restricts the plant growth and allows it to face unfavorable conditions (Kurepin et al. 2013). A key cross talk among IAA, JA, and ABA phytohormones has been described. In the leaves of rice seedlings, low temperatures induce both JA and IAA accumulation by inducing IAA biosynthetic gene expression (Du et al. 2013). The potential cross talk between IAA and ABA in cold tolerance was explored in *OsGH3-2*-overexpressing rice lines (an enzyme that catalyzes IAA conjugation into amino acids) where a reduction in free IAA content caused a concomitant increase in ABA levels and in cold tolerance (Du et al. 2012). It is generally considered that intracellular AUX transport and, hence, AUX gradient play a major role controlling hormonal cross talk with other phytohormones and therefore in the regulation of plant growth and development under cold stress conditions (Rahman 2013).

Similar to what occurred with GAs and AUXs, exposure of *Triticum* seedlings to low temperature (4 °C) causes a fast decrease in CK levels as well as in its direct precursors (CK phosphates). The existence of a multistep two-component signaling system was reported as a key element in the cold-CK-mediated changes in *Arabidopsis* plants. AHK2 and AHK3 HKs act as CK receptors in plants and were found to be primarily involved in mediating cold induction of A-type ARRs. The majority of A-type ARRs are partially redundant negative regulators of CK signaling (Jeon et al. 2010). Moreover, the *ahk2-2*, *ahk3-2*, and *ahk3-2 cre1-12* double mutants display enhanced freezing tolerance without affecting the CBF expression, indicating that CK receptors function as negative regulators in the plant responses to low temperatures (Jeon et al. 2010). However, cold stress has no obvious effect in the expression of *AHK2*, *AHK3*, and *AHK4/CRE1*, indicating that cold stress might alter CK receptor activity through a still unknown mechanism (Shi et al. 2014).

2.4 Plant Development and Cold Tolerance Mechanisms

2.4.1 Hormones and Cold Tolerance Mechanisms: Seed and Bud Dormancy

Good examples of plant adaptation to low temperatures include specialized mechanisms such as seed or bud dormancy, which prevent from premature germination or sprout during unfavorable conditions for plant growth and development. Seed dormancy, which refers to the inability to germinate, is regulated by an intricate cross talk among several groups of hormones that induce, maintain, and release seeds from dormancy. Seeds are able to integrate many environmental cues to detect the optimal spatiotemporal conditions to avoid cold detrimental effects (de Casas et al. 2012), and the exposition to low temperatures is responsible for the release of this dormancy phase, leading to higher germination rates (Fu et al. 2014).

Induction of seed dormancy is mainly governed by a tight relationship between ABA and GAs, where ABA is responsible for dormancy induction and maintenance (Kermode 2005), whereas GAs conduct to dormancy release (Nadjafi et al. 2006). Above all, the ratio between the hormones (ABA/GA) seems to be more relevant than the individual effect of each molecule (Kendall et al. 2011; Fu et al. 2014). Since the cold exposure is gradual during winter, different dormancy stages could be differentiated based on sensitivity to ABA and GA. This fact implicates a continuous decrease in ABA content and in seed sensitivity to this hormone. Concomitantly, GA content and seed sensitivity increase in response to cold (Tuttle et al. 2015). Dormancy release is stimulated by cold- and GA-induced enzymatic activity, a higher protein hydrolysis and sugar availability, and key energy sources to further sustain seed germination (Fu et al. 2014).

During the seed maturation process, low temperatures upregulate ABA biosynthetic genes such as nine-*cis*-epoxycarotenoid dioxygenases (*NCEDs*) and strongly

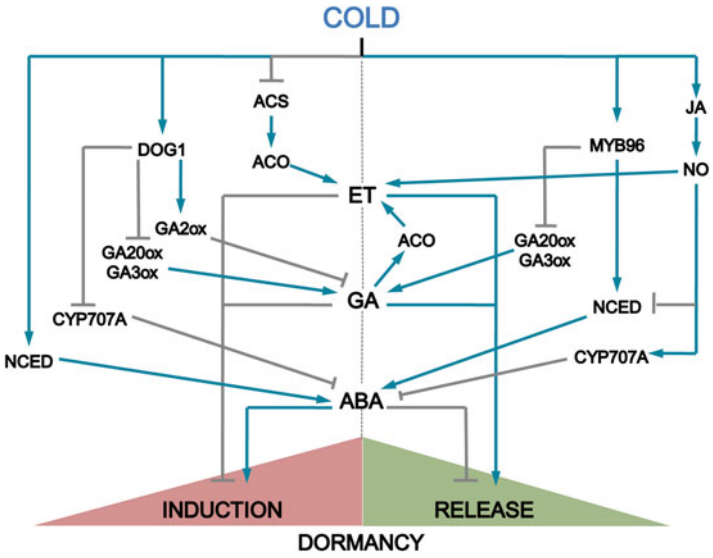

Fig. 2.2 Influence of low temperature and phytohormonal regulation in seed dormancy induction and release processes. Of special relevance is the differential response of hormones depending on the dormancy stage. ABA, abscisic acid; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ACO, ACC oxidase; CYP707A, ABA 8' hydroxylase; DOG1, DELAY OF DORMANCY1; ET, ethylene; GA, gibberellin; GA20-ox, GA 20-oxidase; GA2-ox, GA 2-oxidase; GA3-ox, GA 3-oxidase; JA, jasmonate; NCED, nine-cis-epoxycarotenoid dioxygenase; NO, nitric oxide

downregulate the ABA-catabolic *CYP707A* gene (Fig. 2.2), leading to a final increase in the ABA content, maintaining seed dormancy (Kendall et al. 2011). Overexpression of *NCEDs* inhibits normal seed germination, reinforcing the key role of ABA in this mechanism (Nonogaki et al. 2014). After the dormancy is established, low temperatures are responsible for the progressive decrease in ABA content due to its conversion to inactive forms such as ABA-GE (Chiwocha et al. 2005) or to the stimulation of its catabolism by the induction of *CYP707A2* gene (Sasaki et al. 2015). ABA is able to govern its own catabolism by a tight autoregulatory mechanism. *ABA INSENSITIVE4* (*ABI4*), an ABA-inducible gene associated with dormancy (Shu et al. 2013), and *DELAY OF GERMINATION1* (*DOG1*) gene, which stimulates the expression of *ABI4* leading to an enhanced responsiveness to ABA, are key regulators in this process (Matilla et al. 2014). Apart from inducing *ABI4*, DOG1 also binds to the promoter regions of ABA hydroxylase genes, *CYP707A1* and *CYP707A2* (Fig. 2.2), downregulating ABA catabolism and assuring high levels of this phytohormone during the first stage of seed dormancy (Nonogaki et al. 2014). The accumulation of DOG1 proteins reaches its maximum during seed maturation and remains stable throughout the dormancy period (Nakabayashi et al. 2012). DOG1 proteins also play a role in GA metabolism since they can inhibit the expression of GA biosynthetic genes such as *GA20ox*, favoring seed dormancy during seed maturation while upregulating catabolic genes such as

GA2ox (Kendall et al. 2011; Graeber et al. 2014), determining low GA levels. Interestingly, it appears that the promotion of dormancy induced by DOG1 could be overcome by high levels of GAs, which are only achieved after the exposure to low temperatures. Hence, DOG1 appears to be a key common regulator of ABA and GA balance during seed dormancy (Kendall et al. 2011).

Apart from *DOG1*, the *MYB96* TF is also involved in the response to several abiotic stresses mediated by ABA (Seo et al. 2009a), including low temperatures (Lee and Seo 2015). As DOG1, *MYB96* is implicated in seed dormancy by exerting a role in the regulation of ABA and GA metabolism. *Arabidopsis myb96-1*-deficient mutants show a reduced seed dormancy, explained by the downregulation of *NCED* genes and the upregulation of GA biosynthetic genes *GA3ox1* and *GA20ox1*, indicating that *MYB96* also plays a key role in controlling both hormone metabolisms in seeds (Fig. 2.2). It has been demonstrated that MYB96 directly binds to the promoters of the *NCED2* and *NCED6* genes and activates ABA metabolism, which contributes to the establishment of seed dormancy by adjusting the balance between ABA and GA (Lee et al. 2015).

Other phytohormones such as ET could be interacting in this process since a reduced ACS activity is observed in seeds during low-temperature exposition, reducing ACC levels and leading to a general decrease in ET metabolism (Arc et al. 2013), favoring seed dormancy (Fig. 2.2). It was recently described that during the establishment of seed dormancy, two members of the histone deacetylation complex in *Arabidopsis*, SIN3-LIKE1 (SNL1) and SNL2, decrease histone acetylation levels, leading to an upregulation of several genes from the ABA metabolism and to the downregulation of ET biosynthesis and perception genes (*ACO1*, *ACO4*, *ACO5*, *ERF6*, *ERF9*, *ERF105*, and *ERF112*). The former stimulates seed dormancy showing that SNLs are key mediators in the interaction between both phytohormones (Wang et al. 2013). During the advanced dormancy stage, ET is involved in dormancy release. Using ET mutants and by the exogenous application of ET (Corbineau et al. 2014) as well as hydrogen cyanide, a coproduct of ACC oxidation, it was demonstrated that ET could release seed dormancy (Kępczyński et al. 2014). Et also appears to interplay with GAs in regulating seed dormancy since exogenous GA application upregulates the expression of *AtACO* (Ogawa et al. 2003); meanwhile paclobutrazol (a GA biosynthesis inhibitor) downregulates it (Calvo et al. 2004).

It is thought that nitric oxide (NO) could be interacting with some phytohormones and inducing the release of seed dormancy (Fig. 2.2). Kępczyński and coworkers (2014) suggested that NO could interact directly with ABA and/or ET. Other authors suggest that NO is needed to induce ET production (Gniazdowska et al. 2010) and also favors the decrease in ABA during dormancy release (Liu et al. 2009, 2010a, b) by upregulating the *CYP707A2* expression (Liu et al. 2009). Low temperature is also associated with increased levels of JA-Ile in embryos, suggesting a possible involvement of the jasmonates in dormancy release process (Tuttle et al. 2015). Moreover, it has been postulated that dormancy is reduced by the action of methyl jasmonate (MeJA) in a NO-dependent manner (Fig. 2.2), which led to the downregulation of *NCEDs* and the upregulation of *CYP707A,* causing a decrease in ABA content (Jacobsen et al. 2013). More studies are needed to unravel the specific

role of these compounds and other novel hormones, such as SLs, under cold conditions since it was demonstrated that SLs upregulated the *CYP707A1* gene in the obligate root parasitic *Phelipanche ramosa* (Lechat et al. 2015), suggesting a potential involvement of these metabolites in ABA metabolism.

Similar to the dormancy process as described in seeds, plants developed strategies to avoid cold-damaging effects during the winter. The process of endodormancy involves bud sprout inhibition in a process mainly regulated by low temperatures. It is widely recognized that bud dormancy is initiated once growth cessation occurs under cold and/or short photoperiod conditions (Doğramaci et al. 2013) and that high temperatures during the autumn inhibit endodormancy establishment (Pagter et al. 2015) or could even delay the spring budburst (Luedeling et al. 2011; Fu et al. 2012). The former corroborates that low temperatures are needed to trigger bud dormancy, a key mechanism to assure cold and freezing tolerance.

It is believed that phytohormone cross talk regulates bud dormancy induction, maintenance, and release, although the available information is focused on the individual effect of each phytohormone. In this sense, it is largely accepted that ABA plays a pivotal role essentially during dormancy induction and maintenance (Gómez-Cadenas et al. 2015), whereas a decrease in ABA is needed to release buds from it (Zheng et al. 2015). Moreover, exogenous application of ABA during the final stages of endodormancy is capable of maintaining buds in a dormant state (Bai et al. 2013). During dormancy induction, ABA biosynthetic genes (*NCED3*, *ABA1*, and *ABA2*) and ABA signal transduction components (*PP2C*, *ABI1*, *AREB3*, among others) are induced (Ruttink et al. 2007). Low temperatures during the winter progressively decrease the levels of free ABA until dormancy release is achieved (Zheng et al. 2015). This decline is more pronounced in cultivars with low chilling requirements (Atkinson et al. 2013). The reduction in ABA levels during dormancy has been related to a decrease in *NCED* transcription and to an increase in the ABA catabolism mediated by *CYP707A* (Gai et al. 2013; Zheng et al. 2015).

GAs are considered as ABA antagonists in this process and in some species could be directly inducing dormancy release (Gai et al. 2013), partially replacing the effect of cold. Moreover, cold induces several genes from the GA biosynthesis pathway (van der Schoot and Rinne 2011). It is suggested that during the autumn the remnant leaves could cause a delay in bud dormancy induction due to high levels of GAs (Atkinson et al. 2013), although a lower level of GAs by itself is not sufficient to induce bud dormancy (Mølmann et al. 2005). Once endodormancy is established, low temperatures upregulate *GA20ox* and *GA2* and downregulate *GA2ox* (Karlberg et al. 2010; Gai et al. 2013), stimulating GA accumulation. However, since it is extremely difficult to determine the exact moment of endodormancy release and the onset of ecodormancy (dormancy state that could be released by warm temperatures after chilling requirements are fulfilled), it is still not clear if GA increase at this stage is relevant for endodormancy release process (Bai et al. 2013).

Similar to its function in seed dormancy, ET plays a role in bud dormancy induction possibly in a tight cross talk with ABA. In fact, ET seems to precede ABA during the onset of dormancy. Several ET biosyntheses and response genes are induced just before endodormancy (Horvath et al. 2008) which leads to a transient increase

in ET levels (Ruttink et al. 2007). Moreover, the increase in ET production has been proposed as a previous requirement for the induction of *NCED* genes, finally stimulating ABA accumulation (Doğramaci et al. 2013). Actually, ET-insensitive plants are unable to increase ABA production during this stage (Ruttink et al. 2007). However, the cross talk between ET and ABA is yet poorly understood since exogenous ACC induces several ABA-mediated signaling genes such as *ABI1* and *ABA2*, which could also downregulate the expression of certain ABA biosynthetic genes such as *ABA1* and *NCEDs*, suggesting that ET could exert a role in regulating both ABA biosynthesis and signaling (Doğramaci et al. 2013).

Despite AUXs also seem to be related to dormancy process, their exact role is still unknown (Gai et al. 2013). Lateral buds, which are under the influence of the apical bud in a process known as paradormancy, present higher chilling requirements to be released from dormancy (Atkinson et al. 2013; Pagter et al. 2015). Some reports show that AUX levels, as well as some genes involved in IAA biosynthesis, are reduced during the endodormancy establishment (Doğramaci et al. 2013). It is believed that ET could be mediating this process by which AUXs inhibit shoot growth during the autumn (Doğramaci et al. 2013). Low temperatures perceived during the endodormancy stage reduce AUX transport; meanwhile certain AUX response factors (ARFs) and AUX transporters are also responsive to low temperatures (Gai et al. 2013). Indeed, the expression of *YUCCA* genes, which catalyze the rate limiting step in the AUX biosynthesis pathway, is also induced during mid-endodormancy and decreases afterward (Bai et al. 2013).

BRs appear to be implicated in bud dormancy since the transcription of the *CYP85A* gene, involved in the BR biosynthesis, increases during dormancy (Bai et al. 2013). Other genes related to JA metabolism, such as *3S-lipoxygenase*, are downregulated under dormancy, whereas *jasmonate-O-methyltransferase*, whose product deactivates jasmonate, decreases (Bai et al. 2013). This suggests a possible role of JA in cold stress responses during bud dormancy. CK appears to be also involved since exogenous CKs are able to break bud dormancy. Actually, it has been proposed that CKs must be decreased to induce dormancy (Atkinson et al. 2013). Other signaling molecules such as H_2O_2 could be related to bud break; however, it seems that H_2O_2 is not the primary signal to break the dormancy since exogenous application of H_2O_2 or paraquat is unable to stimulate bud sprout (Gai et al. 2013).

2.4.2 Cold Stress During the Reproductive Phase

The products of sexual reproduction of flowering plants represent the majority of our foods. Fertilization occurs within a narrow window of time, and stress during this phase can be fatal to reproductive success. The reproductive phase involves a series of hierarchical events or many parallel processes that are being controlled by a robust signaling pathway. AUX plays a prominent role, but other hormones are integrated in a synergistic or antagonistic way (McAtee et al. 2013; Dorcey et al. 2009; Sotelo-Silveira et al. 2014).

The reproductive phase begins with the apical floral transition in which the leaf and internode pattern is modified to produce a determinate floral structure. Flowering induction genes like *FT*, *SOC1*, and *AGL24* are highly expressed in the meristem in response to external (vernalization and light) and internal (GA) signals. These proteins promote afterward the expression of *LFY* and *API*, flower meristem identity genes (reviewed in Alvarez-Buylla et al., 2010). Vernalization process regulates the transition to the sensitive reproductive stage until the onset of milder temperatures. In *Arabidopsis* two of the key genes involved in this process are *FRIGIDA (FRI) and FLOWERING LOCUS (FLC;* Michaels and Amasino 1999; Alexandre and Hennig 2008). *CBFs* are induced in response to cold (Thomashow 1999) and this causes the activation of *FLC*, which represses the two flowering pathway integrators *FT* and *SOC1*, thereby delaying flowering. On the other hand, a decreased level of *SOC* causes derepression of cold-inducible genes (Seo et al. 2009b).

Cold temperature can affect reproductive tissues in different manners that ultimately cause poor seed setting and yield (Thakur et al. 2010). Studies in soybean showed that cold stress suppresses flower initiation (Borthwick et al. 1941), decreases the number of splikelets in maize (Bechoux et al. 2000), and promotes the abortion of flowers in chickpea (Nayyar et al. 2005). At the reproductive stage, high levels of ABA have been negatively correlated with cold stress tolerance and overlapped with sugar signaling to control sink-source relationships in the anthers (Thakur et al. 2010; Oliver et al. 2005, 2007). Increase in ABA concentrations was associated with flower abortion and sterility during low-temperature stress (Nayyar et al. 2005; Croser et al. 2003; Gunawardena et al. 2003). Low temperatures can cause pollen sterility by a disruption of sugar metabolism in the tapetum and transport to the pollen grains (Oliver et al. 2005). Exogenous ABA represses cell wall invertase gene expression (*OsINV4*) and reduces anther sink strength in rice, evidence that suggests that this process is tightly regulated by ABA (Oliver et al. 2007).

Little is known about the role that other hormones play in cold response. Evidence of the accumulation, in wheat developing anthers, under low temperature of two cold stress-responsive smRNAs that play an important role in the regulation of AUX signaling, suggested that cold stress-induced male sterility can be regulated by AUX metabolism (Tang et al. 2012). Given the leading role of ABA in responses to cold, the search for cold tolerance by selecting genotypes with reduced ABA expression particularly in the reproductive structures may be included in programs to improve agricultural productivity (Thakur et al. 2010).

2.5 Phytohormones and Cold Tolerance During Fruit Ripening and Postharvest Storage

Ripening in certain fruits is tightly related to a decrease in temperature which triggers different signals that finally cause a rise in ET production with the concomitant changes in fruit color and texture during maturation (Makkumrai et al. 2014). High

levels of ET production in response to cold stress are linked to an increase in ACC levels and ACC oxidase (ACO) activity in cold-stored pears, stimulating fruit ripening based on an autocatalytic ET production (Chiriboga et al. 2012). Similarly, temperatures below 13 °C are necessary for the induction of chlorophyll degradation, carotenoid biosynthesis, and therefore color change in citrus fruit during maturation (Barry and Van Wyk 2006; Rodrigo et al. 2013). In this crop, a cold shock after harvest induced coloration in a similar way that of ET application (Barry and Van Wyk 2006). These evidences suggest that in certain plants an important interaction between cold and ET production occurs, controlling the changes in color during fruit ripening.

Low-temperature storage is a worldwide used technology to extend the postharvest life of fruits and vegetables. However different species are sensitive to cold, developing a diverse range of lesions in both peel and pulp tissues (Lafuente and Zacarías 2006; Sevillano et al. 2009). Fruit response to cold has been the focus of different studies with the aim of alleviating possible detrimental effects on the final product quality (Lafuente et al. 2005; Sevillano et al. 2009; Singh 2011; Villa-Rodriguez et al. 2014; Puig et al. 2015) and revealed the role of different phytohormones and the existence of species-dependent responses. Mainly, ET, ABA, JA, and SA are linked to changes in chilling sensitivity in different fruits and vegetables during postharvest storage.

As for plant cold acclimation, the involvement of ET in fruit cold damage is controversial and depends on the plant species since ET may play either a preventive (Zhou et al. 2001; Dong et al. 2001; Lafuente et al. 2001, 2004; Zhao et al. 2009) or an inductive role (Dou et al. 2005; Edagi et al. 2010; Orihuel-Iranzo et al. 2010). In Marsh grapefruits and Fortune mandarins, chilling-induced ET production is coincident with the initiation of chilling injuries (McCollum and McDonald 1991), whereas exogenous ET induces cold damage in Fortune mandarin and Shamouti oranges (Porat et al., 1999; Lafuente et al. 2001; Gosalbes et al., 2004). The inhibition of ET perception by the application of 1-MCP in Murcott (Edagi et al. 2010), Ortanique, and Nova mandarins (Salvador et al. 2006) favors cold tolerance and stimulates ET biosynthesis in different citrus species (Dou et al. 2005; Lado et al. 2014). By contrast, in the cold-sensitive Fortune mandarin, the continuous ET application during cold storage induces cold tolerance through the increase of CAT and PAL activities (Lafuente et al. 2004). Similarly, ET application or the induction of its biosynthesis in nectarines reduces cold damage (Zhou et al. 2001), being related to a higher expression of *ACO* and *ACS* genes in this fruit (Puig et al. 2015). However, the induction of *ACS* and *ACO* gene expression during low-temperature storage does not represent an accurate indicator of cold tolerance since it has been described in cold-sensitive citrus fruit (Maul et al. 2011; Lado et al. 2014, 2015), papaya (Zou et al. 2014), and zucchini (Megías et al. 2014), whereas it was not observed in grapefruit cold-resistant tissues (Lado et al. 2015). In tomato fruit, 1-MCP reduces cold tolerance, whereas ET exogenous application enhances it, suggesting a positive effect of ET on cold adaptation during postharvest storage (Zhao et al. 2009). Therefore, ET role in cold tolerance during postharvest appears to be strongly dependent on the species and could be influenced by the interaction

and cross talk with other phytohormones. An interaction between AUX and ET has been proposed in peaches under cold storage since a cross-regulatory point between both routes (*VAS1* gene) was specially induced in cold-sensitive varieties (Puig et al. 2015).

Moreover, evidences about the induction of certain isoforms of ET receptors (ETRs) and ET response factors (ERFs) in response to cold in sensitive grapefruit suggest a tight relation between ET perception and cold-induced peel damage (Lado et al. 2014, 2015). *ETR1* and *ETR3* citrus isoforms are stimulated by low temperatures but only in chilling-sensitive grapefruit tissues (Lado et al. 2015). Similarly in tomato fruit, *ETR* expression pattern is erratic and strictly dependent on the isoform (Rugkong et al. 2011). A marked induction in *ETR1* expression is registered in loquat fruit stored at low stressful temperatures, being directly correlated with chilling injury development; however other isoforms showed different patterns of expression in response to cold stress (Wang et al. 2010). Therefore, it appears that ET receptors display a key role in chilling responses in different cold-sensitive fruits, which is also strongly dependent on the isoform considered. These changes in the expression of ET receptors may be relevant to determine tissue sensitivity to the hormone, since ET action is thought to be negatively regulated by ET receptors (Gapper et al. 2013) and high receptor level would repress ET response, while low amount would enhance ET sensitivity (Agarwal et al. 2012).

Alleviation of cold and chilling damage is also associated with jasmonic acid metabolism since exogenous postharvest application of MeJA in tomato (Zhang et al. 2012), loquat (Cao et al. 2009; Jin et al. 2014), papaya (González-Aguilar et al. 2003), banana (Zhao et al. 2013), pomegranate (Sayyari et al. 2011), peach (Jin et al. 2013), sweet pepper (Fung et al. 2004), and mango (Gonzalez-Aguilar et al. 2001) induced cold tolerance. This could be mediated by the induction of heat shock proteins (HSPs) which regulate arginine metabolism and by a higher arginase activity (Zhang et al. 2012). These HSPs also display a function in membrane protection, in the refolding of denatured proteins and in preventing protein aggregation (Al-Whaibi 2011). A lower MDA content and a higher antioxidant enzymatic activity and also a higher level of unsaturated fatty acids were described in MeJA-treated fruit (Cao et al. 2009), which also showed an increased antioxidant activity (Sayyari et al. 2011; Villa-Rodriguez et al. 2014).

An interaction with arginine metabolism (Zhang et al. 2012) and a positive feedback with ABA biosynthesis (Wang and Buta 1994) have also been proposed in different cold-sensitive fruits. The application of MeJA to zucchini squash fruit induces chilling tolerance through the induction of ABA accumulation and the maintenance of levels of polyamines such as spermine and spermidine (Wang and Buta 1994). Therefore, MeJA-induced tolerance to cold stress could be in part mediated by the stimulation of ABA biosynthesis. Molecular and signaling mechanisms behind this response have been explored in banana stored at low temperatures. MeJA induced cold tolerance through the induction of *MYC2*, which may act together with *ICE1* in the activation of CBFs (Zhao et al. 2013). Because the function of *ICE1* in the cold response appears to be conserved among different species (Miura and Furumoto 2013), the overexpression of *Arabidopsis ICE1* also improves

chilling tolerance and enhances the accumulation of soluble sugars and proline in cucumber (Liu et al. 2010a, b).

Similar to MeJA, postharvest application of methyl salicylate (MeSA) in tomato fruit induced HSP accumulation and reduced chilling sensitivity (Ding et al. 2001). In sweet pepper, MeSA application was linked to a higher cold tolerance during fruit storage (Fung et al. 2004). In cucumber fruit, the application of exogenous SA or chitosan-g-SA coating alleviated cold damage through the induction of antioxidant enzymatic activity (CAT, SOD, APX, and GR) as well as enhanced ascorbic acid levels (Zhang et al. 2015). Similarly, the exogenous application of MeSA mitigates cold damage in pomegranate, reducing electrolyte leakage and maintaining fruit firmness, which was associated with an increase in total phenolics and anthocyanin (Sayyari et al. 2011). Low temperatures induce PAL activity that is directly related to a higher cold tolerance (Sanchez-Ballesta et al. 2000; Lafuente et al. 2001). Moreover, when PAL function is impaired through the application of inhibitors, chilling damage is enhanced (Lafuente et al. 2001), suggesting a preventive role of PAL enzymatic activity in Fortune mandarin towards cold damage. Since PAL activity is notably enhanced by cold and is a key regulatory step in SA biosynthetic route (Miura and Tada 2014), this phytohormone appears to contribute to cold tolerance in this fruit.

2.6 Conclusions and Future Perspectives

Different approaches have been used to improve cold and freezing tolerance in different crops including conventional breeding and genetic engineering (Gusta and Wisniewski 2013; Wisniewski and Gusta 2014). However, the complexity of cold hardiness or cold adaptation mechanisms limits the advancement of trait improvement (Wisniewski and Gusta 2014).

How plants can be adapted to challenging environments? Is cold tolerance compatible with plant growth and therefore with biomass development? If and how phytohormones play a decisive role in this balance remains to be elucidated. In order to improve cold tolerance, the key pathways that interact in this process should be taken into consideration by the plant breeders. ABA appears to be a key cold stress response hormone as its level is normally correlated with a higher cold tolerance in different plant species. However, involvement of other phytohormones in cold stress response appears to be highly plant species specific and sometimes tissue specific. In this sense, the ratio and cross talk with other phytohormones could determinate the failure or success of cold survival. It is worth noting that cold tolerance should be pursued in coordination with other stresses (water deficit, high salinity, metal presence, etc.) that could occur simultaneously.

Plants are complex organisms composed of multiple cell types, tissues, and organs as well as a myriad of components within a cell. This complexity results in a multitude of different responses to the environment and involves a great variety of changes within a plant that may limit plant growth. Systems biology, which attempts

to integrate the vast amount of data obtained through recent advances in transcriptomic, proteomic, and metabolomic technologies, has allowed us to obtain a more comprehensive picture of the response of plants to abiotic stress (Cramer et al. 2011). Plant's cold tolerance has been an elusive objective partially addressed in different works and with diverse approaches; however only taking into account the whole integrated responses, where phytohormones play a key regulatory role, it could be understood from a global perspective and provide a reliable tool for plant breeding (Gusta and Wisniewski 2013).

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Chapter 3
Hormonal Interactions Underlying Plant Development under Drought

Maria Elizabeth Abreu*, Paulo Tamaso Mioto*, and Helenice Mercier

Abstract Plants are often exposed to stresses, such as drought, and need to respond accordingly in order to maximize survival. Drought alters the developmental pattern of virtually all plant tissues. For example, the root system generally shows an elongation of the primary root, growing toward water, and a reduction in lateral root formation. In the shoots, the formation and expansion of new leaves are usually reduced under drought. Phytohormones act as integrators of the environmental stimuli and the developmental responses, forming an intricate signaling network. Although plant hormones have been studied for a long time, many questions still remain. In this chapter, the possible interactions between hormones will be addressed, focusing on those that may be important in the alteration of the developmental pattern under drought. Hormones may interact by modifying the amounts, distribution, or sensitivity of others. Examples of each case will be provided throughout the text. Finally, a brief review of hormonal action in plants that shows peculiarities when dealing with drought will be presented.

Keywords Abscisic acid • Drought • Hormonal interaction • Signaling network

3.1 Introduction

When a plant is exposed to drought, numerous responses are triggered. According to Claeys and Inze (2013), plants deal with milder or shorter stresses by reprogramming the metabolism to allow growth under these conditions. An important component of this response is elongation of the primary root associated with a reduction in the formation of lateral roots, which allows access to new regions in the soil that may contain water. When the duration or the intensity of the stress increases, growth stops and responses shift toward ensuring survival until the stress subsides. It is

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important to note that, depending on the species, the definition of mild or severe stress may vary.

Plants have developed an intricate biochemical net in order to perceive and respond to environmental challenges. The signaling processes involved in drought responses are often studied, yet many questions remain unanswered. It has already been established in various plant species that several, if not all, hormonal classes are somehow altered when the plants are exposed to drought. Since it is very likely that these alterations are coordinated, there must be strong interactions between all of these signals. In this chapter we intend to explore some possible connections between different classes of hormones that may be involved in drought stress.

Although phytohormones have been extensively linked to drought stress responses, the cross talk mechanisms between them are only now beginning to be unraveled, and much of the work done on this subject is being made using the species *Arabidopsis thaliana*. In a very rough generalization, it is possible to divide the five classic hormonal classes into the hormones that increase under stress, i.e., abscisic acid and ethylene, and those that are reduced, i.e., auxin, cytokinin, and gibberellins. However, this is not always the case. In fact, the interactions between hormones and other signaling compounds can be quite complex; simple answers are rarely found.

This review will be divided into two parts. In the first part, the main root and foliar growth responses to drought will be discussed, focusing on how hormones influence each event. Then, based on the relations presented in the first part, we will focus on some mechanisms by which the hormones can interact.

3.2 Hormonal Regulation of Growth Responses Under Drought

3.2.1 Root System Growth and Development

Since roots are frequently the first organ to sense drought, they are an important sensor for the whole plant. After perceiving the declining soil water potential, roots trigger a series of signals that spread to the other parts of the plant (Sengupta and Reddy 2011; Cassab et al. 2013). Interestingly, primary root elongation is a common response when the plant is exposed to mild drought, and roots are also one of the last organs to stop growing when the stress becomes more severe (Claeys and Inze 2013). It has been widely described that hormones play important roles in root growth regulation under drought-stressed conditions, but the precise involvement of most of these compounds has not been elucidated (Yamaguchi and Sharp 2010). Below, we summarize the most recent knowledge on the hormonal regulation of root systems under drought.

When the external water supply is limited, the development of root system is essential for the acclimation and survival of plants (Pierik and Testerink 2014). In

fact, according to Xu et al. (2013), the growth of roots in response to drought stress is more adaptive than that of shoots. A previous study demonstrated that primary root length is stimulated by small negative water potentials, although the rate of primary root growth decreases when the drought becomes more severe (Wiegers et al. 2009). Primary root growth under drought is likely influenced by the cross talk between abscisic acid, ethylene, auxin, and cytokinin (Harrison 2012). These hormones appear to play a key role in delineating the transition zone position and regulating root expansion in the cell elongation zone, which contribute to primary root growth (Werner et al. 2010; Moubayidin et al. 2010; Petricka et al. 2012; Pacifici et al. 2015). Each hormone, such as auxin, cytokinin, gibberellin, brassinosteroids, abscisic acid, ethylene, and strigolactones, has specific biosynthetic and signal transduction pathways, and much evidence has been collected showing that correct root growth depends on their cross talk. Apparently, a key point regulating primary root elongation is the auxin flux through the root apex (auxin polar transport), and several hormones can control, at least in part, auxin transport in the roots, as will be seen later in this chapter.

Abscisic acid (ABA) is one of the most studied plant hormones because of its well-described role in plant adaptation to abiotic stresses. In response to drought, endogenous ABA levels increase rapidly (Sengupta and Reddy 2011; Xu et al. 2013), activating specific signaling pathways and the upregulation of several genes under drought stress. Recently, it has been suggested that ABA is essential for the maintenance of primary root, root hair growth, and PM H^+-ATPases activity under moderate water stress conditions by modulating auxin transport in both *Arabidopsis* and rice (Kazan 2013; Xu et al. 2013). Interestingly, it has been shown that auxin is involved in modulating both ABA sensitivity and ABA-responsive genes, which might contribute to auxin-mediated drought stress resistance, suggesting that auxin might also act as a positive regulator in an ABA-dependent pathway (Shi et al. 2014). Other studies have demonstrated that the interaction between abscisic acid and auxin may be regulated via ROS production during ABA signaling (Hong et al. 2013; He et al. 2012). In addition, redox regulation has been implicated at nearly every stage of root development, as well as in the development of lateral roots and root hair expansion (Considine and Foyer 2014).

ABA can regulate primary root growth in *Arabidopsis* since various mutants with altered sensitivity to ABA also showed alterations on primary root elongation. These responses may be related to changes in the pathways of other hormones, including auxin. In fact, recent studies have described that auxin also plays a crucial role in plant responses to drought (Du et al. 2012). Expression and genome-wide analyses of plants under drought stress have indicated differentially expressed auxin-responsive genes (Kazan 2013). It has been recently identified that some genes encoding various members of the ARF transcription factor family are differentially expressed during dehydration in soybean roots, which suggests that these genes may be potential candidates for increased drought tolerance (Van Ha et al. 2013). For example, *DR5::GUS* transgenic plants, which have the auxin-responsive promoter *DR5* fused to a reporter gene, showed a significant decrease in relative *GUS* activity under drought conditions (Shi et al. 2014). On the other hand, mutants

defective in carotenoid (and, consequently, ABA) biosynthesis showed lower levels of YUCCAs (enzymes related to auxin biosynthesis) and higher levels of GH3 (responsible for auxin conjugation) indicating that in some cases ABA may increase the amounts of auxins (Du et al. 2013).

Along with auxin, cytokinins also seem to be implicated in the regulation of root development (Ruzicka et al. 2009a). In fact, the cross talk between these hormones antagonistically influences several developmental processes. Ethylene also may regulate root growth, in part by affecting the level of gibberellins in the endodermis (Shani et al. 2013).

The molecular mechanism underlying root growth and development depends on many auxin-signaling genes, which can be induced by auxin and brassinosteroids (Vanstraelen and Benková 2012). Initially, it has been described that brassinosteroids promote primary root growth at low concentrations, but inhibit it at higher concentrations (Müssig et al. 2003). Interestingly, brassinosteroids promote acropetal auxin transport in the root, suggesting that its effects are dependent on auxin (Fukaki and Tasaka 2009). Recently, it has been suggested that brassinosteroid perception in the epidermis is sufficient to control root growth (Hacham et al. 2011). On the other hand, it has been shown that strigolactones may repress auxin action by reducing its transport and accumulation, repressing lateral root formation (Da Costa et al. 2013). However, further research is necessary to explain the mechanism by which brassinosteroids and strigolactones interact with other hormones in root growth and development in response to drought stress. Figure 3.1a presents a summary of the role of various hormones in directing root architecture, including primary root elongation and lateral root formation.

3.2.2 Hydrotropism Regulation

Previous studies have reported that the development and establishment of the root system in the soil may be influenced by hydrotropism, which is defined as the ability of the roots to bend and grow toward soil moisture (Kazan 2013; Pierik and Testerink 2014). This phenomenon appears to improve plant survival under water-limited conditions (Saucedo et al. 2012; Iwata et al. 2013).

According to Moriwaki et al. (2013), recent genetic and physiological studies of hydrotropism with *Arabidopsis* roots provide some clues to help understand the molecular mechanisms underlying this process. Previous studies have demonstrated that the genes *MIZU-KUSSEL 1* (*MIZ1*) and *GNOM* are somehow responsible for responses related to hydrotropism (Miyazawa et al. 2009; Moriwaki et al. 2011; Miyazawa et al. 2012; Iwata et al. 2013).

When *MIZ1* is overexpressed, the plant shows both a reduction in lateral root formation and an exacerbated curvature of the primary root (Miyazawa et al. 2012; Moriwaki et al. 2012). Some studies have confirmed that *MIZ1* overexpression caused a decrease in auxin levels, suggesting that MIZ1 might act as a negative

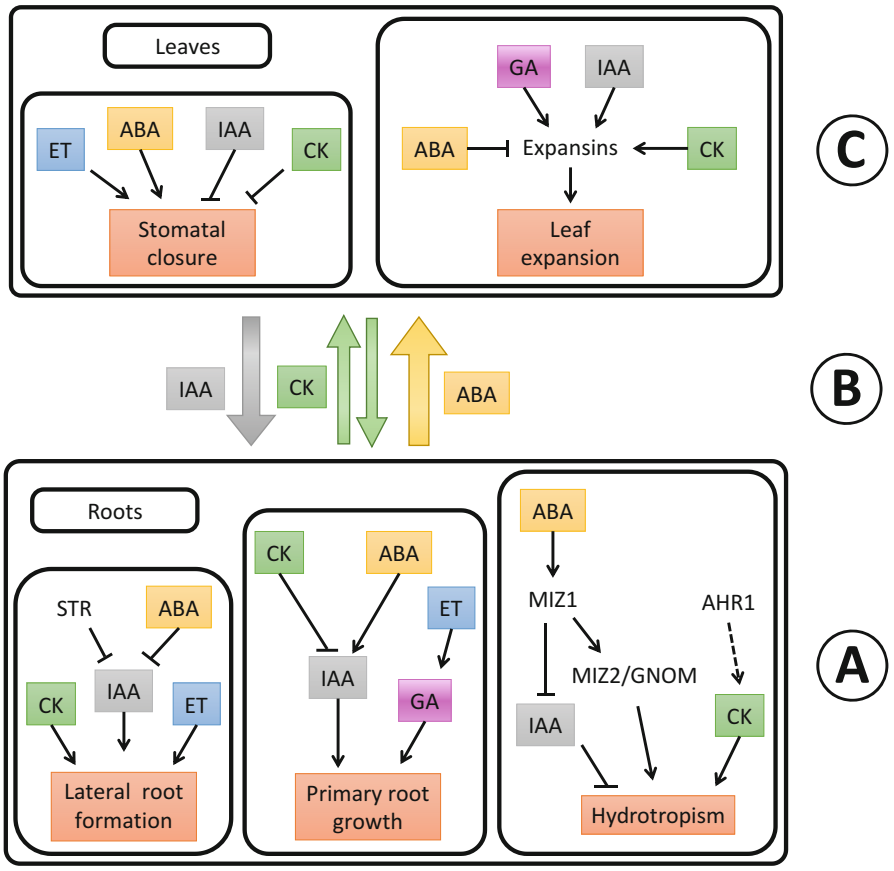

Fig. 3.1 Plant responses to drought as influenced by hormones. (**a**) Under drought, primary root tends to elongate and grow toward a source of water (hydrotropism), while lateral root formation is reduced. (**b**) ABA, cytokinins, and auxins were described to be transported to long distance, integrating both root and shoot responses. (**c**) Reduction of leaf expansion and stomatal closure are common responses in the aboveground plant parts to drought. *IAA* auxin, *ET* ethylene, *CK* cytokinins, *GA* gibberellin, *STR* strigolactones. *Dashed arrows* indicate non-confirmed interactions

regulator of auxin in plants (Moriwaki et al. 2011; Miyazawa et al. 2012; Iwata et al. 2013; Cassab et al. 2013). In addition, the control of auxin levels by MIZ1 may result in the exacerbated root curvature and lateral root inhibition observed in plants overexpressing *MIZ1* (Moriwaki et al. 2011; Miyazawa et al. 2012; Iwata et al. 2013). Interestingly, the *MIZ1OE* phenotype is reversed when crossed with *miz2*, a weak allele of *GNOM*, indicating that it acts downstream of *MIZ1* (Moriwaki et al. 2011). GNOM is capable of regulating lateral root formation by altering PIN location, but the *miz2* mutant shows normal PIN distribution, indicating that its role in the cascade mediated by MIZ1 is not related to its function in auxin redistribution (Moriwaki et al. 2011).

Exogenous application of ABA has been shown to increase *MIZ1* transcription in the ABA-deficient mutant *aba1* (Moriwaki et al. 2012). Cytokinins, in conjunction with abscisic acid, can interact to influence the responses of hydrotropic roots of *Arabidopsis* (Cassab et al. 2013; Hong et al. 2013). This has been evidenced by the fact that application of exogenous cytokinin reverts the incapacity of the roots of the *altered hydrotropic response 1* (*ahr1*) mutant to curve toward the source of water (Saucedo et al. 2012). Curiously, it has been previously demonstrated that there is no correlation between the expression of cytokinin genes and those of hydrotropic inducible genes, suggesting that the action of this hormone in hydrotropism does not depend on gene transcription (Moriwaki et al. 2010). Taken together, these results indicate that abscisic acid and cytokinin signaling participate in hydrotropism regulation through either MIZ1 or AHR1. Figure 3.1a shows the role of various hormones in directing hydrotropism.

3.2.3 Root-to-Shoot Signaling

Roots can directly respond to drought by signaling the shoots via xylem, resulting in physiological changes, which may regulate the level of adaptation to the stress (Anjum et al. 2011). As expected, hormones play a crucial role in the communication between roots and shoots.

Cytokinins are involved in various aspects of plant growth and development, which are recognized, along with ABA, as one of the key signals transported from roots to shoots (Schachtman and Goodger 2008; Ko et al. 2014). The cytokinin translocation via the xylem is controlled both by environmental and endogenous signals (Kudo et al. 2010). According to Perez-Alfocea et al. (2011), cytokinin concentrations in the xylem decrease under drought stress, while abscisic acid concentrations increase. A recent study demonstrated that AtABCG14 (ABCG subfamily of ABC proteins) is essential for translocation of cytokinins into the xylem from roots to shoots and can have a significant impact on the coordination of both root and shoot development (Ko et al. 2014).

Under drought, abscisic acid plays an important role in the long-distance signal, being synthesized in the roots and transported via the xylem stream to the shoots (Dodd 2009; Tardieu et al. 2010; Davies et al. 2011; Perez-Alfocea et al. 2011; Puértolas et al. 2013). Generally, in dry soil conditions, an increase in ABA concentration in the root and a reduction in stomatal conductance have been observed, even when leaf water potentials were kept constant (Parent et al. 2010; Puértolas et al. 2013). On the other hand, other studies have suggested that abscisic acid acting on stomatal closure is synthesized locally in leaves but requires a long-distance hydraulic signal in xylem vessels (Christmann et al. 2007; Christmann et al. 2013). According to Li et al. (2014), abscisic acid not only induces stomatal closure but also regulates aquaporin function in the whole plant. A previous study has shown that ABA promotes an increase in gene expression and protein content of some aquaporins (Parent et al. 2009; Chaumont and Tyerman 2014). Therefore, drought

generally leads to an increase in ABA present in the transport tissues and a reduction in cytokinins.

However, not only is the root-to-shoot transport important for the plant to function correctly; the flux of assimilates and cytokinins from shoots to roots is also important in ensuring normal root function and continued resource uptake by the roots (Perez-Alfocea et al. 2011). In fact, the long-distance transport of cytokinins to the root tip also influences polar auxin transport, maintaining the correct formation of vascular tissue near the meristematic zone (Bishopp et al. 2011). Clearly, more studies are necessary to elucidate the long-range communication between plant tissues under drought. Figure 3.1b summarizes the role of various hormones in the root-to-shoot signaling.

3.2.4 Leaf Growth and Development

Leaves are formed in the flanks of the shoot apical meristems, a process tightly regulated by plant hormones, transcriptional regulators, and mechanical properties of the tissue (Bar and Ori 2015). According to Gonzalez et al. (2012), leaf growth can be influenced by the following parameters: number of cells recruited from the meristem to the primordium, rate of cell division, degree of cell proliferation, cell expansion, and the duration of meristemoid division. Some studies have addressed the effects of water limitation on cell proliferation and cell expansion in leaf primordia (Skirycz and Inzé 2010; Andriankaja et al. 2012; Rymen and Sugimoto 2012; Claeys and Inze 2013). According to Aguirrezabal et al. (2006), water limitation promotes reduction in the leaf area by reducing the expansion rate as well as the duration of the expansion. Moreover, it has been demonstrated that changes in stomatal conductance or carbon assimilation rate occur after a reduction on leaf expansion (Hummel et al. 2010). Although there have been several reports of hormones interacting to regulate guard cell aperture, this was already the theme of various excellent reviews and will not be addressed here (Acharya and Assmann 2009; Daszkowska-Golec and Szarejko 2013; Pantin et al. 2013a; Pantin et al. 2013b; Negi et al. 2014; Osakabe et al. 2014; Tardieu et al. 2015). In any case, ethylene and ABA usually cause stomatal closure, while auxins and cytokinins have the opposite effect (Mioto et al. 2014).

Although several studies have contributed to the broad understanding of leaf development, many questions remain regarding the hormonal cross talk underlying leaf growth in limited water conditions. Since leaf growth is intimately related to expansin activity, mechanisms controlling these proteins might also be related to the reduction in leaf growth under drought.

Expansins are cell wall proteins that act as regulators of cell wall extension during plant growth (Sloan et al. 2009; Zhao et al. 2012; Kuluev et al. 2014). Regarding this, Goh et al. (2012) showed that decreased expansin gene expression leads to a marked repression of growth during the later stage of leaf development. Recently, it has been suggested that some expansins are crucial for responses to many

environmental stresses (Zhao et al. 2011; Zhao et al. 2012; Li et al. 2015). Overexpression of the stress-induced genes *BrERF4* and *WRKY44* in *Arabidopsis* increases tolerance to drought conditions by reducing the expression of expansins and, consequently, leaf cell expansion (Park et al. 2012). However, under moderate drought conditions, expansin genes may be upregulated (Harb et al. 2010). According to Sharova (2007), several hormones are involved in the regulation of the expression of expansin genes, such as auxins, cytokinins, and gibberellins. Additionally, another study showed that ABA also influences expansin activity, but the mechanisms behind it are not yet clear (Zhao et al. 2012). Since the expression of several expansins is regulated by DELLA proteins (Hou et al. 2008), this could be one of the reasons why so many hormonal classes impact the levels of expansins. Figure 3.1c shows a summary of the role of various hormones in leaf expansion and stomatal closure.

In this part of the chapter, we presented a rough view of the hormone fluctuation in a plant subjected to drought. This shifts the developmental pattern of the plant, leading to an increase in primary root elongation and a reduction in lateral root formation, leaf expansion, and stomatal conductivity (Fig. 3.1). However, the interactions between hormones are much more complex than this simplified overview, as the action of each one is dependent on a huge multitude of factors (e.g., tissue, ontogeny, input of simultaneous environmental signals, among many others). In the following section, we will provide some examples of how hormones may interact.

3.3 Mechanisms of Hormonal Cross Talk Under Drought

In the previous sections, we described how the plant architecture changes in response to drought, hinting at some interactions between the hormones. This section will focus on the mechanisms by which the abovementioned interactions may occur. These data were gathered from studies involving drought, whenever possible. Although many mechanisms presented here were not confirmed to occur under drought, we believe that they may be important targets for future research.

Cross talk between hormones can happen at different levels. These interactions can be very complex and development or tissue specific. However, there are some points at which these interactions are more likely to occur. One possibility is that one hormone may modify the levels of another by increasing or decreasing the rate of biosynthesis or removal (including both degradation and conjugation). Alternatively, one hormone may regulate the distribution of another by directly or indirectly influencing transport. Complex interactions may also result in altered sensitivity of a given hormone due to another. This interaction can happen at several levels. For example, hormone "A" can regulate the amount of receptors of hormone "B." If this is the case, the whole signaling cascade related to that receptor would be altered. At the signal transduction level, both hormones can influence the same component in a signaling pathway and, therefore, control the same group of responses. In this case, however, other responses not involved in this component would function

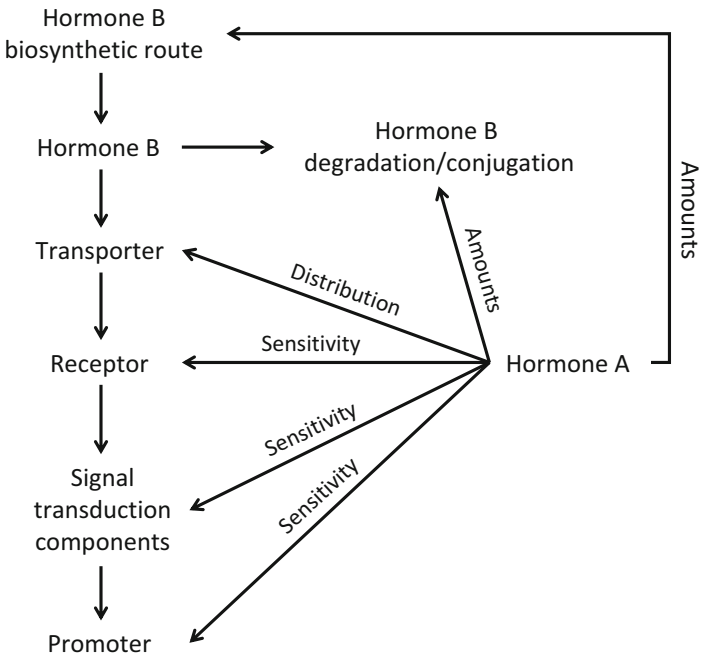

Fig. 3.2 Schematic representation of potential interaction between two hormones. Hormone A may regulate the amounts of hormone B by altering its biosynthesis, conjugation, or degradation. Alternatively, hormone A may interact with hormone B transporters and modify its distribution. Hormone A may also modify the sensitivity to hormone B by interacting with receptors or components of the signaling cascade or by sharing a promoter region of a given gene with hormone B

independently. Regarding the gene expression level, specific promoters may have motifs responsive to transcription factors controlled by more than one hormone. If so, this gene will respond to all these hormones. A schematic view of these relations is presented in Fig. 3.2.

Below, we summarize some mechanisms of interaction that may somehow be related to drought stress responses. We roughly divided them into the levels of interaction presented here.

3.3.1 Interactions Controlling the Biosynthesis or Removal of Hormones

It is very common that one of the genes controlled by a hormone is a key enzyme in the biosynthesis of another. This is also frequent for hormones that act in opposite ways. ABA and cytokinins, for example, are usually antagonists in response to various stress conditions. It has been widely described that ABA levels increase, while cytokinins decrease in response to drought, cold, and/or salinity (Freschi et al. 2010;

Nishiyama et al. 2011; O'Brien and Benková 2013). In fact, ABA application reduced the expression of genes involved with cytokinin biosynthesis (Nishiyama et al. 2011). Strangely, mutants overproducing cytokinin oxidases/dehydrogenases (CKX), which caused a decrease in bioactive cytokinins, also showed lower activity of ABA biosynthetic enzymes and a consequent reduction in ABA production (Nishiyama et al. 2011). Intriguingly, these authors also showed that sensitivity to ABA somehow increased with lower cytokinin levels. The free radical nitric oxide (NO) is commonly related to ABA responses and also has the potential to bind to zeatin (a cytokinin) and inactivate it by conjugation (Liu et al. 2013).

ABA also controls the biosynthesis rate of other hormonal classes. Lee et al. (2012) found an increase in the transcription of *YUCCA7* (a gene related to auxin biosynthesis) in response to drought. Moreover, this increase was ABA dependent, indicating that ABA, in some cases, may upregulate auxin production. In other cases, however, ABA leads to auxin removal. *A. thaliana* MYB96 transcription factor appears to be an interesting integrator of ABA and auxin responses, mainly in the root system. Apparently, ABA-induced MYB96 is capable of arresting the development of lateral roots after the meristem is formed (Seo et al. 2009). Conversely, MYB96 increases the transcription of *GH3* genes that code for enzymes involved with auxin conjugation (Seo et al. 2009; Du et al. 2011). In the aerial parts of the plant, MYB96 promotes drought tolerance via stomatal closure, among other responses, but there seems to be less interaction with auxin (Seo et al. 2009).

ABA is capable of reducing the expression of GA20ox1, an enzyme related to gibberellin biosynthesis, through the transcription factor ATHB12 (Son et al. 2010). As a result, mutants that lack ATHB12 have longer inflorescence stems than the wild type. Achard et al. (2007) found that ethylene is capable of negatively regulating *GA20ox1* and *GA3ox1*, both genes involved in gibberellin biosynthesis. Additionally, two drought-induced ethylene-responsive transcription factors (ERF5 and ERF6) are capable of increasing transcription of *GIBBERELLIN 2-OXIDASE6* (*GA2ox6*) and, consequently, reducing gibberellin levels under drought (Dubois et al. 2013).

3.3.2 Interactions Influencing Hormonal Transport

As mentioned previously, interaction between hormones can occur at the transport level. The action of auxins, for example, is highly dependent on transport, which is governed mainly by PIN-FORMED (PIN) proteins (Wisniewska et al. 2006). These proteins mediate auxin efflux from the cells, leading to organized fluxes that can determine the specificity of several processes, including changes in root architecture.

PIN protein synthesis is often regulated by several hormones. In most cases, cytokinins and ABA decrease PIN levels, while ethylene increases them (Růzicka et al. 2007; Swarup et al. 2007; Ruzicka et al. 2009b; Liu et al. 2010; Shkolnik-Inbar and Bar-Zvi 2010). Apparently, ABA and cytokinins negatively regulate PIN1 by increasing the levels of ABSCISIC ACID INSENSITIVE 4 (ABI4), a transcription

factor usually related to ABA signaling (Shkolnik-Inbar and Bar-Zvi 2010). Gibberellins, on the other hand, are capable of increasing the amount of PIN in the plasma membrane (Willige et al. 2011; Löfke et al. 2013; Habets and Offringa 2014). PIN responses to ethylene show some degree of tissue specificity. Ruzicka et al. (2009) showed that PIN1, PIN2, and PIN4 were upregulated by ethylene application in the roots. Apparently, exogenous ethylene application to *A. thaliana* roots is capable of increasing auxin content in the root tip while decreasing it in the elongation zone, reducing lateral root formation (Muday et al. 2012). This auxin redistribution appears to rely on changes in proteins related to both influx (AUX1) and efflux (PIN3 and PIN7) of this hormone in response to ethylene (Lewis et al. 2011). For instance, application of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) reduces AUX1 in the elongation zone while increasing both PIN3 and PIN7. In the root tip, all three transporters are increased. In this way, the flux of auxin is directed to the root tip, not allowing the accumulation of this hormone in the elongation zone and consequently reducing the number of lateral roots.

The interactions between hormones and PIN, however, are not restricted solely to reducing or increasing PIN transcription. Cytokinins are able to increase endocytosis and, consequently, reduce the levels of PIN-FORMED1 (PIN1) in the plasma membrane (Marhavý et al. 2011). This mechanism does not require gene transcription and is independent of ethylene. Recently, Marhavý et al. (2014) showed that cytokinins are also capable of altering the cellular distribution of PIN1 by degrading these proteins preferentially in the basal portion of cell membrane. The differentiation between basal and apical PIN1 appears to lie in the level of phosphorylation: when in the apical part of the membrane, PIN1 is frequently phosphorylated and, consequently, less sensitive to cytokinin-driven degradation than in the basal portion (Friml et al. 2004; Michniewicz et al. 2007; Huang et al. 2010; Marhavý et al. 2014).

Xu et al. (2013) showed that ABA enhances auxin polar transport and, consequently, H^+-ATPase activity in the root tip in order to maintain elongation in moderate stress simulated by polyethylene glycol (PEG). This mechanism may also be related to PIN phosphorylation. Michniewicz et al. (2007) showed that one of the factors controlling PIN phosphorylation is the balance between protein phosphatase 2A (PP2A) and PINOID proteins: while PINOID phosphorylates PIN, PP2A dephosphorylates it. Consequently, PP2A directs PIN proteins to the basal portion of the cell and protects these transporters from cytokinin degradation. Interestingly, PP2A appears to be involved with drought response in several species (País et al. 2009). Additionally, PP2A is also related to ABA signaling cascade and ethylene biosynthesis and affects responses in which auxin redistribution is vital, such as root elongation and lateral root formation (Kwak et al. 2002; Larsen and Cancel 2003; Pernas et al. 2007; Lyzenga and Stone 2012). Pernas et al. (2007) implicated *PP2A* as a negative regulator of ABA sensitivity, showing that its transcription increased after about 20 min of ABA application. These authors suggest that this may represent a negative feedback mechanism to reset ABA signal. It was also shown that PP2A dephosphorylates ethylene-producing ACS2 and ACS6 proteins, promoting the degradation of these proteins by 26S proteasome (Skottke et al. 2011). Moreover, a subunit from PP2A appears to interact directly with CTR1, a component in ethyl-

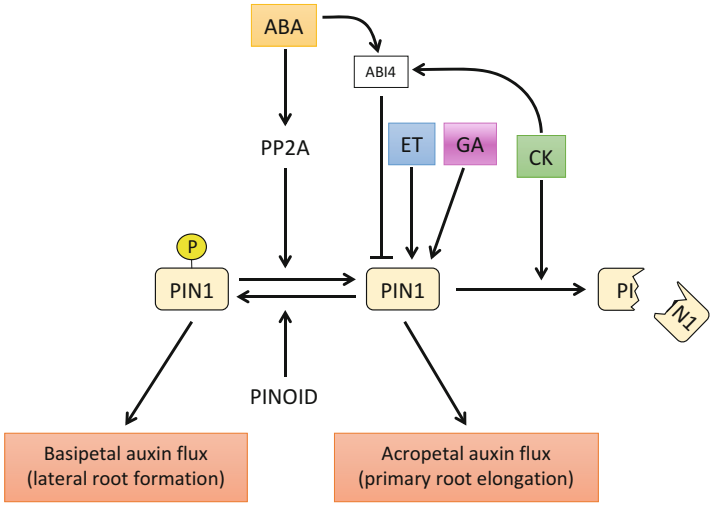

Fig. 3.3 Hormones regulating PIN-FORMED1 (PIN1) amounts and distribution. When phosphorylated, PIN1 tends to localize in the apical membrane of the cell and promote basipetal auxin transport in roots. On the other hand, dephosphorylated PIN1 localizes for the most part in the base of the cell and promotes the acropetal transport of auxin. PINOID phosphorylates PIN1, while PP2A, a component of ABA signaling, dephosphorylates PIN1. When dephosphorylated, PIN1 is more sensitive to cytokinin (CK)-driven degradation. PIN1 production is positively regulated by ethylene (ET) and gibberellins (GA) and negatively by ABA and CK, possibly through ABI4 transcription factor

ene signaling pathway, but the effect of such interaction remains to be characterized (Larsen and Cancel 2003). In conclusion, PP2A may represent an interesting converging point between ABA and ethylene, which, in turn, may regulate PIN phosphorylation.

As shown in Fig. 3.3, besides controlling PIN1 production by several hormones, the increase in amounts of ABA may dephosphorylate PIN1 through PP2A and render it less sensitive to degradation by cytokinins. Therefore, drought may increase acropetal auxin transport, which subsequently increases primary root elongation and reduces the formation of lateral roots.

3.3.3 Interactions Influencing Hormone Sensitivity

After a hormone is perceived by the receptor, a signal transduction cascade begins. Although some components are commonly associated with only one hormonal class (or even named after them), it is possible to see that this perspective may not be the most precise. For example, ethylene and cytokinins may regulate auxin response factors (ARFs). ARFs form dimers with AUX/IAA, and this interaction disrupts the binding of ARFs to the DNA molecule. Upon auxin signaling, AUX/IAAs are targeted for degradation, allowing ARFs to act.

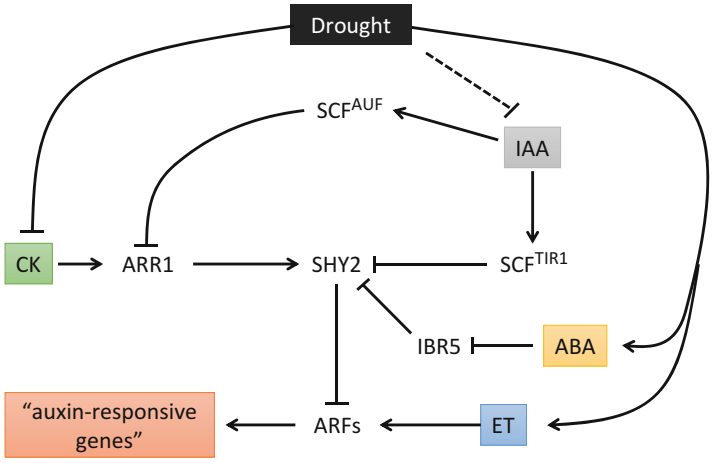

Fig. 3.4 Influence of other hormones in the "auxin signaling pathway." The interaction between auxin (IAA) and SCF^{TIR1} complex leads to degradation of AUX/IAAs, such as SHY2. The degradation of SHY2 allows the dimerization of auxin response factors (ARFs) and expression of "auxin-responsive genes." Ethylene (ET) is capable of increasing transcription of several ARFs, enhancing the sensitivity to auxin. ABA may upregulate SHY2 amounts through reductions in IBR5 levels and, therefore, reduce "auxin responses." Through ARR1, cytokinins (CK) also upregulate SHY2 and decrease sensitivity to auxin. Auxin, on the other hand, can target ARR1 for degradation through the SCF^{AUF} protein complex. Drought increases ABA and ET action, while reducing CK and, presumably, IAA. The dashed line indicates a probable interaction

Li et al. (2006) showed that ethylene upregulates ARF7 and ARF19, while *<arf7,arf19>* double mutants have reduced sensitivity to ethylene in the roots. On the other hand, ethylene may reduce ARF2 levels in the apical hook through expression of *HOOKLESS1* (*HLS1*) (Li et al. 2004; Li et al. 2006). Moreover, ARF2 is also involved in ABA signaling as the knockout of this transcriptional suppressor leads to high sensitivity to ABA in roots (Wang et al. 2011).

Through the action of ARR1 (ARABIDOPSIS RESPONSE REGULATOR 1), cytokinins are capable of increasing transcription of *SHY2* (*SHORT HYPOCOTYL 2*), an AUX/IAA that represses auxin responses (Dello Ioio et al. 2008). On the other hand, AUXIN UP-REGULATED F-BOX PROTEIN1 (AUF1) and AUXIN UP-REGULATED F-BOX PROTEIN2 (AUF2) can assemble with SKP1-Cullin1-F Box (SCF) ubiquitin ligases to target ARR1 for degradation by the 26S proteasome (Zheng et al. 2011). A possible interaction between auxin and ABA may occur through IBR5 (Monroe-Augustus et al. 2003; Strader et al. 2008; Lee et al. 2009; Jayaweera et al. 2014). IBR5 is a MAPK phosphatase that appears to be involved in auxin signaling, acting downstream of SCF^{TIR1} and interacting with MPK12 (a MAPK) and resulting in increased expression of some AUX/IAAs (Lee et al. 2009; Jayaweera et al. 2014). However, salinity, osmotic, and drought stresses, as well as ABA application, are capable of reducing IBR5 contents (Jayaweera et al. 2014). A general overview of how drought may regulate these components is presented in Fig. 3.4.

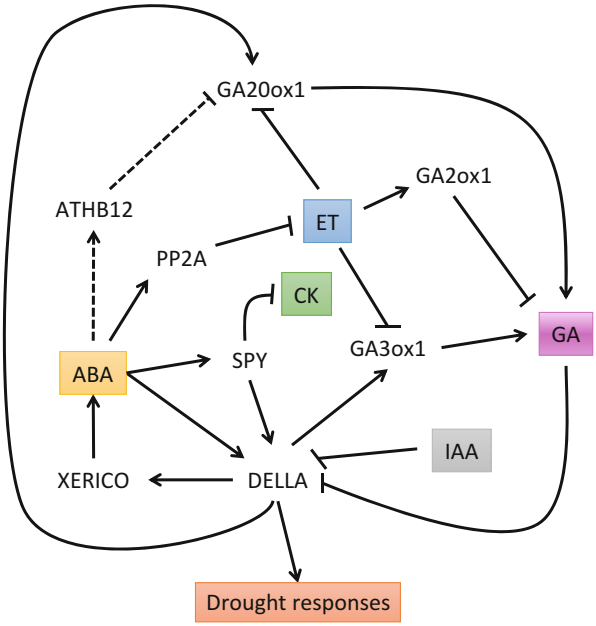

Fig. 3.5 Hormone interactions governing DELLA protein stability and, consequently, several responses to drought. DELLA stability is reduced by gibberellins (GA) and auxins (IAA) and increased by ABA directly. Ethylene (ET) and ABA can reduce GA levels by reducing the levels of GA20ox1, indirectly stabilizing DELLA. ET can further decrease the levels of GA3ox (an enzyme related to GA biosynthesis) and increase GA2ox (related to GA degradation). ABA is also capable of negatively regulating ethylene biosynthesis through PP2A. Spindly (SPY) is upregulated by ABA and is capable of reducing cytokinin (CK) levels and stabilizing DELLAs. Some genes regulated by DELLAs code for both GA and ABA biosynthetic enzymes. Dashed lines indicate relations not yet established for plants under drought

Interestingly, *ahk2*, *ahk3*, and <*ahk2,3*> mutants, which lack the histidine kinase receptors for cytokinins, are more sensitive to ABA, as well as more resistant to drought and salinity, showing the antagonistic effects between these two hormones (Tran et al. 2007; Tran et al. 2010). In fact, the balance between ABA and cytokinins in the xylem sap seems to be very important for root-to-shoot signaling under drought stress (Alvarez et al. 2008; Schachtman and Goodger 2008).

DELLA proteins are intimately related to growth inhibition in virtually all the plant tissues, a typical response to various stresses, such as drought. They are usually constitutively expressed and need to suffer 26S proteolysis in order to lift their inhibition (Sun 2010). The degradation of DELLAs through ubiquitination by SCF^{SLY} complex is classically considered a part of the gibberellin signal transduction pathway, but it is possible to see them as integrators of several hormonal responses (Fig. 3.5).

Auxin, for example, appears to decrease DELLA stability in the roots of *A. thaliana*, increasing gibberellin-like gene responses. Fu and Harberd (2003) showed that mutants lacking DELLA proteins are less sensitive to root shortening induced by 1-N-naphthylphthalamic acid (NPA). Since NPA is an inhibitor of auxin polar transport, this indicates that auxin-mediated root elongation depends partially on the degradation of DELLA. Achard et al. (2006) found that a quadruple DELLA mutant appeared to be insensitive to some responses of ABA and ethylene, as well as high salinity, indicating that these hormones may act through DELLA stabilization to trigger stress responses.

De Grauwe et al. (2008) analyzed several responses of a <*gai, eto2-1*> double mutant and found curious results. The *gai* single mutant lacks a portion of GAI (a DELLA protein) that mediates its degradation in response to gibberellins, while *eto2-1* has a more stable ACC synthase, resulting in increased ethylene levels (De Grauwe et al. 2008). Interestingly, the <*gai, eto2-1*> double mutant showed increased sensitivity to gibberellins when compared to the *gai* single mutant. Apparently, ethylene overproduction increased DELLA sensitivity to gibberellins even when the portion of the protein that should be responsible for the interaction with the gibberellin pathway was defective (De Grauwe et al. 2008). Based on these results, it was proposed that, through posttranslational modifications, ethylene may somehow stabilize the binding between DELLA proteins and the SCF^{SLY} complex and, therefore, enhance gibberellin responses.

Besides DELLA proteolytic degradation, these proteins also appear to have other methods of regulation. Silverstone et al. (2007) suggested that SPINDLY (SPY), an O-linked N-acetylglucosamine (GlcNAc) transferase, may interact directly with DELLA proteins and increase their activity independently of the protein levels, possibly by the binding of GlcNAc. Further research has shown that SPY is negatively related to gibberellin and positively related to cytokinin signaling in several developmental processes (Greenboim-Wainberg et al. 2005). SPY also appears to be induced by drought in an ABA-independent way, but, curiously, the resistance to drought of *spy* mutants was increased (Qin et al. 2011). These authors attribute part of this enhanced resistance to a higher activity of CKX, a cytokinin-degrading enzyme in the *spy* mutants. Certainly, more research is necessary to elucidate the precise roles of SPY in drought resistance and the interaction with DELLAs. In any case, stress can upregulate SPY levels and through it decrease gibberellin-associated responses.

Besides DELLA stability and activity, their downstream targets also provide ample space for hormonal cross talk. Zentella et al. (2007) identified several putative targets of DELLA proteins. Among them is *XERICO*, a gene that codes an enzyme important for ABA accumulation. *GA3ox1* and *GA20ox2*, enzymes responsible for gibberellin biosynthesis, are also upregulated by DELLA. Therefore, DELLA proteins are a very interesting group of transcription factors that may be a central point in the interaction between gibberellins, auxins, cytokinins, ABA, and ethylene, as shown in Fig. 3.5.

3.4 Conclusion and Perspectives

The vast majority of research on hormonal interaction was performed using the model plant *A. thaliana*. Although the importance of such works is indisputable, there are some plants that have responses to drought not observed in *A. thaliana*. The studies focusing on these plants are still in their infancy, but some interesting parallels are beginning to arise.

A trait that involves a drastic reduction in water loss is crassulacean acid metabolism (CAM). CAM plants are capable of closing their stomata during the day and still perform photosynthesis, thanks to a prefixation of CO_2 during the previous night (for review, see Lüttge 2004; Matiz et al. 2013; Winter and Holtum 2014; Mioto et al. 2015). Some species are capable of regulating the intensity of CAM in response to drought, with hormones playing an important part in the process. ABA and the free radical nitric oxide (NO) are the positive effectors of CAM, while cytokinins appear to be negative regulators of CAM (Taybi 1999; Taybi and Cushman 2002; Freschi et al. 2010; Mioto and Mercier 2013).

Extreme cases in processes of tolerance are the so-called resurrection plants, which are capable of remaining viable for two years in equilibrium with an atmosphere of 2 % humidity (Gaff and Ellis 1974; Griffiths et al. 2014). Seeds and pollen grains also present mechanisms that enhance tolerance to low levels of tissue water. Apparently, resurrection plants are capable of maintaining these mechanisms throughout the entire life cycle, coupled with a suppression of dehydration-triggered senescence (Griffiths et al. 2014). Unfortunately, there is little information on how hormones influence and interact during the process of desiccation in these plants. Djilianov et al. (2013) performed a quantification of ABA, jasmonic acid, salicylic acid, auxin, and cytokinins along with their derivatives. Interestingly, there was an increase in ABA, JA, SA, and cytokinins in both leaves and roots of these plants during 24 h of air-drying, followed by a decrease after rehydration. Auxin showed relatively constant levels. Recently, it was proposed that an increase in cytokinin levels when these plants are dried allows both the inhibition of senescence and the accumulation of sugars, which confers drought resistance (Griffiths et al. 2014). Unfortunately, the mechanisms regarding how these hormones interact still remain unknown for the resurrection plants.

In conclusion, work on both model and non-model plants is vital to our understanding of how plants cope with drought. The integration between these two fronts of research is also important to develop new technologies in agriculture.

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Chapter 4
Participation of Phytohormones in Adaptation to Salt Stress

Agnieszka Waśkiewicz, Olimpia Gładysz, and Piotr Goliński

Abstract Salt stress is one of the major abiotic stresses limiting productivity and quality of agricultural crops. The adverse effects concern germination, plant vigor, and crop yield in arid and semiarid regions. Most crops are salinity sensitive or even hypersensitive and they are described as glycophytes. In contrast, high salinity is tolerated by halophytes, which are present in very small numbers, accounting for approx. only 1 % of the world's flora. Glycophytes develop some adaptation mechanisms to monitor salt stress and regulate plant physiology and metabolism in order to cope with this stress. Phytohormones are recognized as vital agents in the adaptation process during salt stress. Plant hormones including abscisic acid, salicylic acid, jasmonic acid, ethylene, brassinosteroids, and the others can regulate that cross talk and responses to salt stress. Molecules, such as transcription factors and MAP kinases, are main agents involved in stress signaling pathways and phytohormone cross talk. This chapter provides an explanation of the salt stress mechanism, while salt stress tolerance and the roles of different plant hormones are presented. The effects of endogenous and exogenous phytohormones on adaptation to salt stress are characterized.

Keywords Glycophytes • K^+/Na^+ • Plant hormones • Cross talk • Salinity

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4.1 Introduction

Plant hormones are small molecules that regulate plant growth and development, as well as responses to changing environmental conditions. By modifying the production, distribution, or signal transduction of these hormones, plants are able to regulate and coordinate both growth and stress tolerance to promote survival and establishment under environmental stresses such as drought, salinity, UV, ozone, and high or low temperature. Hormones are produced in one part of plant and translocated to other parts, where at very low concentrations, they stimulate physiological response (Kaya et al. 2009). It is suggested that phytohormones act as signals to communicate the stress between roots and shoots. This chapter is focused on the involvement of abscisic acid, salicylic acid, jasmonic acid, ethylene, brassinosteroids, nitric oxide, cytokinins, auxins, gibberellins, and strigolactones in plant tolerance to salinity.

4.2 Salinity

Under optimal conditions plants grow and reproduce, but often face a changing environment that may cause unfavorable conditions. In such an environment, plants are considered to be "stressed"; it prevents them from expressing their full ability to reproduce. The consequence of stress in plants can vary from impeded growth to death. Among a wide range of environmental stresses (such as drought, salinity, high and low temperature, UV stress), salt stress is one of the major abiotic stresses limiting the productivity and quality of agricultural crops, with adverse effects on germination, plant vigor, and crop yield, especially in arid and semiarid regions (Koca et al. 2007; Munns and Tester 2008; Parvaiz and Satyawati 2008). These factors are the most problematic in arid and semiarid regions and the area affected by salinity is estimated at as much as 800 million ha (Pessarakli and Szabolcs 2010). Salinity is found both in irrigated and nonirrigated croplands. According to Yadav et al. (2011), salinity is defined as an excess of salts above the level required by plants. This phenomenon occurs due to dissolved salts and their tendency toward excessive accumulation in soil water. Sources of salinity include natural processes, e.g., rock weathering. Also winds and rains carry sea salts such as NaCl, $CaCl_2$, $MgCl_2$, sulfates, and carbonates. These natural processes are long lasting and may span even millions of years. In contrast, human agricultural activity causes salinity much faster. Improper irrigation systems (salt-rich irrigation, insufficient drainage), clear-cutting, and cultivation of annul crops instead of perennial crops, leaching, land clearing, and deforestation are mainly responsible for salinity (Zhang et al. 2006; Yadav et al. 2011).

Soil salinity is determined by the measurement of electrical conductivity (EC) in decisiemens per meter (dS/m). Electrical conductivity below 2 dS/m at a soil depth

up to 60 cm is a standard for nonsaline soil. Other criteria are 2–4 dS/m for weak salinity, 4–6 dS/m for moderate salinity, 6–8 dS/m for strong salinity, and below 8 dS/m for very strong salinity (Alberta Agriculture 2001). In general, soil is considered saline when electrical conductivity is 4 dS/m or more. It corresponds to 40 mM NaCl (Cabot et al. 2014).

4.3 Glycophytes and Halophytes

Most crops are salinity sensitive or even hypersensitive and they are described as glycophytes. They develop some adaptation mechanisms to monitor salt stress and regulate plant physiology and metabolism in order to cope with this stress (Sairam and Tyagi 2004). Glycophytes rapidly inhibit the root and shoot growth (Parvaiz and Satyawati 2008). Salinity tolerance depends on the stage of development. For example, one of the main food crops is rice, which is thought to be highly sensitive to salinity. In fact, its seedlings are highly salt sensitive, which greatly affects crops, whereas at the germination stage, rice is relatively resistant (Singh et al. 2008).

In contrast, high salinity is tolerated by halophytes, which constitute natural flora in saline regions (Keshtehgar et al. 2013; Kosova et al. 2013). Adaptation mechanisms are varied, ranging from biochemical reactions to specialized morphologies (Rajaravindran and Natarajan 2012; Shabala et al. 2012). Unfortunately, halophytes, adapted to high salinity (about 200 mM NaCl), are present in very small quantities, accounting for approx. 1 % of the world's flora (Sairam and Tyagi 2004; Sulian et al. 2012). Natural halophytes grow in sea shallows, salt marshes, salt lakes, and saline desserts. Typical examples are sea grasses and mangroves forests, as well as *Salicornia bigelovii* (dwarf glasswort), *Anemopsis californica* (lizard tail), *Panicum virgatum* (switch grass), *Atriplex* (saltbush), *Attalea speciosa* (babassu), *Spartina alterniflora* (smooth cordgrass), and *Tetragonia tetragonioides*. At the end of the scale (in terms of salt tolerance), there are euhalophytes. They are adapted to extreme exposure to seawater in the root zone (Flowers and Colmer 2015). Halophytes are studied as models of salt tolerance, but they may also be potentially cultivated as sources of antioxidants such as phenols, flavonoids, ascorbate, reduced/oxidized glutathione, and reactive oxygen species (ROS)-scavenging enzymes. Production of secondary metabolites is induced by salinity in plants from the families Amaranthaceae, Brassicaceae, Plantaginaceae, and Rhizophoraceae. Species *Tripolium pannonicum*, *Plantago coronopus*, *Lepidium latifolium*, and *Salicornia europaea* seem to be potential functional foods and nutraceuticals (Flowers and Muscolo 2015).

Most halophytes and glycophytes reveal similar survival mechanisms during salt stress. Some halophytes are able to adapt to extreme salinity due to their very special anatomical and morphological characteristics.

4.4 Plant Responses to Salt Stress

Salinity causes salt stress, one of abiotic stresses and the main reason of decreasing crop yields. Excessive salt concentrations in soils affect plants as a result of water stress, ion toxicity, nutritional disorders, oxidative stress, disturbance of metabolic processes, membrane disorganization, reduction of cell division, and genotoxicity (Carillo et al. 2011).

Salt stress affects the integrity of cellular membranes and enzymatic activities and disrupts the photosynthetic apparatus (Jithesh et al. 2006). Inhibition of microtubule polymerization and skewed root growth may also be observed (Dinneny 2015). Roots may be the first place to initiate the protection and adaptation mechanism. Under salt stress, phospholipid signaling results in differential auxin response. As a result roots are curved to avoid soil of the highest salinity. *Arabidopsis*, tomato, and sorghum roots grow avoiding salinity, even against the gravity axis. Such a phenomenon is called halotropism and it is a response of plant roots in order to reduce their exposure to salinity (Galvan-Ampudia et al. 2013).

The main stressor in saline soil is NaCl. Firstly, it causes osmotic imbalance and changes in K^+ and Ca^{2+} concentrations. As a result, nutritional imbalance is observed and toxic effects of NaCl lead to disorders affecting membranes and enzymes. Then subsequent oxidative stress is manifested, as ROS, such as superoxide radicals ($\bullet O^{2-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$), are produced, which are scavenged by both enzymatic and nonenzymatic antioxidants (Qureshi et al. 2007; Roychoudury et al. 2008). Excessive Na^+ accumulation manifests itself by damage to old leaves. Other negative effects include disturbed root growth, water consumption, nutrient uptake (microelements P, Fe, and Zn), and a negative influence on mycorrhizal fungi (Parvaiz and Satyawati 2008). When salt stress occurs, salt-tolerant plants are able to retain a higher K^+ ion concentration and a lower Na^+ ion concentration in the cytosol of cells (Li et al. 2014).

Two distinct kinds of effects could be noticed in plants following salt stress, which are categorized as osmotic (physical) and toxic (chemical) (Ellouzi et al. 2014). During plant response to salt stress, two phases are distinguished (Munns and Tester 2008; Parvaiz and Satyawati 2008) and they are presented in Fig. 4.1. The first phase is fast and the growth is inhibited due to the osmotic response to salt outside the roots. Hyperosmotic stress (Gupta and Huang 2014) limits water absorption by roots, while water loss from leaves is increased. Consequently, shoots stop growing. Next stomata become closed for a short time to protect the plant against ion flow. Afterward, stomata are opened for continuing carbon assimilation. The growth of younger leaves is inhibited.

The second phase is slower and salt concentrations inside plants become toxic. Plant growth is reduced. The response is salt specific (Munns and Tester 2008) and is considered to be hyperionic stress (Gupta and Huang 2014). Toxic ions are accumulated in plant tissues (Gupta and Huang 2014); mainly Na^+ ions are accumulated in leaf blades (Munns and Tester 2008). Old leaves are more exposed to ion toxicity.

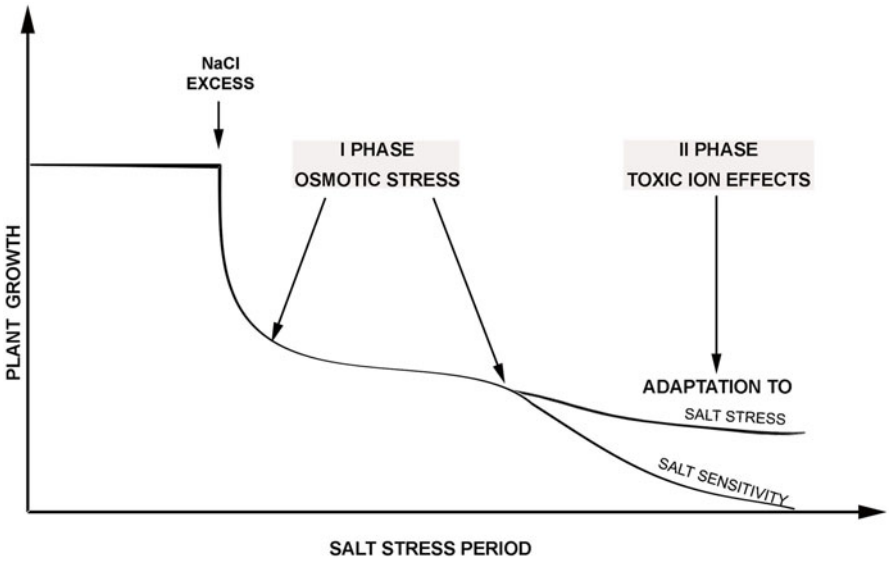

Fig. 4.1 Plant response to salt stress proposed by Munn and Tester (2008)

Less resistant plants lose old leaves, demonstrating necrosis, and photosynthesis is reduced, and the carbohydrate supply is limited. To protect and save plants, their older tissues die back (Gupta and Huang 2014). A negative effect of salt stress is connected with the production of ROS, such as singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide that cause oxidative damage to proteins, lipids, and DNA (Gupta and Huang 2014).

Physiological and biochemical mechanisms of salt tolerance are complex. One of them is biosynthesis of osmoprotectants. They are simple sugars (fructose, glucose), sugar alcohols (glycerol, inositol, mannitol, pinitol), complex sugars (trehalose, raffinose), quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine), and sulfonium compounds (choline-o-sulfate, dimethyl sulfonium propionate) (Yokoi et al. 2002). They make membranes more stable and protect enzymes from denaturation (Carillo et al. 2011).

The additional mechanisms are based on the activation of antioxidant enzymes and synthesis of antioxidant compounds, polyamines, and nitric oxide (NO). Also ion uptake, compartmentalization, and transport are essential for ion homeostasis (Gupta and Huang 2014). The excess of salt is transported to vacuoles or separated in older parts of plants. Toxic ion transport is mediated by the Na^+/H^+ transporter. There are two types of H^+ pumps in vacuolar membranes such as vacuolar V-ATPase and vacuolar pyrophosphatase V-PPase, the major H^+ pump inside plant cells (Gupta and Huang 2014).

Plants under salt stress conditions use the signaling system, which consists of the perception of stress signal and response for adaptation through gene expression. These genes particularly osmotic stress-responsive genes (OR) are not activated unless plants face stress. Water loss through stomata or cuticles is minimized by osmotic adjustment, which is most important in osmotic homeostasis. Signaling induced by stress includes ionic and osmotic stress signaling, detoxification signaling, and cell division signaling (Aryadeep et al. 2013).

Moreover, salt stress is accompanied by the production of plant hormones such as abscisic acid (ABA). Other phytohormones, which are potentially helpful in improving salt tolerance, include salicylic acid (SA) and brassinosteroids (BRs) (Gupta and Huang 2014).

Investigations providing insight into the mechanisms of plant response to salt stress are crucial, as it could improve salt tolerance and have practical implication in crop improvement.

4.5 Phytohormones

Phytohormones are hormones that are produced by plant cells in low concentrations. They act as plant growth regulators (PGR), while they also regulate development and differentiation of plant cells and tissues (Argueso et al. 2009; Santner and Estelle 2009; Messing et al. 2010). Plant growth regulators include the five classical phytohormones, abscisic acid (ABA), ethylene (ET), cytokinin (CK), auxin (IAA), and gibberellin (GA), as well as jasmonic acid (JA), brassinosteroids (BRs), salicylic acid (SA), nitric oxide (NO), and strigolactone (SL). It is also likely that additional growth regulators are yet to be discovered (Srivastava 2002; Peleg and Blumwald 2011). Some of them are recognized as vital agents in the adaptation process during abiotic stress (Khan and Khan 2013).

The evolution process in the plant kingdom brought a variety of mechanisms developed for their survival under environmental stresses, e.g., salt stress. Some molecules, such as transcription factors and MAP kinases, are main agents involved in stress signaling pathways and phytohormone cross talk. Transcription factors (sequence-specific DNA-binding factors) are proteins, which are attached to specific DNA sequences. Mitogen-activated protein kinases (MAP kinases) are protein kinases which are specific to amino acids. MAP kinases play a role in cellular responses to environmental factors, including osmotic and salt stress, and they regulate cell functions. Plant hormones including abscisic acid, salicylic acid, jasmonic acid, ethylene, and brassinosteroids can regulate that cross talk and responses to salt stress (Fujita et al. 2006). The plant signaling cascade is presented schematically in Fig. 4.2 (adopted from Bajguz and Hayat 2009).

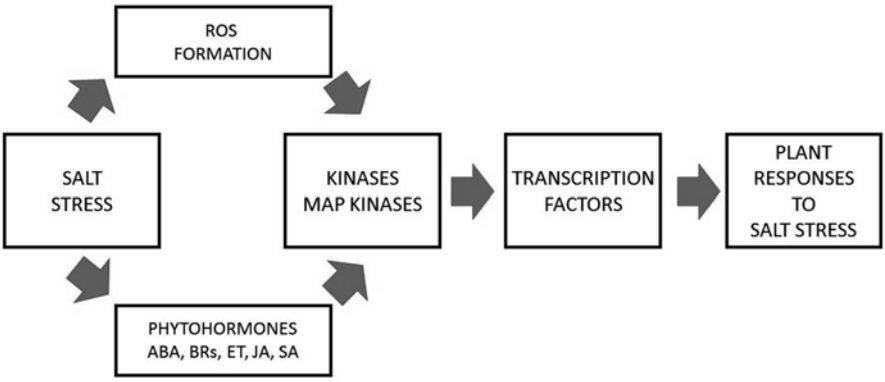

Fig. 4.2 Schematic representation of plant signaling (adopted from Bajguz and Hayat 2009)

4.5.1 Abscisic Acid

4.5.1.1 Biosynthesis

Firstly (in the 1960s), this compound was identified in abscised and fallen leaves and as a compound responsible for seed dormancy. Abscisic acid (5-(1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-y1)-3-methylpenta-2,4-dienoic acid) is a sesquiterpenoid (C15H20O4), with one asymmetric, optically active carbon atom in position 1′ (Fig. 4.3). In plants, it is found as S-ABA, while R-ABA, the mirror-image form, has not been reported in nature (Zaharia et al. 2005). ABA biosynthesis consists of two enzymatic steps starting from C40 carotenoids (e.g., zeaxanthin). The first interconversion is catalyzed by zeaxanthin epoxidase (ABA1). All-trans-violaxanthin is converted to 9-cis-violaxanthin and 9-cis-neoxanthin. These C40 carotenoids are split to C15 aldehyde xanthoxin and C25 apocarotenals by means of violaxanthin de-epoxidase (VDE). Xanthoxin is exported to the cytosol and it is converted to abscisic aldehyde by dehydrogenase/reductase (ABA2). Next abscisic aldehyde is oxidized to abscisic acid by abscisic aldehyde oxidase (AAO) (Christmann et al. 2006).

4.5.1.2 ABA in Physiological Plant Development

Apart from abiotic stresses, abscisic acid (ABA) plays a role during physiological and optimal plant development as well as seed germination. ABA protects from premature growth under adverse conditions, as it affects bud and seed dormancy. Physiological dormancy is described as the mechanism, which inhibits and prevents

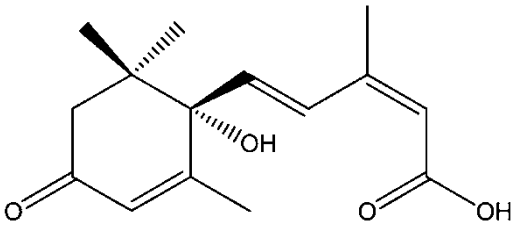

Fig. 4.3 Chemical structure of abscisic acid

the formation of radicles. The role of ABA in dormancy is well known and both ABA content and sensitivity are essential (Finkelstein 2013). There is genetic evidence on the role of ABA in seed dormancy in several plant species such as *Arabidopsis*. ABA mutants which are unable to produce ABA or insensitive to ABA do not exhibit dormancy. In contrast, dormancy is prolonged with excess ABA biosynthesis and accumulation (in ABA-oversensitive species) (Dekkers and Bentsink 2015).

There are complex interactions between ABA and sugar glycolysis. The metabolic pathway of glycolysis converts glucose into pyruvate and free energy is released, which forms the high-energy compounds – ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). In this way carbohydrate metabolism provides essential energy for plant growth and development. The interactions between glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPCp) and ABA signal transduction in *Arabidopsis* have been investigated. However, the detailed mechanism is still unknown. GAPCp deficiency causes ABA insensitivity and weak ABA signal transduction (Muńoz-Bertomeua et al. 2011). Glyceraldehyde-3-phosphate is the substrate of the precursor of GAPCp, while it is also the precursor of the ABA biosynthetic pathway (methylerythritol phosphate pathway). In plants, glyceraldehyde-3-phosphate dehydrogenase interacts directly with protein kinase (OSAK), which is activated by osmotic stress during salinity (Muńoz-Bertomeua et al. 2011).

4.5.1.3 Participation ABA in Salt Stress

Abscisic acid is a phytohormone which sends an endogenous signal during drought and salt stress. The mode of ABA action depends on its content. Nanomolar concentrations promote the growth of plants, while micromolar concentrations inhibit growth (Dinneny 2015). When plants recover from salt stress, ABA, via the signaling system, enables primary root growth and ABA concentration becomes low. Thus ABA plays a vital role in plant response to unfavorable environmental conditions.

ABA biosynthesis is enhanced with the change in water status that eventually induces the closure of stomata. Thus stomatal closure is mediated by ABA with the change in ion fluxes in guard cells. ABA-activated protein kinase open stomata 1

(OST1) is a regulator of stomatal closure; it activates the anion channel (slow anion channel associated 1, SLAC1) and inhibits the cation channel (KAT1) by phosphorylation. The two channels are regulated by the ABA signaling pathway and Ca^{2+} (Raghavendra et al. 2010).

The activity of ABA and its protective role during salt stress are connected with increasing the contents of K^+, Ca^{2+}, and other osmolytes. ABA-induced signaling may also be responsible for regulating the expression of selected genes in response to salt stress. For instance, ABA regulates the expression of the H^+ pump and Na^+/H^+ antiporter genes, key determiners of salt tolerance (Fukuda and Tanaka 2006).

A concept has been proposed that ABA signaling is based on intracellular messengers. The plasma-membrane-localized perception site, soluble receptor, and ABA-binding proteins are constantly studied. ABA-induced stomatal closure is associated with signaling molecules such as nitric oxide, reactive oxygen species, and cytosolic free calcium. ABA perception and signaling have been studied using genetic approaches. As a result of salt stress, ABA induces osmotic stress-responsive genes (OR). These genes encode the LEA proteins and enzymes. They are involved in the biosynthesis of osmolytes and detoxification. The OR genes also encode ion transporters and regulatory molecules (TFs), protein kinases, and phosphatases. Apart from ABA-dependent responses to salt stress, there are also ABA-independent pathways that are activated in response to salt stress. Both pathways affect each other in a cross-talk network (Aryadeep et al. 2013).

The analysis of ABA/stress-responsive genes revealed that a DNA sequence element consisting of ACGTGGC is essential during ABA regulation (Qureshi et al. 2007). The abscisic acid response element (ABRE) is used to describe transcription factors regulating the expression of ABA/stress-responsive genes. *Arabidopsis* bZIP proteins are related to ABFs (ABRE-binding factors). Their expression is initiated by ABA and high salt stress (Qureshi et al. 2007).

ABA perception is based on the binding of ABA to ABA receptors, referred to as RCARs/PYR1/PYLs, which stands for regulatory components of ABA receptor/pyrabactin resistance protein1/PYR-like proteins. These binding proteins are found in the chloroplast and nucleus. The binding of ABA to the receptors leads to inactivation of type 2C protein phosphatases, such as ABI1 and ABI2. There are other ABA-binding proteins, designated as ABAR/CHLH/GUN5 and located in the chloroplast and nucleus, GCR2, GTG1, and GTG2 in plasma membranes (Raghavendra et al. 2010).

Abscisic acid plays a key protective role in all halophyte species under salinity conditions. *Atriplex leucoclada*, *Suaeda fruticosa*, and *Salicornia virginica* exhibit higher concentrations of ABA under salt stress as compared to indole acetic acid (IAA), which plays a major role in regulating plant growth (Bano and Bano 2011). Salinity stress is a major factor inducing carotenogenesis in some species of halotolerant green algae *Dunaliella*, and it also induces *Dunaliella* to produce high levels of ABA. There has been little information concerning the mechanism, through which ABA responds to environmental stress in algae (Sarmad et al. 2007).

Concentrations of ABA were studied in salt-tolerant and salt-sensitive tomatoes under salt stress (Amjad et al. 2014). Three sodium salt concentrations (0, 75,

150 mM NaCl) and two potassium salt concentrations were tested (0, 4.5 M KCl) in climatic chamber. It turned out that salt-tolerant species produce more ABA and ethylene in comparison to salt-sensitive species and to control cultivation (0 mM NaCl). When K^+ ions were applied (4.5 mM), the phytohormone concentrations were lower. K^+ ions compete with Na^+ ions under salt stress, resulting in an increased chlorophyll content and stomatal conductance and better plant growth. The chlorophyll content index (CCI) can be a good measure of photosynthetic activity in plants under salt stress. It has been applied as a good indicator of salt tolerance in some species such as cotton, rice, soybean, wheat, quinoa, and radish (Amjad et al. 2014). Summing up, salt stress decreased CCI, xylem sap K^+, and K^+/Na^+ and increased xylem sap Na^+ and ABA concentrations which can be considered as a biomarker of salt tolerance.

4.5.1.4 The Effect of Exogenous Abscisic Acid on Adaptation to Salt Stress

The response of two canola cultivars (*Brassica napus*), Fornex (salt tolerant) and Okamer (salt sensitive) to ABA foliar application, was investigated under salinity stress (0 and 120 mM NaCl). The results showed that shoot dry matter, photosynthetic rate, peroxidase and catalase activity, and shoot K^+ concentration increased in the salt-sensitive variety, while Na^+ concentration in shoots decreased. However, too high concentration of ABA treatments inhibited growth. The adverse effects of ABA foliar application were observed in the case of the salt-tolerant cultivar (Farhoudi and Saeedipour 2011).

Foliar application of ABA was studied in turfgrass species, creeping bent grass (*Agrostis stolonifera*) and Kentucky bluegrass (*Poa pratensis*). Abscisic acid was effective in mitigating physiological damage resulting from drought or salinity for both grass species, but effects were more pronounced in Kentucky bluegrass. The effects of ABA application included the suppression of membrane electrolyte leakage (EL) and membrane lipid peroxidation (expressed as malondialdehyde (MDA) content) and an increase in ascorbate peroxidase (APX), peroxidase (POD), and superoxide dismutase (SOD) activities after 35 days of salinity stress. These results suggest that foliar application of ABA mitigates salinity stress in turfgrass (Yang et al. 2012).

Induced salinity stress and foliar ABA applications were studied in wheat genotypes. High salinity stress (10 dS/m) had a positive effect on proline synthesis of wheat genotypes. The results showed that exposure of plants to both salinity stress and foliar application of ABA resulted in higher levels of proline and even the yield was better (Bakht et al. 2012). Also, exogenous application of ABA alleviated osmotic stress effects on wheat seedlings (Marcinska et al. 2013).

Exogenous application of small molecules, such as ABA (as well as NO, $CaCl_2$, H_2S, polyamine, and melatonin), is able to alleviate the adverse effects of salt stress. During warm season application of such small molecules influenced the accumulation of osmoprotectants and antioxidants in Bermuda grass. They enabled the maintenance of cell membrane integrity, increased photosynthesis, and helped to keep

ion homeostasis. These actions protected Bermuda grass against salt stresses (Chan Zhulong and Shi Haitao 2015).

Plants react to environmental changes through very complex signaling networks. Phytohormones, which are involved in these processes, can generate positive and negative effects. It is well accepted that salinity induces an increase in ABA content and ABA has been linked to plant susceptibility to bacteria, fungi, and oomycetes (DiLeo et al. 2010). The influence of salt stress on infection of tomato and chrysanthemum roots by *Phytophthora* spp. was studied. Roots in hydroponic cultures were exposed to NaCl stress. The increase in root ABA contents in tomato was related to stress-induced susceptibility. Exogenous ABA could substitute for salt stress and intensify pathogen colonization. ABA-deficient tomato mutants did not exhibit susceptibility, and it could be reversed by supplementation with exogenous ABA. This phytohormone seems to be an important factor in predisposition to *Phytophthora* spp. infection induced by salt stress (DiLeo et al. 2010).

Abscisic acid decreased Na^+ exclusion in leaves of *Phaseolus vulgaris* L. under salt stress. Short-term NaCl treatment in beans caused higher contents of leaf ABA, and ABA concentration was released by Na^+, not by Cl^-. When fluridone (an ABA inhibitor) pretreatment was used, beans under salt stress showed a lower Na^+ uptake and a higher leaf Na^+ exclusion in comparison to beans without fluridone pretreatment. Na^+ uptake was higher and leaf Na^+ exclusion was lower when an ethylene inhibitor – aminooxyacetic acid (AOA) – and ABA were applied. Such a non-ion-specific increase in ABA concentration may be a signal of the osmotic component of salt. A higher ABA concentration influences leaf Na^+ concentrations due to a lower Na^+ exclusion or an increased root-shoot Na^+ translocation (Cabot et al. 2009).

4.5.2 Ethylene

4.5.2.1 Ethylene in Plant Development

Ethylene (ET) is a gaseous phytohormone which is involved in various processes in plants, e.g., inhibition of growth and fruit ripening (Hahn and Harter 2009). It is also a modulator of auxins, gibberellins, cytokinins, and ABA when seeds ripen. It is well known that ethylene stimulates dormancy (the primary, secondary, and light-induced dormancy) (Khan et al. 2009). Salinity is one of the factors which causes an increased ethylene production. Excessive content of ethylene leads to premature decay of plants (Siddikee et al. 2012).

4.5.2.2 Biosynthesis

The biosynthesis of ethylene in plants starts from methionine, which is converted to S-adenosyl-methionine (SAM). Next, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) using ACC synthase (ACS). Finally, ACC is converted to

ethylene by ACC oxidase (ACO). All these compounds and enzymes are produced when salt stress occurs. The last step (conversion to ACC) is referred to as the rate-limiting process during ethylene synthesis. Salt stress might be reduced by inhibition of ethylene production by halotolerant bacteria which produce ACC deaminase. Plant growth-promoting rhizobacteria (PGPR) transform ACC into ammonia (Siddikee et al. 2012).

4.5.2.3 Receptors

In the endoplasmic reticulum, five receptors encoded by various ethylene-responsive genes such as ethylene response1 (ETR1), ethylene response2 (ETR2), ethylene resistant1 (ERS1), ethylene resistant2 (ERS2), and ethylene insensitive4 (EIN4) have been found. The main part of the ethylene response pathway is a constitutive triple response1 (CTR1), which is a signaling regulator acting downstream of ethylene receptors (Li et al. 2014). Ethylene insensitive2 (EIN2) is a downstream component of CTR1, while ethylene response factor1 (ERF1) is an upstream component. When there is no ethylene, CTR1 acts with ethylene receptors ETR1 and ERS1 and reduces the ethylene signal response. When ethylene is produced, then CTR1 becomes inactive. Ethylene formation is directed by the mitogen-activated protein kinase (MAPK) cascade. One of the causes for the activation of this cascade is salt stress (Hahn and Harter 2009).

4.5.2.4 Ethylene During Salt Stress

Ethylene is an active agent in ion homeostasis when salt stress occurs. It regulates the H^+-ATPase gene expression (Amjad et al. 2014). Also ethylene acts as a downstream signal of cGMP, which stimulates plasma membrane PM H+−ATPase. cGMP (3′,5′-cyclic guanosine monophosphate) is a messenger in response to salt stress and its concentration increases even ten times. The evidence that ethylene participates in signaling is provided by the fact that ethylene-insensitive mutants are more exposed to the effects of salt stress. The mechanism of ethylene signaling under salt stress is still unknown (Li et al. 2014).

Ethylene is one of the molecules of the alternative respiratory pathway (AP). AP is active during responses to different stresses and helps plants to adapt to adverse environmental conditions. AP is the second pathways, additional to the cytochrome pathway (CP). AP may stop the production of reactive oxygen species (ROS) during salt stress, when CP is not efficient. Ethylene and hydrogen peroxide induce AP in plants. It seems that ethylene is needed for AP. There is a hypothesis that ethylene, NO, and H_2O_2 are signaling agents under salt stress in *Arabidopsis*. When salt stress occurs, nitric oxide (NO) is produced using nitric oxide synthase (NOS). Also plasma membrane (PM) NADPH oxidase induces H_2O_2 production. Hydrogen peroxide activates ACC synthase (ACS), and it results from ethylene production as well

as AOX activity, AOX1a expression, and pyruvate production. Finally, alternative respiratory pathway (AP) is induced (Wang et al. 2010).

There is an integrating role of ethylene and ABA in tomato plant adaptation to salt stress (Amjad et al. 2014). Ethylene increases the K^+/Na^+ ratio by increasing plasma membrane H^+-ATPase activity. Since elevated levels of ABA and ethylene are associated with salt stress, their levels decreased as plants were supplied with potassium.

4.5.2.5 The Effect of Exogenous Ethylene on Adaptation to Salt Stress

The application of ethylene to some halophyte seeds reduces negative effects of salt stress (Khan et al. 2009).

The influence of ethylene and nitric oxide on germination of *Arabidopsis* seeds under salt stress was studied, but the detailed nature of their interaction and the effect on germination have not been specified (Yingchao et al. 2013). These molecules modulate seed germination under unfavorable environmental conditions. Application of 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene biosynthesis, helps to mitigate the inhibition of germination when salinity occurs. Aminoisobutyric (AIB) acid is an inhibitor of ethylene biosynthesis and this compound decreases the positive influence of exogenous ethylene. This is the evidence that ethylene, together with nitric oxide, is involved in the protection against salt stress. Ethylene and NO cooperate in the germination process during excessive salinity. It was shown that the precursor of ethylene (ACC) is able to increase NO content (Yingchao et al. 2013).

Ethylene has been studied in terms of alleviation of salt stress-induced inhibition of seed germination in cucumber (*Cucumis sativus* L.). As it was expected, seed germination was significantly inhibited by salt stress, but this negative effect was reduced by the precursor of ethylene biosynthesis (ACC). On the other hand, ACC did not affect the growth of radicles under salt stress. Also, exogenous glutamate (Glu) was observed as an alleviating agent during the inhibition of seed germination and radicle growth induced by salt stress. The positive effect of L-Glu on seed germination was smaller when two antagonists of ethylene synthesis, aminoethoxyvinylglycine (AVG) and $CoCl_2$, were applied. This fact indicates that ethylene is involved in the suppression of seed germination under salt stress and that L-Glu interacts with ethylene and modulates seed germination under salt stress (Chang Chenshuo et al. 2010).

Ethylene and nitric oxide (NO) were studied in terms of their protective action that modulates ion homeostasis in *Arabidopsis* calli under salt stress. The ethylene-insensitive mutant was more prone to salt stress than the wild variety. The treatment in the ethylene-insensitive mutant using 100 mM NaCl caused a greater Na^+/K^+ ratio and a lower plasma membrane H^+-ATPase activity in calli in comparison to calli in the wild variety. Under NaCl stress the NO accumulation and ethylene production appeared at the beginning. NO production induced ethylene emission in wild calli. Application of exogenous ACC or sodium nitroprusside (a NO donor) mitigated the

negative effects of NaCl only in the ethylene-sensitive variety. The results showed a lower Na^+/K^+ ratio and a higher plasma membrane H^+-ATPase activity in calli of the wild variety, but not in calli of the mutant. Additionally, the expression of PM H^+-ATPase genes was induced by ethylene when salt stress started. Summing up, ethylene and NO act together to increase H^+-ATPase activity under salt stress. They both promote maintenance of ion homeostasis (Wang Huahua et al. 2009).

4.5.3 Jasmonates

4.5.3.1 Jasmonates in Plant Development

Jasmonates, i.e., jasmonic acid (JA) and its derivatives, e.g., methyl jasmonate (MeJA), play a crucial role in plant development, in biotic and abiotic stresses. Figure 4.4 presents the chemical structure of jasmonic acid. Jasmonic acid (JA) and its conjugates with amino acids, especially with L-isoleucine, are involved in numerous plant responses to almost all types of stresses (drought, salinity, UV, ozone, cold, high temperatures, osmotic stress). They act in arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria (PGPR). Also jasmonates are vital in the development of plant organs, embryos, seedlings, roots, trichomes, tuber, and seed germination. Moreover, gravitropism, leaf movements, and plant deterioration with age are dependent on these compounds (Wasternack and Hause 2013; Wasternack 2014).

4.5.3.2 Biosynthesis of Jasmonate

The main pathway of jasmonate biosynthesis starts from membrane lipids. α-Linolenic acid is released from the membrane by means of phospholipase A1. Linolenic acid is oxygenated to 13-(S)-hydroxy linolenic acid (13-HPOT) by lipoxygenase. 13-HPOT is transformed to 12-oxophytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is reduced and follows three steps of α-oxidation and jasmonic acid (JA) is produced. JA is converted to methyl jasmonate by JA carboxyl methyltransferase (JMT) (Dar et al. 2015).

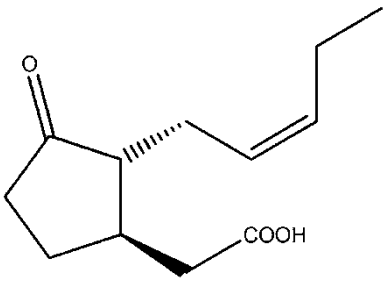

Fig. 4.4 Chemical structure of jasmonic acid

4.5.3.3 Jasmonates During Salt Stress

Jasmonates are active as signaling molecules during signal transduction when abiotic stresses take place (Wasternack 2014). During salt stress, the content of endogenous jasmonates increases (Javid et al. 2011a, b). In tomato cultivars salt stress induced a higher level of JA in the salt-tolerant variety at the beginning of salt stress. In the case of the salt-sensitive variety, JA content was decreased after one day of salt stress (Javid et al. 2011a, b). Similarly, in salt-sensitive rice, the content of JA was higher than in salt-tolerant rice (Javid et al. 2011a, b). Besides the levels of jasmonic acid, the content of its conjugates with L-isoleucine is increased as well (Wasternack 2014). The signaling pathway which involves jasmonates is connected with a specific protein family, known as jasmonate ZIM domain (JAZ) proteins. They are negative regulators of jasmonic acid-induced gene expression. JAZ proteins undergo ubiquitination via the SCF ^{COI1} complex. The abbreviation COI1 stands for coronatine insensitive1 and it is an F-box protein. The abbreviation SCF is a Skp1/Cullin/F-box complex, which is an E3 ubiquitin ligase. The F-box protein is able to indicate specific target proteins, which are proteasomal degraded. The SCF ^{COI1} complex consists of the bond of the ligand for the COI1-JAZ interaction, an enantiomer of jasmonate and (+)-7-iso-L-isoleucine. This enantiomeric form is accumulated under physiological and stress conditions. When JA/JA-Ile content is low, the promoters of JA-responsive genes are not activated by transcription factors (MYC2), because repression takes place. When JAZ proteins undergo proteasomal degradation, transcription factors, such as the MYC or MYB families, are released, and they are linked to promoters of JA-responsive genes. During JA signaling, there is an important SCF^{COI1}-JAZ co-receptor complex. This complex has a function of the JA receptor, the JAZ protein, and the transcription factors (MYC2) (Wasternack 2014). The mitigation of salt stress by jasmonates was studied in species *Glycine max*, *Oryza sativa*, *Pisum sativum*, *Hordeum vulgare*, and *Iris hexagona* (Dar et al. 2015). The JA signaling cooperates with other hormones, e.g., salicylic acid, auxins, or gibberellins via cross talk.

4.5.3.4 The Effect of Exogenous Jasmonates on Adaptation to Salt Stress

It has been observed that exogenous application of jasmonates can help plants to cope with salinity and the effects of salt stress are less severe (Javid et al. 2011a, b). The application of exogenous JA (or an increased level of endogenous JA) is assisted by the synthesis of large amounts of proteins, known as JIPs.

The results of salt stress and the application of JA were studied in the case of two different rice (*Oryza sativa* L.) cultivars, i.e., Dongjinchalbyeo (DJC, salt tolerant) and Dongjinbyeo (DJ, salt sensitive). As a result of salt stress, their roots were shorter and the concentration of abscisic acid was lower. The ABA concentrations in the salt-tolerant cultivar progressively increased when NaCl amounts increased, whereas in the salt-sensitive cultivar, the ABA concentrations decreased at 80 mM NaCl. In shoots JA concentrations in the salt-tolerant cultivar were lower than in the

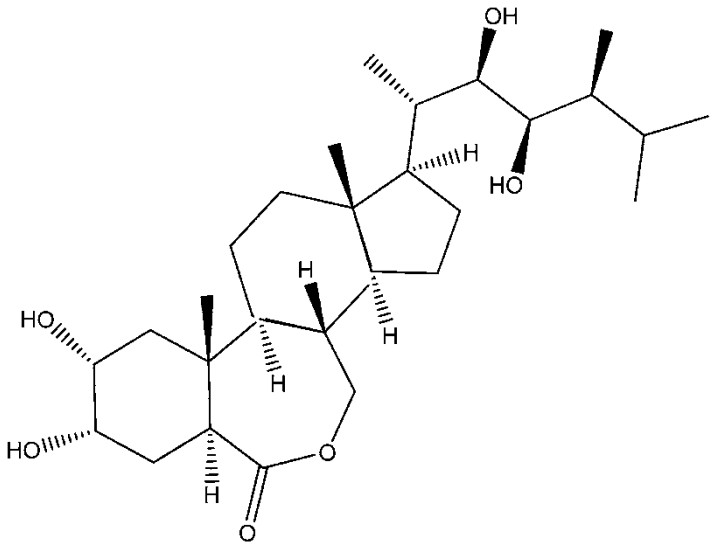

Fig. 4.5 Chemical structure of brassinolide

salt-sensitive cultivar. Post-application of JA (24 and 48 h after NaCl) was more beneficial for the recovery from salt stress than the application at 24 h and 48 h before salt stress or in the middle of salt stress. Also JA treatment caused a decrease of Na uptake in the salt-sensitive cultivar and an increase in Ca and Mg contents. Leaf water potential, leaf photosynthetic rate, and maximum quantum yield of photosystem II (PSII) improved after the JA application. The exogenous JA post-application is helpful in the recovery of rice from salt stress, more effectively in the case of the salt-sensitive cultivar. The influence of JA application on the balance of other endogenous plant hormones has been suggested as well (Kang et al. 2005).

4.5.4 Brassinosteroids

4.5.4.1 Brassinosteroids in Physiological Processes

Brassinosteroids (BRs) are a large group of phytohormones with the structures of polyhydroxysteroids. They are present in most parts of plants as free compounds or bounded up with sugars or fatty acids. About 70 of brassinosteroids have been extracted from plants (Bajguz and Hayat 2009). One of the first discovered representatives is brassinolide, which was extracted from *Brassica napus*. Its chemical structure is presented in Fig. 4.5.

Brassinosteroids are agents in various physiological processes such as growth, seed germination, rhizogenesis, senescence, and leaf abscission. The effects of brassinosteroids are pleiotropic (Javid et al. 2011a, b). Plant varieties, which are

lacking BR biosynthesis and signaling, are scrubby, with short hypocotyls, stems, and dark green leaves, and delayed aging is observed (Chung and Choe 2013).

Besides the participation in plant physiology, e.g., plant development, cell division, and cell elongation in stems or roots, brassinosteroids are active in stress responses (Ahammed et al. 2015; Fariduddin et al. 2013).

4.5.4.2 Brassinosteroids During Salt Stress

The mechanism of the stress response and the regulation of stress-responsive gene expression require further investigations. Probably, brassinosteroids interact and give impulses for other hormones. The cross talk between brassinosteroids and other hormones takes place in plants (Ahammed et al. 2015). The expression of hormone biosynthetic genes and signaling intermediates varies. BRs increase the ethylene content. Also additive effects of BRs were observed with gibberellins and synergistic with auxin on stem segment elongation (Bajguz and Hayat 2009).

For example, abscisic acid (ABA) is a signaling molecule of salt stress, which inhibits the action of brassinosteroids. The mechanism is not fully understood, although it is known that ABA and BRs act antagonistically on their target genes at or after the BIN2 (BR-insensitive 2) step in BR signaling, and as a result plants adapt to salinity conditions. BRs suppress the expression of the ABA-responsive genes to improve ABA-dependent stress tolerance (Chung et al. 2014).

Physiological responses of BRs under salt stress include influences on photosynthesis, the antioxidant system, and ion homeostasis (Fariduddin et al. 2013). First of all, BRs defend the photosynthetic system against any reduction in the activity of photosynthetic enzymes such as RuBisCO. Secondly, photosystem II (PSII) is protected and its yield is increased by BRs.

The application of BRs reduces the excessive levels of ROS and minimizes lipid peroxidation under salt stress. BRs protect plant cells against the accumulation of high concentrations of sodium ions. BRs activate the high-affinity K^+ transporters, so the K^+/Na^+ ratio is higher. Also the uptake of Ca^{2+} and K^+ is increased by BRs; as a result the ratios of Ca^{2+}/Na^+ and K^+/Na^+ are higher as well.

4.5.4.3 The Effect of Exogenous Brassinosteroids on Adaptation to Salt Stress

One of the techniques to reduce salt stress is exogenous application of some plant growth regulators (Ashraf et al. 2010). When brassinosteroids are used, they reduce the effects of salt stress and modify antioxidant enzymes. For example, the activity of catalase in *Arachis hypogaea* was higher following foliar treatments of 28-homobrassinolide (28-HBL) and 24-epibrassinolide (24-EBL) (Fariduddin et al. 2013). When salt stress occurs, BRs maintain the content of chlorophyll. Also the activity of nitrate reductase is increased by brassinosteroids (Javid et al. 2011a, b). This enzyme is responsible for nitrogen supply in plants. 24-Epibrassinolide (EBR)

influences positively the growth and photosynthesis in wheat under salt stress (Fariduddin et al. 2013).

It was observed that BR application increased growth and seed yield of rapeseed, cotton, and rice. Moreover, germination is improved in *Eucalyptus camaldulensis* and *Oryza sativa* (Bajguz and Hayat 2009). In general, the application of BRs helps plants to grow at excessive salt concentrations (Javid et al. 2011a, b).

Wheat germ agglutinin (WGA) is a cereal lectin; its content is higher when biotic and abiotic stresses, such as salt stress, occur. The treatment using BRs reduces WGA concentration in roots; thus BRs might serve a protective role (Bajguz and Hayat 2009).

EBR was studied as a potential remedy for salt stress in salt-tolerant and salt-sensitive pea genotypes. EBR treatment significantly increased the leaf water status and production of osmolytes (Shahid et al. 2015).

The results of brassinolide (BL) treatment on cotton growth were analyzed. Cotton roots, both in cv. Sumian 12 (salt sensitive) and cv. Sumian 22 (salt tolerant), were subjected to NaCl stress (200 mM). Too high salinity caused an increase in Na^+, proline, and malondialdehyde concentrations, while protein contents in the roots decreased in both varieties. The application of BL alleviated growth inhibition of cotton, reduced the accumulation of Na^+, and increased proline concentration. The positive effects of BL were more evident in the salt-sensitive variety. The analysis showed that BL influenced the gene expression in roots of the salt-sensitive variety under salt stress. Summing up, BL applied to cotton roots is able to ameliorate NaCl stress by improving the activity of roots, physiological processes, and gene expression (Shu Hongmei et al. 2015).

There are a variety of papers concerning advantages of BR application in order to ameliorate salt stress (reviewed by Ahammed et al. 2015). The beneficial effects were observed in the case of oilseed rape (Efimova et al. 2014), *Cucumis sativus* L. (Fariduddin et al. 2014), basmati rice (Sharma et al. 2013), eggplant *Solanum melongena* L. (Ding et al. 2012), lettuce (Ekinci et al. 2012), and strawberry (Karlidag et al. 2011).

4.5.5 Salicylic Acid

4.5.5.1 Role of Salicylic Acid in Biological Processes

Salicylic acid (SA, 2-hydroxy benzoic acid, Fig. 4.6) – produced by a wide range of prokaryotic and eukaryotic organisms – is a phenolic compound of hormonal nature, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesized by plants (Chen et al. 2009; Seyfferth and Tsuda 2014). SA acts as a key regulator of the signaling network in plants under abiotic and biotic stresses, such as low and high temperature, salts, and oxidative conditions (Gunes et al. 2007; Noreen et al. 2009; Hara et al. 2012; Fu and Dong 2013; Miura and Tada 2014). Indeed, SA exerts stimulatory effects on various physiological processes

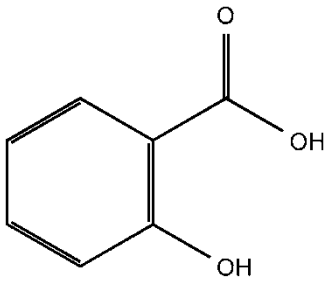

Fig. 4.6 Chemical structure of salicylic acid

related to plant growth and development and plays key signaling roles in thermogenesis and disease resistance (Vlot et al. 2009; Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; Chandran et al. 2014). The SA signaling pathway is highly interconnected with other phytohormone signaling, such as jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; Derksen et al. 2013). For example, JA and ET signaling negatively regulates SA biosynthesis at the transcriptional level (Chen et al. 2009; Zheng et al. 2012).

Szalai et al. (2005) tested maize plants under NaCl conditions (50 and 100 mM). In leaves and roots collected after the 1st, 3rd, and 7th days of salt treatment, there were no changes in endogenous free and bound salicylic acid levels and in catalase and ascorbate peroxidase activities.

Recently, SA-induced expression has been shown for 59 proteins in cucumber, which were identified for their involvement in various cellular responses and metabolic processes, including antioxidative reactions, cell defense, photosynthesis, carbohydrate metabolism, respiration and energy homeostasis, protein folding, and biosynthesis (Hao et al. 2011).

4.5.5.2 Exogenous Application of Salicylic Acid Under Salinity

Several studies refer the application of exogenous salicylic acid to improved resistance to salt stress in various types of plants (Szalai et al. 2005; Hussein et al. 2007; Kaydan et al. 2007; Azooz 2009; Noreen et al. 2009; Khan et al. 2010; Shahba et al. 2010; Noreen et al. 2011; Ghafiyehsanj et al. 2013; Ismail 2013; Karlidag et al. 2009; Khan et al. 2015; Singh et al. 2015).

The impact of salinity (140 mM NaCl) and SA application (0.2 mM) on physiological processes in two faba bean genotypes (115 and 125) was tested (Azooz 2009). Salt stress induced high K^+/Na^+ ratios in shoots and roots with changes in dry weight and tissue water contents, while the SA application alleviated the effect of salinity in both cultivars, and in some cases better results were obtained than in the untreated plants. In a similar study, Khan et al. (2010) examined the role of SA (0.1, 0.5, and 1.0 mM) in tolerance of salinity (50 mM NaCl) in mung bean. The best results manifested in the maximum decrease in the contents of Na^+, Cl^-, H_2O_2, and thiobarbituric acid-reactive substances (TBARSs) and in an increase of N, P, K, and

Ca contents; activities of antioxidant enzymes, glutathione content, photosynthesis, and yields were recorded in the case of plants treated with 0.5 mM SA.

Hussain and coworkers (2007) investigated the addition of salicylic acid (200 ppm) on growth parameters in maize plants under salinity conditions (2000 and 4000 ppm NaCl). Spraying plants with SA improved all tested parameters, i.e., plant height, the number and area of green leaves, stem diameter, and dry weight of stems, leaves, and the whole plants. In other studies – apart from growth parameters – the level of phenolic compounds was also tested in 2-week-old maize plants growing under salinity conditions (0, 50, 100, 150, and 200 mM NaCl) and treated with salicylic acid (0.5 mM) (Singh et al. 2015). After salt treatment, the results showed reduction in plant dry weight, leaf relative water content and contents of photosynthetic pigments, and an increase in total phenolics. Exogenous application of SA resulted in an increase of growth parameters and decreased phenolic contents. Among all phenolics, ferulic acid was dominant. Also Ismail (2013) investigated the effect of exogenous application of salicylic acid (200 ppm) in maize plants under different salt concentrations (20, 40, 60, 100 mM NaCl) by analyzing such parameters as shoot and root lengths, fresh and dry weights, leaf area, antioxidant enzymatic activities, chlorophylls a and b, total chlorophyll, and chlorophyll stability index. Salinity reduces the abovementioned growth parameters and enzyme contents, while SA treatment improved them. In the case of a low NaCl concentration (20 mM), the toxic effects were completely eliminated by SA application.

The biochemical characteristics of salt-stressed wheat plants after the application of salicylic acid were demonstrated by Ghafiyehsanj et al. (2013) and Kaydan et al. (2007). In the first study, two levels of salinity (75 and 150 mM NaCl) and two SA concentrations (200 and 400 mg/L) were tested. With increasing salinity the protein and insoluble sugar contents and shoot and root weight were reduced, while soluble sugar, proline, and malondialdehyde (MDA) concentrations were increased. After the application of SA, the above parameters were improved, while an exogenous application of SA without salt stress caused no changes in the levels of proline, soluble and insoluble sugars, and MDA. Kaydan et al. (2007) tested the addition of salicylic acid (10^{-2}, 10^{-4}, 10^{-6} mol/L) under salinity (8 dS/m) and its impact on growth parameters, osmotic potential, and photosynthetic pigments in wheat plants. Exogenous SA increased emergence percentage, osmotic potential, shoot and root dry weight, the K^+/Na^+ ratio, and the contents of photosynthetic pigments (chlorophylls a and b, carotenoids), thus indicating a positive role of hormone application.

In a study conducted by Shahba et al. (2010), the effect of salicylic acid (0.5, 1.0, and 1.5 mM) on tomato germination, growth, and photosynthetic pigments under different salt stress levels (25, 50, 75, and 100 mM) were investigated. The germination rate decreased with increasing salinity. At low salt concentrations (25 and 50 mM), SA application leads to a decrease in germination percentage.

Studies concerning the effect of salicylic acid on physiological parameters under salinity were also carried out in two sunflower cultivars (Hisun-33 and SF-187) (Noreen et al. 2009, 2011). After foliar application of SA (10, 200, and 300 mM) under salt stress conditions (120 mM), an increase was observed in the levels of pigments (chlorophylls a and b), activities of antioxidant enzymes (superoxide

dismutase, catalase, and peroxidase), growth, leaf turgor potential, and leaf and root Ca^{2+} concentrations. Among applied SA levels, 200 and 300 mM were relatively more effective than the level of 100 mM in improving chlorophyll content, leaf turgor potential, and leaf and root Ca^{2+} concentrations.

Karlidag and coworkers (2009) assessed the impact of different applications of SA (0.25, 0.50, 1.0 mM) on growth and yield parameters of strawberry under salt stress (35 mM NaCl) and greenhouse conditions. In stressed plants treated with SA, an increase was demonstrated in root and shoot fresh and dry weight, levels of chlorophyll, and all nutrients (N, P, K, Ca, Mg, Zn, Cu, Fe) in leaves and roots, with the highest values at 1.0 mM salicylic acid.

Recent available literature includes studies on less popular plants in the context of NaCl and SA treatment, such as fenugreek (Babar et al. 2014), vetch (Namdari and Baghbani 2013), violet (Hussain et al. 2011), garden cress (Habibi and Abdoli 2013), or basil (Delavari Parizi et al. 2011; Mohammadzadeh et al. 2013).

In their study Babar et al. (2014) demonstrated that foliar application of salicylic acid (100 mg/L) on two cultivars (Deli Kabul and Kasuri) of fenugreek under salt stress conditions (100 mM NaCl) mitigated reduction in growth biomass, gas exchange attributes, and chlorophyll content.

Low seed germination and seedling emergence are the main problems in saline areas. Seed priming treatment with SA (0.5 mM) was tested on the early growth of smooth vetch under different levels (50, 100, 150 mM NaCl) of salt stress (Namdari and Baghbani 2013). After SA application a significant emergence and growth of 18-day-old seedlings were observed, which was associated with lesser oxidative damage. In addition, the activity of ascorbate peroxidase, superoxide dismutase, and glutathione reductase was increased, while catalase activity was not dependent on the SA application. To investigate seed germination rates, the seedling vigor index, and growth parameters of garden cress, three levels of NaCl (50, 100, and 150 mM) and SA (500, 1000, and 1500 μM) were applied (Habibi and Abdoli 2013). Application of different levels of salicylic acid did not significantly increase seedling fresh weight and length. At a low SA concentration (500 μM), the germination percentage was increased, but with the increasing SA concentration, germination percentage was decreased.

The role of exogenous application of salicylic acid (30 mg/L) under salinity conditions (5 dS/m) in violet plants was investigated using three treatments: the control (unstressed plants), NaCl, and NaCl + SA (Hussain et al. 2011). In the combination with NaCl + SA, a strong reduction was observed in the accumulation of Na^+, K^+, Ca^{2+}, Cl^-, glycine betaine, and total soluble sugars, and an increase was recorded in plant and root lengths and plant fresh and dry weights.

Mohammadzadeh et al. (2013) tested the response of four basil cultivars (Shandabad Tabriz, Shiraz, Isfahan, and Sabzevar) to the application of salicylic acid (0.5 mM) under salt stress conditions (50, 100, and 150 mM NaCl) by analyzing morphological parameters. After SA addition all traits (root and stem lengths, stem diameter, the number of branches and leaves, and leaf area) were positively affected, except for internode length and root fresh weight/shoot fresh weight. In turn, Delavari Parizi et al. (2011) investigated the effect of SA (0.01, 0.1, 0.5, 1.0, 1.5, 2.0, and 3.0 MM) and salinity (100 and 200 mM NaCl) on Na^+ and K^+ contents

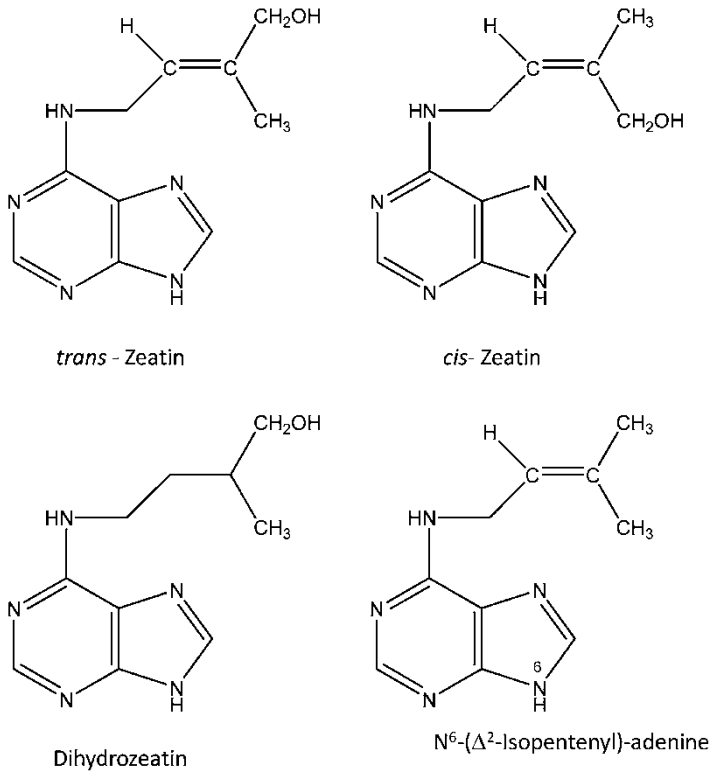

Fig. 4.7 Chemical structures of some naturally occurring cytokinins

in basil plants. In plants treated with both levels of NaCl, the level of the Na^+ ion increased and that of K^+ decreased. Among the SA levels applied, the concentrations of 0.01 and 0.1 mM were selected for further analyses. When basil plants were treated with SA (0.01 and 0.1 mM), Na^+ concentration decreased and K^+ concentration increased in their experiment.

4.5.6 Cytokinins

4.5.6.1 Cytokinins in Biological Processes

Cytokinins (CKs) are phytohormones, which regulate numerous biological processes, including responses to environmental stresses, via a complex network of CK signaling (Ha et al. 2012). Cytokinins found in plants are adenine derivatives substituted at the N6-position with either an isoprenoid or an aromatic side chain (Fig. 4.7). In both groups, there are small variations in side-chain structure, such as the absence or presence of hydroxyl groups and their stereoisomeric position (Sakakibara 2006; Dolezal et al. 2007). Cytokinins play an important role in the development and

growth of both root and shoot systems. Processes regulated by cytokinins include senescence, apical dominance, branching, flowering, and seed germination. These molecules also regulate responses to various stimuli such as water and nutrient availability, light conditions, and infection (Werner and Schmülling 2009).

Cytokinins are major leaf senescence-inhibiting hormones, since senescence is delayed after the exogenous application or the overproduction of CKs in transgenic plants (Cowan et al. 2005; Rivero et al. 2007; Ghanem et al. 2011). Also cytokinins are especially important in regulating cell division and expansion (Kurakawa et al. 2007; Argueso et al. 2009) and delaying senescence (Guo and Gan 2007).

Studies conducted by Javed and coworkers (2011a, b) indicated that an increase in the rice grain yield, 1000-grain weight, and filled-grain percentage are associated with an increase in the contents of starch, sucrose, glucose, and fructose in grain caused by the exogenous application of indole-3-acetic acid and kinetin. Salinity-induced leaf growth inhibition and premature senescence were correlated with decreased root, xylem sap, and leaf bioactive CK concentrations (Albacete et al. 2008; Ghanem et al. 2010). Root-synthesized CKs may regulate shoot responses under salinity, conferring better growth and fruit yield and increasing shoot xylem CK concentrations (Albacete et al. 2009).

4.5.6.2 Exogenous Application of Cytokinins Under Salinity Conditions

The significance of endogenous cytokinin alteration for growth and development of salt-stressed maize and pea plants (50, 100, 150 mM NaCl) was investigated by Atanassova et al. (1996). Zeatin and isopentenyl adenine and their ribosides were measured in roots and leaves under control and salinity conditions. The high concentration of cytokinins may be the decisive factor influencing plants during early formation and flowering at different stress conditions.

Ghanem et al. (2011) tested root-synthesized cytokinins and whether specific root-localized transgenic *IPT* (a key enzyme for cytokinin biosynthesis) gene expression could substantially improve tomato (*Solanum lycopersicum* L.) plant growth and yield under salinity (100 mM NaCl). Research showed that a transient root *IPT* induction increased root, xylem sap, and leaf bioactive cytokinin concentrations two- to threefold without shoot *IPT* gene expression. In addition, the induced root IPT gene expression increased root-to-shoot CK transport improving salt tolerance by increasing vegetative and fruit growth and also delaying leaf senescence and maintaining stomatal conductance. This approach may be applied in the cultivation of other crops.

4.5.7 Auxins

4.5.7.1 Role Auxins in Biological Processes

Indole-3-acetic acid (IAA, Fig. 4.8) is the most frequently found natural auxin and plays a major role in plant morphogenesis, including tropistic growth, root patterning, vascular tissue differentiation, auxiliary bud formation, and flower organ

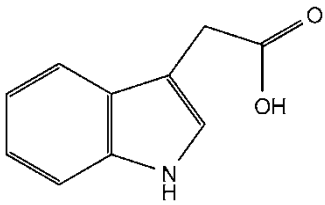

Fig. 4.8 Chemical structure of indole-3-acetic acid

development (Hamdia and Shaddad 2010, Zhao 2010, Javid et al. 2011a, b; Jung and Park 2011; Mano and Nemoto 2012; Rosquete et al. 2012; Nakhooda et al. 2012; Du et al. 2013; Sauer et al. 2013). IAA is produced in meristematic tissues through tryptophan-dependent and tryptophan-independent biosynthetic pathways (Kazan 2013). Auxin signaling interacts with the signaling pathways of all other known plant hormones and is also proposed to have a role in regulating the communication both within the same plant (root-shoot communication) and between different plants (root-root communication) (Kazan 2013).

4.5.7.2 Exogenous Application of Auxins Under Salinity

Akbari et al. (2007) showed that the application of auxin increased hypocotyl length, seedling fresh and dry weights, and hypocotyl dry weight in three wheat cultivars under salinity conditions. A similar study conducted by Khorasani and coworkers (2015) showed that auxin increased plumule length, seedling fresh and dry weight, and plumule dry weight, but did not influence seed germination percentage or radicle length. Many auxin-signaling genes are responsive to stress responses (Javid et al. 2011a, b; Du et al. 2013).

Auxin also plays a role in seed germination, a crucial stage in the life history of plants, and salt tolerance during germination (Birgit et al. 2005; Akbari et al. 2007). In a study conducted by Jung and Park (2011), transgenic plants overexpressing microRNA160 (miR160) or its target transcription factor gene *Auxin Response Factor10* (*ARF10*) were used to support the contribution of auxin to seed germination. To verify the notion that auxin plays a negative regulatory role in seed germination under high salinity, the transgenic plants overexpressing the *YUCCA3* (*YUC3*) gene, which encodes an auxin biosynthetic enzyme, were generated. It was observed that seed germination of the *YUC3*-overexpressing plants was more sensitive to high salinity. Based on these studies, it was found that auxin signals are incorporated into the NTM2-mediated salt signal transduction pathway in modulation of seed germination under high salinity.

In a similar study, seeds of three wheat cultivars (Mahdavi, Pishtaz, Shiraz) were used to investigate the effects of different salinity levels (0, −0.6, −1.2 MPa) and auxin concentrations (0, 1, 2 mg L^{-1}) on their germination rate, radical and hypocotyl lengths, seedling fresh and dry weight, and radical and hypocotyl dry weight (Akbari et al. 2007). Positive effects of auxin were shown in the case of some germination traits (hypocotyl length, seedling fresh and dry weight, and hypocotyl dry weight). In addition, the IAA hormone did not influence seed germination percentage.

Khorasani et al. (2015) tested three wheat cultivars (Sepahan, c-84-8, and c-83-1) in terms of the effects of salt stress (0, 4, and 8 dSm-1 NaCl) and auxin (0, 0.5, and 1 ppm) on germination factors. Results showed that an increasing NaCl concentration reduced germination percentage, radicle length, plumule length, seedling fresh and dry weight, and plumule dry weight. In turn, auxin increased plumule length, seedling fresh and dry weight, and plumule dry weight, but did not influence seed germination percentage or radicle length. In a similar study, the effects of different levels of salinity (0, 40, 80, and 120 mM of NaCl) and indole-3-acetic acid on the early growth and germination of wheat seedlings were investigated (Abdoli et al. 2013). Application of IAA at the cell division stage of grain growth caused a significant increase in seedling growth parameters under tested salinity levels. The interaction between the application of IAA and salinity levels significantly affected the final germination percentage.

One method of mitigating the effects of stress is exogenous application of phytohormones (alone or in mixture). A foliar application of growth regulators as a commercial mixture with gibberellins, auxins, and cytokinins (0 mL L^{-1} – control – and 5 mL L^{-1} at the beginning of flowering (BF) and 5 mL L^{-1} at the vegetative stage + the beginning of flowering) on bean under different salt stress levels (0, 1000, and 2000 ppm of NaCl) was used (Torres-Gracia et al. 2009). The analysis showed that salinity tolerance could be induced by the addition of exogenous growth regulators such as gibberellins, auxins, and cytokinins, which inhibited the synthesis of ABA and ethylene, increased fruit size, and delayed leaf senescence. Darwesh (2014) also applied SA (at 400 ppm once a week) and IAA (30 ppm once a week) during two successive seasons on date palm *Phoenix dactylifera* L. cv. Bartomouda exposed to salt stress at 14000 ppm. The treatments with SA and IAA enhanced most growth parameters, i.e., plant height, leaf number, fresh and dry weight of leaves, and plant tolerance to salinity.

Kim et al. (2006) investigated the effects of soaking in two plant hormones, gibberellic acid (GA_3) (10 μM) and indole-3-acetic acid (IAA) (20 μM), on dehulled rice seeds exposed to low NaCl (20 mM) stress. The IAA- and GA_3-soaked rice seeds showed relatively high amounts of endogenous IAA under salt stress; in particular, the IAA content increased more markedly in GA_3-soaked rice seeds than in IAA-soaked rice seeds.

The influence of salt stress on plant hormones, ABA and auxin (IAA, indole-3-butyric acid – IBA), in two maize cultivars differing in salt resistance (a salt-resistant hybrid and a sensitive hybrid) was tested by Zorb and coworkers (2013). Results showed that the salt-resistant maize significantly increased IBA concentrations in growing leaves and maintained IAA concentration in roots.

4.5.8 Gibberellins

4.5.8.1 Biosynthesis of Gibberellins and Their Role in Biological Processes

Gibberellins (GAs) are a class of phytohormones, commonly known as gibberellic acids, that impact various aspects of plant growth and development (Fleet and Sun 2005; Gupta and Chakrabarty 2013). Gibberellic acid plays a key role in seed

germination through coordinating interactions with other growth hormones and external signals. These compounds are also produced by some species of lower plants, fungi, and bacteria. Gibberellic acids are tetracyclic diterpenoid compounds (Fig. 4.9) and stimulate trigger transitions from the meristem to shoot growth, juvenile to adult leaf stage, and vegetative to flowering and grain development along with the interaction of different environmental stress factors (Verelst et al. 2010; Gupta and Chakrabarty 2013; Colebrook et al. 2014). Their names – gibberellin A_1, gibberellin A_2, and gibberellin A_3 – are derived from the fungus *Gibberella fujikuroi*, from which they have been isolated (Kawaide 2006). GAs are synthesized via the terpenoid pathway requiring three enzymes, terpene synthase, cytochrome P450 monooxygenase, and 2-oxoglutarate-dependent dioxygenases, located, respectively, in plastids, the endomembrane system, and the cytosol (Hedden and Thomas 2012).

4.5.8.2 Exogenous Application of Gibberellins Under Salinity

Tuna and coworkers (2008) showed that foliar application of gibberellin A_3 (50 ppm) counteracted some of the adverse effects of NaCl (100 mM) salinity with the accumulation of proline, which maintained membrane permeability and increased macro- and micronutrient levels.

The effect of salinity on rice and the role of GA_3 application (150 ppm) were observed in two salt-tolerant rice cultivars (Pokkali and MR219) grown at various salt concentrations (0, 50, 100, 150, and 200 mM) in a greenhouse experiment (Misratia et al. 2013). Results showed the best salinity alleviating the role of GA_3 in the case of moderate salinity stress at 50 and 100 mM NaCl.

In a similar study, the role of gibberellic acid was tested on two wheat cultivars (Sohag 3 and Giza 168) in improving their salt stress tolerance (Shaddad et al. 2013). The plants were grown under different salt concentrations (0.0, 50, 100, 150, 200 mM) and then treated with 100 ppm GA_3. The hormonal supplementation alleviated salt stress as observed from the increase in protein contents in the different organs, improved growth parameters, contents of photosynthetic pigments, and consequently yields of two wheat cultivars (Sohag 3, sensitive to salinity, and Giza 168 – tolerant).

Ghodrat and Rousta (2012) investigated the role of exogenous gibberellic acid (0, 1.5, 2.5, and 5 mg L^{-1}) in germination and growth of corn under different levels of salt stress (0, 5, 10, 12, and 15 dsm^{-1}). Results showed that priming with low concentrations of GA_3 had no effect on seed germination; however, in some concentrations GA_3 could increase shoot and root lengths (the most important parameters for salt stress), dry weight, fresh weight, and tissue water content. Based on these experiments, an appropriate concentration of gibberellic acid may be suggested which has the greatest effect on growth parameters.

Low concentrations of gibberellic acid (5 μM) or spermine (50 μM) were used in tests on mung bean (*Vigna radiata* L. Wilczek) under different NaCl contents (25, 50, and 100 mM) (Ghosh et al. 2015). The combined effect of salinity and low GA_3 concentration in the test plants showed a significant alteration (an increase in seed-

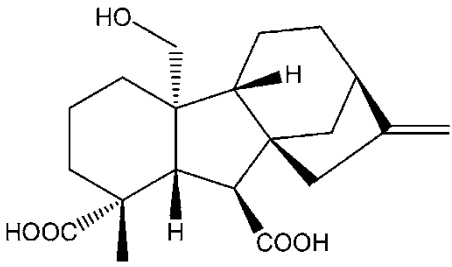

Fig. 4.9 Chemical structure of gibberellic acid

ling elongation, biomass production, chlorophyll content, and a decrease in all antioxidant enzymatic activities) and may play an important role in salt uptake.

Maggio et al. (2010) tested effects of gibberellic acid (0 and 100 mg L^{-1}) application on tomato exposed to three levels of salinity (28, 55, 88 mM Na and 55, 111, 177 mM Cl). GA_3 treatment reduced stomatal resistance and enhanced plant water use (by 30 %) at low salinity. Results showed that exogenous applications of GA_3 may compensate for the salt-induced growth deficiency and consequently facilitate plant adaptation to a saline environment.

The effects of gibberellic acid (5 ml of 10^{-5} M hormone for each plant) on growth, physiology, and yield of mustard (*Brassica juncea* L. Czern and Coss) cv. Varuna under salinity conditions (25 and 50 mM NaCl) were studied by Shah (2007). Hormone treatment mitigated the adverse effects of salt stress, with a greater amelioration response in the case of 50 mM NaCl.

In a hydroponic experiment, it is indicated that supplementing exogenous gibberellic acid – GA_3 (100 ppm) – plays an important role in enhancing nutrient uptake and in counteracting growth inhibition of sugarcane under salt stress (EC 0 and 9 dSm^{-1}) (Shomeili et al. 2011). In a similar study, exogenous GA_3 application in soybean significantly promoted plant length and plant fresh/dry biomass, while it was markedly hindered by NaCl-induced salt stress (Hamayun et al. 2010). Phytohormonal analysis of soybean showed that the level of bioactive gibberellins (GA_1 and GA_4) and jasmonic acid increased in GA_3-treated plants, while the endogenous abscisic acid and salicylic acid contents declined under the same conditions. Also Abdel-Hamid and Mohamed (2014) demonstrated that the application of GA_3 (0 and 100 μM) counteracts salinity (0, 100, and 300 mM) by improving membrane permeability and nutrient levels in leaves and also induced physiochemical changes responsible for the induction of salt tolerance in two cultivars of barley. It is noteworthy that exogenous applications of GA_3 could be a useful tool in promoting seedling growth and establishment under salt stress conditions.

Seed germination and establishment are the most sensitive stages to abiotic stresses (Patade et al. 2011; Ansari et al. 2012). The effect of salicylic acid (50 ppm) and gibberellin (50 ppm) on enzymatic activity and germination characteristics of wheat seeds under salt stress (at osmotic potentials of 0 (as control), – 4, –8, –12, and –16 bar) was tested by Tabatabaei (2013). Hormone priming improved germination percentage, germination index, normality seedling percentage, and seedling length and also increased catalase and ascorbate peroxidase levels as compared to the untreated seeds.

4.5.9 Nitric Oxide

4.5.9.1 Role of Nitric Oxide in Biological Processes

Nitric oxide (NO) is a short-life bioactive gaseous molecule in plants and plays a central role in a variety of physiological processes including germination, senescence, flowering, ripening of fruits, and response to various abiotic and biotic stresses, such as bacterial disease (Delledonne et al. 1998), drought (Gracia-Mata and Lamatina 2001), high temperature (Hasanuzzaman et al. 2012), salinity (Ruan et al. 2002; Uchida et al. 2002; Ruan et al. 2004a; Zheng et al. 2010; Manai et al. 2014), and UV radiation (Mackerness et al. 2001). Studies demonstrated that NO promoted the accumulation of proline and the probable protective effect of NO against salt-induced oxidative damage to wheat seedlings, but poorly documented how NO regulates proline accumulation and its role in the ABA pathway underlying proline accumulation under salinity conditions.

Ruan et al. (2004b) investigated the relationship of NO and ABA in the process of proline accumulation responding to salt stress. It was shown that NO could activate the synthesis of endogenous ABA and the level of proline was significantly elevated by exogenously supplied ABA in wheat seedling leaves under 150 mmol/L NaCl salt stress after 4 days of treatment.

4.5.9.2 Effect of Exogenous Application of Nitric Oxide Under Salinity

Several studies indicate that the application of exogenous nitric oxide alleviates oxidative stress caused by salt. The protective role of NO treatment was tested in various plants such as tomato, pepper, wheat, barley, rice, maize, soybean, mangrove, or chamomile (Li et al. 2008; Zheng et al. 2010; Fallahi and Khajeh Hosseini 2011; Nalousi et al. 2012; Nasrin et al. 2012; Simaei et al. 2012; Kausar et al. 2013; Ali and Ismail 2014; Chen et al. 2014; Egbichi et al. 2014; Habib and Ashraf 2014; Manai et al. 2014; Kaya et al. 2015; Sanam et al. 2015). In studies conducted by Ali and Ismail (2014), the application of NO (10 μM of sodium nitroprusside) improved tomato fruit quality in the face of salinity (100 mM NaCl) by enhancing the synthesis of health-promoting compounds (phenolic compounds, flavonoids, and alkaloids) in tomato fruits along with significant changes in other quality parameters. In addition, exogenous application of NO improves plant K^+ contents, while decreasing Na^+ concentration, thereby maintaining the K^+/Na^+ ratio in plants (Zheng et al. 2009; Chen et al. 2013), and enhances chlorophyll content; activities of CAT, POD, and SOD; and levels of soluble proteins and total free proline in the salt-stressed plants (Kausar et al. 2013).

The protective role of nitric oxide (by spraying with different levels of sodium nitroprusside, SNP – 0.05, 0.10, and 0.15 mM – as the NO source) in wheat plants exposed to salinity (150 mM) was tested by Kausar et al. (2013). The NO treatment of wheat enhanced the activities of antioxidant enzymes (superoxide dismutase,

peroxidase, and catalase) and increased levels of proline, chlorophyll, and soluble proteins, showing a protective role against salt-induced oxidative damage. In addition, growth and yield parameters were improved only in unstressed plants.

To investigate the alleviating role of NO in barley under salinity conditions, Li and coworkers (2008) tested three treatments (50 μM SNP, 50 mM NaCl, 50 μM SNP + 50 mM NaCl) compared to the control. Similarly to previous studies, they found that a simultaneous application of SNP and NaCl increased the activities of antioxidant enzymes, protecting plants against salt stress-induced negative changes. Moreover, NO induced an increase of ferritin accumulation to chelate larger numbers of ferrous ions. Sanam et al. (2015) also observed an increase in activities of enzymes and protein contents when two rice cultivars (Khazar and Goohar) were treated with 50 μM SNP + 50 mM NaCl. Habib and Ashraf (2014) also tested plants of four rice cultivars (KS-282, IRRI-6, Shaheen Basmati, and Basmati PB-95) supplemented with NaCl (80 mM) and three levels of NO (0, 0.1, 0.2 mM). Salinity caused a significant increase in leaf water contents and osmotic potentials and a decrease in leaf turgor potential and relative water content. After seed treatment with nitric oxide, leaf osmotic and water potentials and shoot and root Cl^- and Na^+ concentrations decreased with a simultaneous increase in leaf relative water content, leaf turgor potential K^+ and Ca^{2+} contents, and K^+/Na^+ ratio in shoots and roots of rice cultivars under salinity conditions.

Soybean, one of the most important plants used as a source of protein and oil in its seeds, is moderately sensitive to salinity. High salt stress significantly reduces seed germination, seedling growth, and yield. An effective action in mitigating the effect of salt stress is provided by nitric oxide application, sometimes in combination with other phytohormones (Egbichi et al. 2014; Simaei et al. 2012). In their study Egbichi et al. (2014) tested six experimental variants: the control, 10 μM 2,2'(hydroxynitrosohydrazono)bis-ethanimine – DETA/NO as a donor of NO – 10 μM DETA, 80 mM NaCl, 10 μM DETA/NO + 80 mM NaCl, and 10 μM DETA + 80 mM NaCl. Results showed that long-term salinity over a 16-day period negatively affected soybean plants, while supplementation with DETA/NO increased shoot, root, and nodule weights, nodule number, as well as the enzymatic activity of ascorbate peroxidase.

Simaei and coworkers (2011; 2012) showed a protective role of NO (100 μM SNP) and SA (100 μM) in soybean under salinity conditions (100 mM NaCl). After the application of phytohormones + SNP, the activities of polyphenol oxidase and phenylalanine ammonia lyase increased, thus reducing the damaging effects of salt stress by enhancing the activity of antioxidative systems.

Kaya et al. (2015) tested the effect of NO + thiourea (TU) treatment (two levels of NO + TU: 3 + 400 mg/L and 6 + 500 mg/L) as seed soaking treatment and foliar application in two maize cultivars (Apex 836 and Dk 5783) under salinity conditions (100 mM NaCl). Seed treatment was more effective than foliar application in terms of improvement in fresh weight. In both types of treatment, N and P contents increased and leaf Na^+ contents decreased. Results indicate that exogenous application of NO + TU in maize plants should increase resistance to salinity by improving plant growth.

The role of exogenous application of SNP in ROS production and lipid peroxidation in salt-treated mangrove (as the species with high salinity tolerance) was demonstrated by Chen et al. (2014). In the first stage of the experiment, different levels of SNP (0, 10, 50, 100, 500, and 5000 μM) and NaCl (0, 100, 350, and 500 mM) were tested to select appropriate concentrations. In the second stage, three combinations in three replicates were tested: (1) 100 μM SNP, (2) 350 mM NaCl, and (3) 100 μM SNP + 350 mM NaCl. Results indicated the protective role of NO against oxidative damage in leaves of mangrove by reducing hydrogen peroxide levels, lipid peroxidation, and increase in reduced glutathione and polyphenol contents.

In another study Nalousi et al. (2012) investigated the impact of exogenous SNP as the NO donor (0, 25, 50, and 75 mM SNP) in germination and seedling growth of bell pepper under salinity conditions (0, 4, 6, and 8 ms/cm^3 NaCl). As in the previous research, the presence of NO decreased lipid peroxidation in plant leaves and increased the activities of superoxide dismutases and peroxides. In addition, seed germination was promoted at concentrations between 0.2 and 0.8 mM SNP.

Research conducted by Guo et al. (2008) concerned *Kosteletzkya virginica* – a native species in the southeastern USA – treated with SNP (0.06 mM) and NaCl (100, 200, 300, and 400 mM NaCl) in order to verify their impact on dry weight, antioxidant enzymatic activities, proline accumulation, lipid peroxidation, and distribution of sodium in plants. After SNP application under salinity conditions, the researchers recorded an increase in dry weight, catalase, peroxidase, and superoxide dismutase activities and proline accumulation and a lower ratio of Na^+/K^+, which might protect plants against oxidative membrane damage and translocation of Na^+ from roots to shoots.

Tests with various doses of nitrogen (0, 120, 240, and 360 kg/ha urea) on wheat (cv. Gascogen) under salinity conditions (0, 121.5, 243, 364.5, and 486 mM NaCl) showed that nitrogen at low levels of salt has a positive or negligible effect on germination and growth factors, while at high salinity levels, its effect was negative (Fallahi and Khajeh Hosseini 2011).

4.5.10 Strigolactones, Their Hormonal Functions, and Ameliorative Role Under Salt Stress Conditions

Strigolactones (SLs, Fig. 4.10) are a small group of sesquiterpene lactones produced from carotenoid precursors mainly in plant roots initially identified as seed germination stimulants for parasitic weeds (Dun et al. 2009; Marzec et al. 2013; Cavar et al. 2014; Van Ha et al. 2014). SLs are also known as a chemical signal between plant roots and arbuscular mycorrhizal fungi (AMF) (Akiyama et al. 2005; Lopez-Raez et al. 2011; Aroca et al. 2013); their hormonal functions likely predate the evolution of the signaling role in the rhizosphere (Delaux et al. 2012). In addition, SLs contribute to a number of crucial processes in plant growth and development, namely, in the shaping of plant architecture, which is of major agronomic

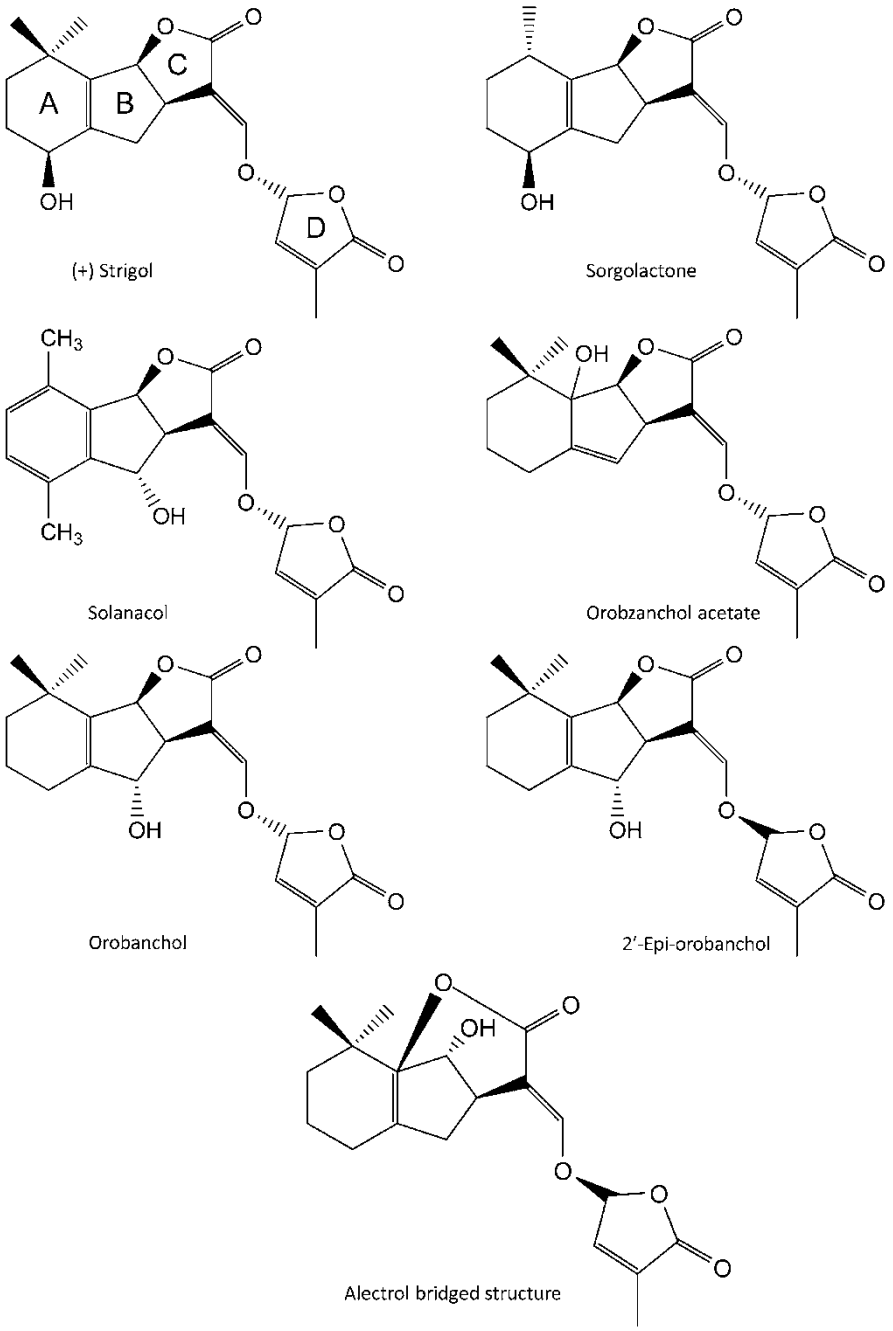

Fig. 4.10 Chemical structure of several naturally occurring strigolactones

importance, as it is closely related to the potential grain yield and adaption to fluctuating environmental conditions. SLs are secondary metabolites derived from a carotenoid precursor (Matusova et al. 2005). To date, more than 15 natural SLs have been characterized from various plant species, and they all share a common four-cycle skeleton (A, B, C, and D), with cycles A and B bearing various substituents and cycles C and D being lactone heterocycles connected by an ether-enol bond (Fig. 4.10) (Liu et al. 2013). Plants produce so small amounts of SLs in the roots and lower part of shoots that they are very difficult to determine (Xie et al. 2010).

Arbuscular mycorrhizal (AM) symbiosis can alleviate salt stress in plants. Aroca and coworkers (2013) investigated the effects of salinity (0, 40, and 80 mM NaCl) on lettuce plant performance and production of strigolactones and assessed its influence on mycorrhizal root colonization under three different salt concentrations. The results show a correlation between strigolactone production, ABA content, AM root colonization, and salinity level, which suggests that AM symbiosis alleviates salt stress by altering the hormonal profiles and affecting physiology in the host plant.

Recent research suggests that genetic modulation of SL content/response could provide a new, potential approach to reduce the negative impact of salt stress on crop productivity (Van Ha et al. 2014).

4.6 Conclusion and Future Prospects

Salt stress is associated with decreases in auxin, cytokinin, gibberellin, and SA in the plant tissues and an increase in ABA and JA. These changes in phytohormone are thought to be an initial process controlling growth reduction due to salinity. Therefore, reduction in plant growth under abiotic stress can be mitigated by exogenous application of plant growth regulators. Different strategies are tested to maximize plant growth under salt stress conditions. One of them is to produce salt-tolerant genotypes of different crops which is much technical and time consuming. In contrast, application of plant growth regulators (plant hormones, minerals, amino acids, quaternary ammonium compounds, polyamines, and vitamins) is economic and effective to improve plant tolerance to salinity. Future studies are to be designed keeping real-world scenario in mind, where more than one biotic and/or abiotic stress may coexist. Understanding complex cross talk between multiple stresses and phytohormones will help to shape future crop management and genotype improvement strategies.

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Chapter 5
Roles of Phytohormones in Morphological and Anatomical Responses of Plants to Flooding Stress

Zhongyuan Hu, Xiaohua Qi, Mingfang Zhang, Xuehao Chen, and Mikio Nakazono

Abstract Waterlogging and submergence stress, which are caused by flooding, often produce injurious effects on plant growth. Plants have some adaptive mechanisms, such as aerenchyma formation, adventitious root formation, and control of shoot elongation, to avoid oxygen deficiency under flooded conditions. Ethylene is involved in most of the morphological and anatomical responses of plants to flooding; thus, this phytohormone is a key factor for plant growth control under flooded conditions. The processes of some morphological responses are controlled by different interactions between ethylene and other phytohormones [such as auxin, gibberellin (GA), and abscisic acid (ABA)]. Ethylene and auxin act synergistically in the control of adventitious root formation. On the other hand, ethylene acts synergistically with GA and antagonistically with ABA in the control of adventitious root formation and submergence-stimulated shoot elongation. Here, we summarize how the morphological and anatomical responses of plants to flooding stress are controlled by ethylene or the interplay between ethylene and other phytohormones.

Keywords Abscisic acid • Anatomical response-Auxin- • Ethylene-Flooding- • Gibberellin • Hormone interplay-Submergence- • Waterlogging

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5.1 Introduction

Water is necessary for plant growth, but excess water, which is caused by flooding, may lead to injurious effects on plant growth. During flooding, plants are often submerged or waterlogged. Submergence is defined as a condition in which plants stand in water with at least part of the terminal shoot remains above the water or are completely covered with water (Catling 1992). Waterlogging is defined as a condition in which only plant roots are exposed to excess water, which limits gas diffusion in soil (Armstrong 1980; Setter and Waters 2003). Oxygen diffusion in water is approximately 10,000 times slower than in air (Armstrong 1980); thus, oxygen deficiency is induced by submergence or waterlogging and may cause damage to plants (Colmer and Voesenek 2009). Plants have a variety of adaptive mechanisms to avoid oxygen deficiency under submerged or waterlogged conditions, such as aerenchyma formation, adventitious root (AR) formation, and control of shoot (e.g., leaf, petiole, or internode) elongation.

Aerenchyma, which consists of longitudinally interconnected gas spaces, contributes to the transport of gases (O_2, CO_2, ethylene, and methane) between and within the aerial and submerged parts (Jackson and Armstrong 1999; Colmer 2003; Evans 2003; Colmer et al. 2006). Aerenchyma can be classified into two types, primary aerenchyma and secondary aerenchyma (Jackson and Armstrong 1999; Yamauchi et al. 2013; Takahashi et al. 2014). Primary aerenchyma and secondary aerenchyma form in primary tissues and secondary tissues, respectively. Primary aerenchyma is formed by schizogeny, lysigeny, or a combination thereof (Justin and Armstrong 1987; Jackson and Armstrong 1999; Seago et al. 2005; Takahashi et al. 2014). Schizogenous aerenchyma is formed by cell separation and expansion of existing spaces by differential cell divisions and/or cell expansion in particular directions (Takahashi et al. 2014). However, to date, little is known about the molecular mechanism of schizogenous aerenchyma formation. On the other hand, lysigenous aerenchyma is formed by spatially selective programmed death in parenchyma tissues such as the root cortex (Fig. 5.1a, b) (Justin and Armstrong 1987; Seago et al. 2005; Steffens et al. 2011; Voesenek and Bailey-Serres 2015).

During long-term flooding, the primary root system easily deteriorates because of a lack of oxygen, which consequently leads to energy shortage. To replace the root system under flooded conditions, some plants develop ARs, which have more aerenchyma than primary roots, from the hypocotyl or mesocotyl, and the basal stem regions (Fig. 5.2a, b) (Visser and Voesenek 2004). Consequently, ARs enhance gas exchange near the water surface and greatly contribute to plant tolerance of poorly aerated soils.

Aerenchyma and AR formation facilitates plant avoidance of oxygen deficiency under waterlogged or partially submerged conditions, but many plants are still sensitive to complete submergence. Some plant species have evolved the ability to rapidly develop elongation of shoots (e.g., leaves, petioles, or internodes) under complete submergence so that the shoots can rise above the water surface. This is an escape strategy [i.e., low-oxygen escape syndrome (LOES)] and enables these

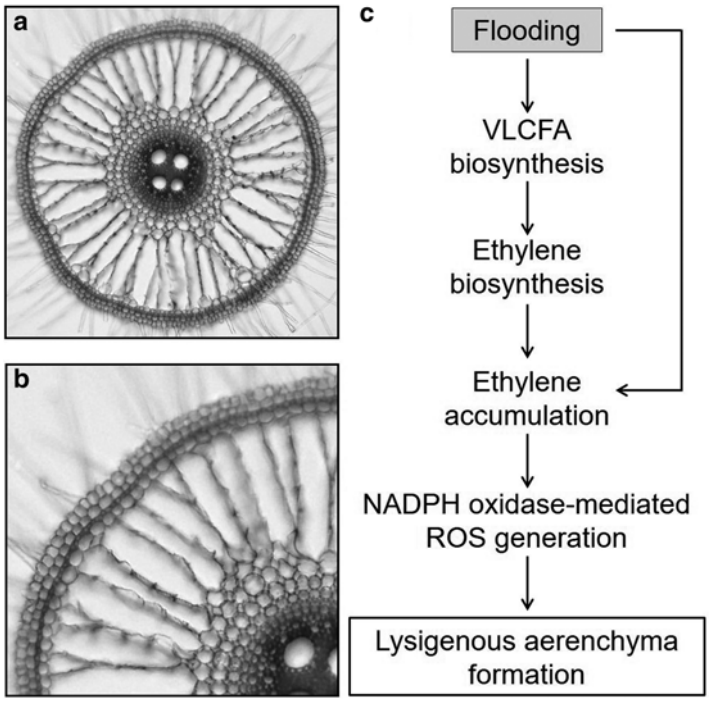

Fig. 5.1 Control of lysigenous aerenchyma formation in roots of gramineous plants (e.g., rice) by ethylene under flooded conditions. (**a**) Cross section of the root of rice seedling grown under flooded conditions. Airspaces in the root indicate lysigenous aerenchyma. (**b**) Higher magnification of the root cross section in (**a**). (**c**) Model of the ethylene-controlled lysigenous aerenchyma formation in rice roots under flooded conditions. Under flooded conditions (i.e., stagnant deoxygenated conditions in waterlogged soil), very long-chain fatty acid (VLCFA) biosynthesis is enhanced in roots. The increased VLCFAs promote ethylene biosynthesis, which results in elevated levels of ethylene, followed by enhancement of NADPH oxidase-mediated reactive oxygen species (ROS) generation. Higher accumulation of ROS activates lysigenous aerenchyma formation by programmed cell death and lysis of the cortical cells

plants to maintain gas exchange between the aerial and underwater parts and to reestablish aerial photosynthesis (Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009). The escape strategy is effective under relatively shallow, long-term flooded conditions (Voesenek et al. 2004; Bailey-Serres and Voesenek 2008). By contrast, some plant species have another strategy, in which shoot elongation is suppressed to avoid unnecessarily losing energy for 1–2 weeks under complete submergence; thus, the plants can restart their growth during desubmergence using preserved carbohydrates. This is a quiescence strategy [i.e., low-oxygen quiescence syndrome (LOQS)] (Colmer and Voesenek 2009). The quiescence strategy is effective under relatively deep, short-term flooded conditions (Voesenek et al. 2004; Bailey-Serres and Voesenek 2008).

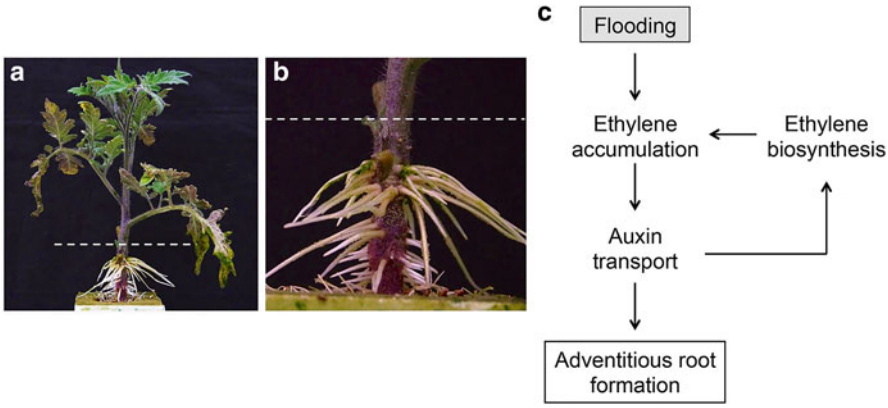

Fig. 5.2 Control of adventitious root (AR) formation in plants (e.g., tomato) by the interplay between auxin and ethylene under flooded conditions. (**a**) AR formation in a tomato plant under flooded conditions. An aerobically grown 28-day-old plant was further grown under flooded conditions for 10 days. (**b**) Higher magnification of the hypocotyl part in (**a**). The dotted lines indicate the water level used for flooding treatment in (**a**) and (**b**). (**c**) Model of the flooding-stimulated AR formation in tomato plants. Flooding induces the accumulation of endogenous ethylene by entrapment. The entrapped ethylene stimulates transport of auxin, thereby increasing the amount of auxin in the stem. The auxin triggers ethylene biosynthesis, which promotes further ethylene accumulation. The accumulated auxin also promotes AR formation by inducing the growth of initially preformed ARs

It is known that ethylene, a gaseous phytohormone, plays important roles in morphological and anatomical responses of plants to flooded conditions (e.g., aerenchyma and AR formation and control of shoot elongation). Ethylene biosynthesis is accomplished by two main successive enzymatic reactions: conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and conversion of ACC to ethylene by ACC oxidase (ACO) (Yang and Hoffman 1984). Both ACS and ACO activities are enhanced in plant tissues under flooded conditions (He et al. 1996). Thus, in addition to rapid accumulation of ethylene by entrapment in the organs/tissues surrounded by water upon waterlogging or submergence (Visser and Voesenek 2004), the amount of ethylene is further increased by enhancement of ethylene biosynthesis (Van Der Straeten et al. 2001; Sasidharan and Voesenek 2015). In this chapter, we summarize the roles of ethylene and some other phytohormones, including auxin, gibberellin (GA), and abscisic acid (ABA), in morphological and anatomical changes of plants in response to waterlogging or submergence.

5.2 Roles of Phytohormones in Lysigenous Aerenchyma Formation

The gaseous phytohormone ethylene is involved in lysigenous aerenchyma formation in the roots of some plants (Drew et al. 2000; Shiono et al. 2008). Indeed, lysigenous aerenchyma formation was induced by ethylene in the roots of

gramineous plants such as maize (*Zea mays*), rice (*Oryza sativa*), or wheat (*Triticum aestivum*), either when endogenously produced or exogenously applied (Drew et al. 1981; Jackson et al. 1985; Steffens et al. 2011; Yamauchi et al. 2014), and was prevented by inhibitors of ethylene action and perception [e.g., silver ion] or ethylene biosynthesis [e.g., aminoethoxyvinylglycine (AVG)] (Drew et al. 1981; Jackson et al. 1985). These findings indicate that ethylene is involved in the signaling pathway of lysigenous aerenchyma formation. However, treatments with higher concentrations of ethylene or the ethylene perception inhibitor, 1-methylcyclopropene (1-MCP), did not result in any changes of lysigenous aerenchyma formation in the roots of a wetland species, *Juncus effusus* (Visser and Bögemann 2006).

The influence of other phytohormones such as auxin, ABA, GA, and cytokinin on the formation of aerenchyma in maize roots was also investigated (Konings and de Wolf 1984). 1-Naphthalene acetic acid (NAA; a synthetic auxin) and ABA prevented aerenchyma formation in maize roots, although NAA (but not ABA) enhanced ethylene concentration in maize roots. By contrast, GA and kinetin (a synthetic cytokinin) stimulated ethylene production and promoted aerenchyma formation in aerated maize roots (Konings and de Wolf 1984). Subsequently, Justin and Armstrong (1991) reported that aerenchyma formation in maize roots was promoted by low levels of NAA, but was reduced by higher NAA levels. Higher NAA levels inhibited root growth and thus possibly resulted in reduced aerenchyma formation. Hence, it was concluded that low levels of auxin promoted ethylene production and aerenchyma formation in maize roots (Justin and Armstrong 1991).

Recently, Yamauchi et al. (2015) found that concentrations of saturated very long-chain fatty acids (VLCFAs) of 24, 26, and 28 carbons were increased in rice ARs under stagnant deoxygenated conditions, which mimic oxygen-deficient conditions in waterlogged soils. The increased VLCFAs upregulated expression of a gene that encodes ACC synthase-1 (ACS1) and thus increased ethylene accumulation, thereby inducing lysigenous aerenchyma formation in rice roots (Fig. 5.1c). Interestingly, expression levels of *ACS1* and the gene that encodes a fatty acid elongase (CUT1L) were predominantly induced in the outer part of the roots under stagnant deoxygenated conditions. These results indicate that VLCFAs increase ethylene production by promoting ACC biosynthesis in the outer part of roots, which, in turn, induces lysigenous aerenchyma formation in the root cortex of rice under oxygen-deficient conditions (Fig. 5.1c) (Yamauchi et al. 2015).

Reactive oxygen species (ROS) are key signaling factors that stimulate abiotic stress responses in plants (Suzuki et al. 2011) and accumulate during aerenchyma formation in roots (Bouranis et al. 2003; 2006), which indicates that ROS are involved in regulating root aerenchyma formation (Rajhi et al. 2011; Yamauchi et al. 2011). A plasma membrane-located NADPH oxidase [i.e., a respiratory burst oxidase homolog (RBOH)] is considered as a key enzyme for ROS generation during lysigenous aerenchyma formation (Steffens et al. 2011; Yamauchi et al. 2011) (Fig. 5.1c). Thus, the genes related to ROS generation (e.g., *RBOH*) as well as Ca^{2+} signaling and cell wall loosening were upregulated in the cortex during aerenchyma formation in the maize primary root (Rajhi et al. 2011). The upregulated expression of these genes was suppressed by pretreatment of the ethylene perception inhibitor,

1-MCP. On the other hand, there is increasing evidence that plant metallothioneins (MTs) regulate ROS accumulation. For example, rice MT2b and cotton (*Gossypium arboreum*) MT3a have higher antioxidative capacity against hydroxyl radicals than other antioxidants in vitro, and many cysteine residues in MTs are remarkably reactive to oxidizing agents (Coyle et al. 2002; Wong et al. 2004; Xue et al. 2009). During aerenchyma formation in the maize primary root under waterlogged conditions, the *MT* expression was specifically downregulated in the cortex, and the downregulation was also ethylene dependent (Rajhi et al. 2011; Yamauchi et al. 2011). Aerenchyma formation in maize roots under waterlogged conditions was prevented by an NADPH oxidase inhibitor, diphenyleneiodonium (DPI) (Yamauchi et al. 2011). Even under aerobic conditions, the treatment of the maize primary root with ethylene induced aerenchyma formation, which was inhibited by DPI treatment (Takahashi et al. 2015). Similarly, in wheat, treatments on ARs with an ethylene precursor, ACC, under aerobic conditions, which was expected to increase the amount of ethylene in roots, upregulated expression of *RBOH* genes and also induced lysigenous aerenchyma formation. In addition, ACC-induced aerenchyma formation was prevented by DPI pretreatment (Yamauchi et al. 2014). These findings indicate that NADPH oxidase-mediated ROS generation contributes to ethylene-induced aerenchyma formation in roots of some gramineous plants (Fig. 5.1c).

In rice, aerenchyma is constitutively formed in leaf sheaths (Parlanti et al. 2011) and internodes (Steffens et al. 2011), but its formation is further enhanced under submerged conditions. In leaf sheaths, the dependency of aerenchyma formation on ethylene is different between rice genotypes. For example, aerenchyma formation was enhanced in leaf sheaths of the rice varieties "FR13A" and "Arborio Precoce" under submergence. However, it is likely that ethylene controls aerenchyma formation in "Arborio Precoce" but not in "FR13A" (Parlanti et al. 2011). In internodes, ethylene promoted ROS generation and exogenously applied H_2O_2 enhanced aerenchyma formation. Moreover, in rice, a mutant of the gene that encodes the ROS scavenging MT2b, in which ROS generation was expected to increase, further enhanced aerenchyma formation in internodes compared with the wild type (Steffens et al. 2011). Taken together, these findings indicate that ethylene-induced ROS generation is commonly involved in lysigenous aerenchyma formation in leaf sheaths, internodes, and roots of rice, although the mechanism may vary between genotypes.

5.3 Roles of Phytohormones in Adventitious Root Formation

Auxin is the major phytohormone that initiates AR formation, because it is an essential regulator of the redifferentiation of shoot cells into root apical meristems (Verstraeten et al. 2013; Agulló-Antón et al. 2014). The first experimental evidence of the effect of auxin on AR formation was found in sunflower, in which flooding-induced auxin accumulation was observed at the base of the shoot at the onset of AR formation (Phillips 1964). A similar role of auxin in AR formation under flooded conditions was also found in other species, such as box elder (*Acer negundo*)

(Yamamoto and Kozlowski 1987) and tobacco (*Nicotiana tabacum*) (McDonald and Visser 2003). Moreover, treatment with N-1-naphtylphtalamic acid (NPA), an inhibitor of auxin transport, suppressed the flooding-induced AR formation in *Rumex*, tobacco, and tomato (*Solanum lycopersicum*) (Visser et al. 1995; McDonald and Visser 2003; Vidoz et al. 2010).

Ethylene is also involved in AR formation, which was first identified in 1933 (Zimmerman and Hitchcock 1933). Since then, it has been well established that ethylene has a positive effect on AR development and emergence (Drew et al. 1979; Verstraeten et al. 2014). In fact, flooding-induced ethylene accumulation is responsible for AR formation (Wample and Reid 1979; Visser et al. 1996a, b; Vidoz et al. 2010). In deepwater rice, exogenous ethylene treatment promoted AR emergence by enhancing the cell division activity in ARs (Lorbiecke and Sauter 1999). Treatment with the ethylene biosynthesis inhibitor, AVG, resulted in reduction of AR formation in waterlogged tomato plants (Vidoz et al. 2010).

The cross talk between auxin and ethylene is important for AR formation under flooded conditions. In tomato, the auxin-insensitive *diageotropica* (*dgt*) mutant reduced the flooding-induced AR number and abolished ethylene production (Vidoz et al. 2010). Exogenous auxin application increased AR formation on stem cuttings of wild-type tomato plants, but had little or no effect on AR development in the ethylene-insensitive *never ripe* (*nr*) mutant tomato plants (Clark et al. 1999), which indicates that ethylene responsiveness is necessary for the promotive effects of auxin on AR formation (Fig. 5.2c). Furthermore, ethylene affected auxin transport efficiency or sensitivity to auxin in flooded plant tissues (Visser et al. 1996b; Vidoz et al. 2010) (Fig. 5.2c). For example, flooding induced the accumulation of endogenous ethylene rather than auxin in the *Rumex palustris* hypocotyl during flooding-induced AR formation (Visser et al. 1996b). The ethylene-stimulated AR formation in the *R. palustris* hypocotyl was almost completely blocked by treatment with the auxin transport inhibitor NPA, whereas auxin-induced AR formation was not prevented by treatment with the ethylene biosynthesis inhibitor AVG. Indeed, ethylene accumulation led to an increase in sensitivity of the root-forming tissue to auxin, which stimulated AR development (Visser et al. 1996b). In tomato, ethylene stimulated auxin transport efficiency to the flooded hypocotyl. Consequently, auxin accumulation in the stem promoted additional ethylene biosynthesis, which further stimulated auxin flux under flooded conditions (Vidoz et al. 2010) (Fig. 5.2c).

In rice, ethylene coordinates promotion of AR growth and induction of local programmed cell death (PCD) of epidermal cells that overlay the AR primordium under submerged conditions (Steffens and Sauter 2005, 2009; Steffens et al. 2012). The epidermal PCD facilitates emergence of AR and prevents injury to the growing root tip (Mergemann and Sauter 2000). The PCD requires ethylene-stimulated, NADPH oxidase-mediated ROS generation in the epidermal cells; thus, downregulation of the gene that encodes an ROS scavenging-related MT2b induces the epidermal PCD (Steffens and Sauter 2009). The ethylene-promoted AR growth generates mechanical force that is exerted on epidermal cells, which indicates that, in addition to ethylene signaling, this mechanical force may promote specificity of the site where the epidermal PCD occurs (Steffens et al. 2012).

In addition to auxin and ethylene, GA and ABA are involved in AR growth under flooded conditions. In rice, submergence resulted in accumulation of bioactive GA within 3 h (Hoffmann-Benning and Kende 1992). GA acted in a synergistic manner together with ethylene to promote AR initiation and elongation (Steffens et al. 2006), but application of only GA showed little or no effect on AR initiation (Lorbiecke and Sauter 1999; Steffens et al. 2006). Paclobutrazol, a GA biosynthesis inhibitor, inhibited AR elongation but not AR initiation in deepwater rice (Steffens et al. 2006). These results indicate that GA does not affect the initiation of AR but determines the growth rate of emerged roots. On the other hand, ABA content decreased in rice under submergence, and the decrease in ABA levels promoted AR growth (Hoffmann-Benning and Kende 1992; Steffens et al. 2006; Bailey-Serres and Voesenek 2008). Furthermore, exogenous ABA strongly inhibited both ethylene-induced AR initiation and GA-promoted AR elongation (Steffens et al. 2006), which indicates that ABA acts as a potent inhibitor of both GA and ethylene signaling. In addition to AR growth, it is known that the ethylene-induced epidermal PCD is also enhanced by GA and inhibited by ABA in rice (Steffens and Sauter 2005; Steffens et al. 2006).

5.4 Roles of Phytohormones in Control of Shoot Elongation Under Submergence

Although the escape and quiescence strategies for shoot elongation under submergence are opposite strategies, both mechanisms are controlled by ethylene-dependent signaling pathway in *Rumex* and rice. Moreover, these strategies can both exist within the same species or genus.

In *Rumex*, *R. palustris* and *R. acetosa* adopt the escape strategy and the quiescence strategy, respectively, under submerged conditions (Voesenek et al. 2004). Upon submergence, *R. palustris* leaves changed their orientation from horizontal to vertical (so-called hyponastic growth), and there was then rapid elongation of their petioles (Cox et al. 2004, 2006), whereas *R. acetosa* petiole elongation was suppressed under submergence (Pierik et al. 2009). Ethylene was highly accumulated in both species (*R. palustris* and *R. acetosa*) under submergence (Benschop et al. 2005, 2006). In *R. palustris*, the accumulated ethylene stimulated decrease in ABA levels in petioles by repressing ABA biosynthesis and enhancing ABA catabolism. By contrast, in *R. acetosa*, rapid decrease in ABA levels was not observed under submergence (Benschop et al. 2005). Indeed, significant downregulation of expression of the gene that encodes 9-cis-epoxycaotenoid dioxygenase-1 (NCED1), which is a key enzyme for ABA biosynthesis, was observed in *R. palustris* but not *R. acetosa* (Benschop et al. 2005; van Veen et al. 2013). However, upregulation of expression of the gene that encodes ABA-8′-hydroxylase, which is responsible for ABA catabolism (Kushiro et al. 2004; Saika et al. 2007), was observed in both *R. palustris* and *R. acetosa* (van Veen et al. 2013). This indicates that, despite induction

of expression of the ABA catabolism gene, the controlled expression of ABA biosynthesis genes (e.g., *NCED1*) contributes to the maintenance of higher ABA levels in *R. acetosa* under submergence.

Increase in a bioactive GA (i.e., GA_1) levels was observed in *R. palustris* only after 6 h of submergence, and the increase in GA_1 levels was dependent on the ethylene-induced decrease in ABA levels in submerged petioles (Benschop et al. 2006). Thus, it was proposed that the submergence-induced petiole elongation response in *R. palustris* was separated into three phases: (1) independence on GA until 4 h after submergence, (2) limitation by GA from 4 to 6 h after submergence, and (3) dependence on but not limitation by GA from 15 h after submergence (Benschop et al. 2006). However, upregulation of expression of the genes related to the GA-mediated signaling pathway was observed at an earlier phase of submergence in *R. palustris*, which indicates that sensitivity to GA is increased prior to the increase in active GA levels (van Veen et al. 2013).

Auxin, together with ethylene, also plays an important role in submergence-induced shoot elongation of some species that inhabit aquatic or semiaquatic environments (Cookson and Osborne 1978; Walters and Osborne 1979; Horton and Samarakoon 1982; Malone and Ridge 1983). In *R. palustris*, the depletion of auxin levels by removal of the leaf blades from the petiole delayed submergence-induced petiole elongation, and the delayed petiole elongation was recovered by submerging the plants treated with a synthetic auxin, 2,4-dichlorophenoxy acetic acid (2,4-D) or NAA (Cox et al. 2004, 2006). Although ethylene is essential for submergence-induced petiole elongation in *R. palustris*, ethylene did not restore elongation in submerged petioles of plants with depletion of auxin levels, which indicates that both auxin and ethylene play active roles in growth responses. The submergence signal, which could increase the amount of internal ethylene, and the following cooperative action of ethylene with endogenous auxin are both necessary for stimulating petiole elongation.

As in the case of *Rumex*, the degree of shoot elongation in rice under submergence differs among varieties. For example, "deepwater rice" varieties employ the escape strategy to survive under deepwater flooding, which last for several months; that is, deepwater rice can rapidly and substantially elongate its internodes to cope with deepwater flooding. Rapid elongation can result in leaf tips extending above the water surface to exchange gases for respiration and photosynthesis (Bailey-Serres and Voesenek 2008). During internode elongation, the amounts of ethylene was increased, and the accumulated ethylene was responsible for the increased GA/ABA ratio by increasing GA levels and decreasing ABA levels, which stimulates internode elongation (Hoffmann-Benning and Kende 1992; Kende et al. 1998; Sauter 2000) (Fig. 5.3). Hattori et al. (2007, 2008) detected three quantitative trait loci (QTLs) for internode elongation on chromosomes 1, 3, and 12 of deepwater rice (variety C9285) and produced three nearly isogenic lines (NILs) that possessed each of the three deepwater rice QTLs (NIL-1, NIL-3, and NIL-12) by backcrossing with a lowland (i.e., non-deepwater) rice variety. Internode elongation of the three QTL pyramiding lines (NIL-1 + 3 + 12) was almost the same as that of deepwater rice C9285, which indicates that lowland (non-deepwater) rice can acquire the same

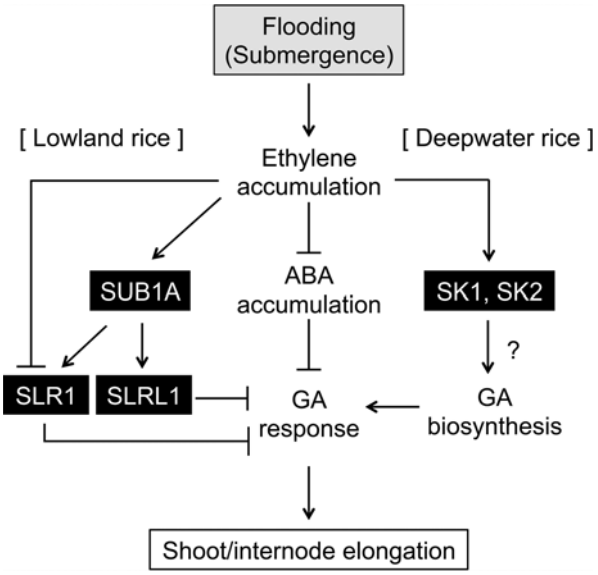

Fig. 5.3 Control of flooding-induced shoot/internode elongation in rice by the interplay among ethylene, ABA, and GA. Flooding induces the accumulation of endogenous ethylene by enhancement of ethylene biosynthesis as well as entrapment. The accumulated ethylene promotes decrease in ABA levels, followed by derepression of GA responsiveness and induction of shoot/internode elongation. SK1/SK2 and SUB1A are key regulators of flooding-induced shoot/internode elongation in deepwater rice and lowland (i.e., non-deepwater) rice, respectively. In deepwater rice varieties, the accumulated ethylene upregulates *SK1* and *SK2* expression under flooding (i.e., submergence). SK1 and SK2 promote internode elongation through possibly enhancement of GA biosynthesis, which stimulates GA responsiveness. On the other hand, in lowland rice varieties that possess *SUB1A*, accumulated ethylene upregulates *SUB1A* expression under flooding (i.e., submergence). SUB1A limits flooding-induced shoot elongation through the enhanced expression of the key GA signaling repressors SLR1 and SLRL1, which reduce GA responsiveness. By contrast, in lowland rice varieties that do not possess *SUB1A*, the accumulated ethylene reduces the amount of SLR1 (but not SLRL1), thereby derepressing GA responsiveness

ability for internode elongation as deepwater rice by introducing the three QTLs (Hattori et al. 2009). Accumulation of several GAs (GA_1, GA_4, GA_9, and GA_{20}) increased in the aerial parts of NIL-1 + 3 + 12 and deepwater rice C9285 under deepwater flooded conditions. The increase in GA levels may be due to upregulation of expression of the GA biosynthesis gene *OsGA20ox2* (Ayano et al. 2014). By positional cloning of the QTL for internode elongation on chromosome 12, two ethylene-inducible genes, *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*), were identified (Hattori et al. 2009). *SK1* and *SK2* encode the group VII ethylene response factor transcription factors, which are responsible for ethylene signaling during internode elongation. Although it is unclear how SK1 and SK2 interact with the products encoded by the other QTLs on chromosomes 1 and 3, SK1 and SK2 may control ethylene-induced GA biosynthesis during internode elongation in deepwater rice (Fig. 5.3).

Generally, many lowland rice varieties also have the ability to elongate their leaves toward the water surface under complete submergence. However, most cannot elongate enough to reach the water surface when they are deeply submerged. Because large amounts of energy are lost during shoot elongation, these plants cannot fully recover after the water recedes and eventually are severely damaged or dead (Jackson and Ram 2003). By contrast, the East Indian rice variety FR13A uses the quiescence strategy; that is, FR13A can repress shoot elongation and reduce energy usage under submergence (Setter and Laureles 1996). Upon desubmergence, growth can be restarted using the energy preserved during submergence (Singh et al. 2001; Fukao et al. 2006). By positional cloning of the *SUBMERGENCE-1* (*SUB1*) locus on chromosome 9 of FR13A, Xu et al. (2006) found that the *SUB1* locus contained *SUB1A*, *SUB1B*, and *SUB1C*, all of which encode the group VII ethylene response factor transcription factors and were upregulated under submergence. Among them, only *SUB1A* was responsible for the suppressed shoot elongation under submergence (Fukao et al. 2006; Xu et al. 2006; Bailey-Serres et al. 2010) (Fig. 5.3). SUB1A controlled repression of expression of the genes involved in starch and sucrose metabolism, thereby preserving energy and carbohydrates under submergence (Fukao et al. 2006). SUB1A also enhanced expression of the genes involved in alcohol fermentation and the genes that encode SLENDER RICE-1 (SLR1) and SLR1 LIKE-1 (SLRL1), which are key repressors of GA signaling in rice (Fukao et al. 2006; Fukao and Bailey-Serres 2008) (Fig. 5.3). Increased amounts of SLR1 and SLR1L may restrict shoot elongation by negatively regulating the GA responsiveness under submergence. Ethylene upregulated expression of *SUB1A*, thereby enhancing *SLR1* and *SLR1L* expression and decreasing GA responsiveness in the shoots (Fukao and Bailey-Serres 2008) (Fig. 5.3). Interestingly, in rice varieties that do not possess *SUB1A*, ethylene reduced the amount of SLR1 (but not SLRL1). On the other hand, rapid decline in endogenous ABA levels under submergence was SUB1A independent (Fukao and Bailey-Serres 2008) (Fig. 5.3).

5.5 Conclusions and Perspectives

Ethylene is involved in aerenchyma formation, AR formation, and shoot growth control under both escape and quiescence strategies, which indicates that ethylene is a key factor in morphological and anatomical responses of plants to flooding stress. Upon waterlogging or submergence, ethylene is entrapped and quickly accumulates in the submerged tissues because water surrounding the tissues prevents its escape (Visser and Voesenek 2004); thus, the rapid accumulation of ethylene may work as a priming factor for these adaptive responses of plants to flooding. By contrast, the downstream processes may be controlled by unique mechanisms in different places (i.e., different cell or tissue types) where the adaptive responses occur. Moreover, some studies found that interactions of ethylene with other phytohormones such as auxin, GA, and ABA were also important for AR formation and shoot growth control under submergence. However, much remains to be elucidated

about cell- or tissue-specific molecular processes that are regulated by the interplay between ethylene and other phytohormones. To answer these questions, transcriptome or proteome analyses combined with methods for isolating specific cells or tissues (e.g., laser microdissection) may be useful. Indeed, many genes associated with lysigenous aerenchyma formation in maize roots were identified using a microarray analysis combined with laser microdissection (Rajhi et al. 2011; Takahashi et al. 2015). The same approach can be applied to identify the genes associated with other traits for morphological and anatomical responses of plants under flooded conditions. Further functional analyses of the identified genes will accelerate our understanding of the molecular mechanisms of plant adaptive responses controlled by phytohormones under flooded conditions.

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Chapter 6
Phytohormonal Responses to Soil Acidity in Plants

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Abstract Phytohormones play an important role in plant growth and adaptation to various environmental stresses. Acid soils are recognized as one of the most important abiotic stresses, limiting plant growth and reducing crop yield. Furthermore, acid soils can cause changes in phytohormone metabolism such as biosynthesis and action. Thus, in this chapter, we provide a critical overview focused on phytohormone responses, plant hormone balance, and cross-interactions to nutrients and acid soils, including low pH stress (or high H^+ toxicity) and low pH-induced aluminum toxicity.

Keywords Aluminum toxicity • Cross-interactions • High H^+ toxicity • Low pH • Phytohormones

6.1 Introduction

Phytohormones play a crucial role in regulation of plant growth, development, nutrient, and source/sink allocations (Davies 2010; Kurepin et al. 2013a; Hüner et al. 2014; Zaman et al. 2015). They are signaling molecules that control many physiological and biochemical processes, affecting various plant physiological

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processes including seed dormancy and germination (Davies 2010; Park et al. 2015). Furthermore, phytohormones are essential for the ability of plants to adapt to abiotic stresses, as they mediate a wide range of adaptive responses (Kurepin et al. 2013b, 2015a). The classical phytohormones such as abscisic acid (ABA), ethylene, cytokinins (CKs), auxins (predominantly indole-3-acetic acid or IAA), and gibberellins (GAs) as well as other signaling molecules such as brassinosteroids (BR), salicylic acid (SA), jasmonic acid (JA), nitric oxide (NO), and strigolactone (SL) are involved in a wide range of plant response to various definite and/or co-occurring abiotic stresses (Peleg and Blumwald 2011; Ahammed et al. 2015).

In response to a numerous abiotic stresses, plants typically reduce the levels of growth-promoting phytohormones such as IAA, GAs, and CK, via reductions in biosynthetic activities and/or increases in catabolic activities. Since these growth-promoting phytohormones are responsible for cell division and expansion, it is not surprising that both processes responsible for plant growth are typically inhibited by abiotic stress. In contrast, abiotically stressed plants typically increase accumulation of "stress-related" phytohormones, such as ABA, SA, JA, and, occasionally, ethylene. The biosynthesis of ABA occurs mainly in the chloroplast, except for the last biosynthetic steps, which take place in the cytosol (Schwartz and Zeevaart 2010). Abscisic acid appears to be a key phytohormone in plant responses to environmental changes and plays a major role in acclimation of plants to multiple abiotic stresses (Zeevaart and Creelman 1988; Cho et al. 2009; Kurepin et al. 2013b, 2015a). For example, drought-stressed plants rapidly increase the de novo synthesis of ABA, which quickly leads to stomatal closure to protect the plant from rapid desiccation (Schwartz and Zeevaart 2010). Furthermore, stress-induced accumulation of ABA and also SA may cause an upregulation in the expression of genes involved in the biosynthesis of compatible osmolytes, such as glycinebetaine (Jagendorf and Takabe 2001). Moreover, exogenous applications of ABA and especially SA have been shown to increase plant stress tolerance to various abiotic stresses and thus their yield (Ivanov et al. 1992, 1995; Kiba et al. 2011). Jasmonic acid is involved mainly in plant defense responses to herbivory insects (Howe 2010). However, it has also been shown to play a role in root development (Kurepin et al. 2011a), in modulation of photosynthesis (Popova et al. 1988; Maslenkova et al. 1992; Ivanov and Kitcheva 1993), and, as discussed later in this chapter, in increasing plant stress tolerance to heavy metal stress. Ethylene plays a key role in multiple growth and developmental processes, as well as plant responses to stress (Abeles et al. 1992).

There are two main hurdles in studying the involvement of each phytohormone group in various growth and developmental processes controlled by environmental signals. First, phytohormones interact extensively in multiple growth and developmental processes, resulting in synergistic or antagonistic interactions (Wang and Irving 2011). These interactions play crucial roles in plant responses to abiotic stress (Peleg and Blumwald 2011). For instance, stress-induced accumulation of ABA, which typically leads to stomatal closure, can be counteracted by endogenous increases in CK or ethylene levels (Wilkinson et al. 2012). Moreover, stress-induced increase of ABA levels can counteract increases in GA and IAA levels in abiotically

stressed plants (Tanada 1973; Kurepin et al. 2011b). Increases in the growth-active GA levels can be additionally stimulated by endogenous increases in IAA levels and therefore further promote plant growth. This IAA-GA interaction is due to IAA ability to increase the expression of key GA biosynthetic genes (O'Neill and Ross 2002). Furthermore, application of growth-active BR increases shoot growth most likely via indirect effects on the IAA-GA interaction (Kurepin et al. 2012a). However, it is important to note that phytohormone interactions may differ across species (Ross et al. 2011), as was shown, for example, when phytohormonal interactions were compared between sunflower and *Arabidopsis* plants subjected to a light signaling stress (Kurepin et al. 2012b; Kurepin and Pharis 2014). Consequently, when studying the effect of abiotic stress signals, such as soil acidity or Al toxicity, it is important to evaluate the involvement of multiple phytohormones, as well as to attempt to deduce their interactions on a species-specific basis.

Second, various phytohormones can be directly produced by certain soil- or plant-associated bacteria or fungi, or these microorganisms can influence *in planta* phytohormone metabolism (Kurepin et al. 2014). Direct biosynthesis of growth-promoting phytohormones, such as IAA, GAs, and CKs, was reported for several bacteria and fungi species (Bottini et al. 1989; Ivanova et al. 2001; Waqas et al. 2012). Direct or indirect effects on the plant endogenous levels of "stress-related" phytohormones by the presence of microorganisms are also very likely. For example, inoculation of plants with plant growth-promoting bacterium *Burkholderia phytofirmans* caused increases in both shoot and root growth, and these increases in growth were associated with endogenous changes in not just growth-promoting phytohormones but also in the levels of ABA, SA, and JA (Kurepin et al. 2015b, c). Furthermore, many species of both soil- and plant-associated bacteria have been shown to carry 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene controlling the biosynthesis of ethylene precursor, ACC (Klee et al. 1991). The product of ACC deaminase gene reduces the pool of available ACC and thus can limit ethylene production (Walton et al. 2012). Introduction of ACC deaminase gene into plant tissues via inoculation with a bacterial species carrying this gene or via genetic engineering can improve plant tolerance to abiotic stresses (Kurepin et al. 2007) but can also negatively affect growth and development of non-stressed plants (Walton et al. 2012). Since soil acidity can also negatively affect the activity and presence of soil- and plant-associated microorganisms (Francis 1982; Rousk et al. 2009), this, in turn, can modify the levels of phytohormones in plant tissues and/or rhizosphere.

6.2 Phytohormonal Responses to Soil Acidity

Soil acidity occurs when soil pH drops below 5.5, having a severe negative impact on crop yield (Kochian et al. 2004). The negative impact of acid soils on low productivity and yields in various crops has been mainly attributed either to nutrient (phosphorus (P), calcium (Ca), and magnesium (Mg)) deficiency or toxicity to

aluminum (Al), iron (Fe), and manganese (Mn) (Soon 1991; Millaleo et al. 2010). Close to 3,950 million ha, or 30 % of total land area and 70 % of the potentially arable land, experience some degree of soil acidity (Von Uexküll and Mutert 1995). Soil becomes acidic due to an increment of H^+ ion concentration. This process occurs when basic cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) are leached from soil and the excess of H^+ ions replaces these cations (Delhaize and Ryan 1995). Soil acidification is influenced by edaphic, climatic, and biological factors, but it can be intensified due to acid rain from pollution and certain farming practices, such as application of ammonium-based N fertilizers (Kennedy 1986; Hede et al. 2001; Zheng 2010). A number of studies have reported that phytohormone biosynthesis and action can be affected by the soil acidity stress (Yuan et al. 2009; Zhou et al. 2014). In most of these studies, the involvement of phytohormones was evaluated and focused on the phytohormonal responses to aluminum (Al) toxicity (Massot et al. 2002; Shen et al. 2004; Reyna-Llorens et al. 2014; Zhou et al. 2014). This is because Al toxicity is recognized as the most important factor limiting crop productivity on acid soils (Soon 1991; Kochian et al. 2004; 2015). However, the negative effects of low pH stress (or high H^+ toxicity) on plant growth and phytohormones are less known and documented (Takahashi 2012). Root growth inhibition by low pH stress has been reported in some plant species (Kinraide and Parker 1987; Kinraide et al. 1994). It has been proposed that apoplastic calcium (Ca^{2+}), an important signaling molecule for cell growth, is the main target of low pH stress, and higher H^+ concentration could cause inhibition of root elongation due to Ca displacement in the apoplast (Kinraide et al. 1994).

ĈÍžková (1990) reported that CK levels increased in oak (*Quercus sessilis* Ehrh.) seedlings exposed to pH of 2.5, whereas the levels of GAs were not affected relative to the control seedlings. Low-pH stress also affected endogenous levels of several phytohormones in *Atractylodes lancea* (Thunb.) DC. plants (Yuan et al. 2009). In particular, the levels of IAA and ABA increased, relative to control, 2.8-fold and 1.4-fold, respectively, in response to low pH treatment by day 30 (Yuan et al. 2009). Furthermore, levels of a CK zeatin riboside increased 2.0-fold by day 60, whereas the levels of GAs were not affected by a low pH stress at either measurement date (Yuan et al. 2009). Interestingly, these increases in CK, IAA, and ABA levels in response to a low pH stress triggered an increase in root hair density and root hair number, but inhibited elongation of the main root. The formation and elongation of root tissues is under tight phytohormonal control (Kurepin et al. 2011a). The results reported by Yuan et al. (2009) fall into the current model of root formation and growth, where localized increases in IAA levels suppress localized CK accumulation and therefore lead to root formation, but later, potentially via negative ABA-IAA interaction, localized IAA increase diminishes, and thus CK levels increase, causing further root development (Kurepin et al. 2011a). In addition, increase in ABA levels in root tissues following soil acidity could be responsible, via localized antagonistic ABA-IAA and ABA-CK interactions, for a decrease in main root elongation (Kurepin et al. 2011a).

Experiments with plant hormone mutant lines have also shown the importance of phytohormones in root growth in response to a low pH stress. For example,

Takahashi and Inoue (2008) reported that auxin and ethylene are essential to stimulate root hair elongation. Interestingly, mutation in CTR1 (*C*ONSTITUTIVE *T*RIPLE *R*ESPONSE *1*), a negative regulator of ethylene signaling and inhibitor of ethylene action, causes fewer root hairs (Masucci and Schiefelbein 1994). Rahman et al. (2002) showed that root hair length of a double mutant *ein2-1 aux1* (ethylene-insensitive and auxin-influx mutant) was reduced compared with wild type in response to a low pH stress. However, the elongation of root hairs was recovered by exogenous auxin application. Therefore, we can suggest that ethylene and auxin interact to inhibit the main root elongation and stimulate root hair formation for plants exposed to a low pH stress. Nonetheless, further research is required to understand the phytohormonal response to a low pH stress. Moreover, several studies dealing with the phytohormonal responses to acid soil were unable to separate the effects caused by Al toxicity and a low pH stress. However, the experimental system developed by Koyama et al. (2001) can be successfully used to study the phytohormonal responses to a low pH stress alone. The use of this experimental system may provide us with more knowledge about phytohormonal responses to acid soils and possibly facilitate in developing hormone-based approaches to increase low pH stress tolerance in crop plants. Luchi et al. (2007) identified a mutant hypersensitive to protons named *Arabidopsis* STOP 1 (*s*ensitive *to p*roton rhizotoxicity 1). The *STOP 1* gene encodes a zinc-finger protein located, by fluorescence, in the nucleus (Sawaki et al. 2009). The STOP 1 is a transcription factor, which is regulated by the expression of *al*uminum-activated *m*alate *t*ransporter *1* (ALMT1) (Kobayashi et al. 2014). The ALMT1 plays a critical role in Al tolerance by malate exudation (Sasaki et al. 2004). However, involvement of phytohormones in signaling pathway and expression of STOP 1 gene are no yet clarified.

6.3 Mineral Nutrients and Phytohormonal Responses to Soil Acidity

Plants require several mineral nutrients for the normal growth and development. These mineral nutrients are classified in two groups: (I) macronutrients, such as nitrogen (N), potassium (K), Ca, P, Mg, and sulfur (S), where each one may exceed 1 % of the total plant dry weight, and (II) micronutrients, such as Fe, chlorine (Cl), copper (Cu), Mn, zinc (Zn), molybdenum (Mo), and boron (B), which constitute about 0.04 % of the total plant dry weight (Barceló and Poschenrieder 2002; Lambers et al. 2008). These nutrients are incorporated to plant tissues mainly from soil, where their abundance can be influenced by soil physical and chemical properties, microorganisms, and certain agronomic practices such as fertilization and irrigation (Lambers et al. 2008).

Agroecosystems are agricultural ecosystems, which include biophysical and human components as well as their interactions (Altieri 1999). Agroecosystems are constantly disturbed by extraction, mobilization, or application of agrochemicals

(such as fertilizers, pesticides, etc.), affecting soil conditions (Altieri 1999; Iqbal et al. 2013). A fertilization deficiency severely limits the growth, development, and productivity of plants in agroecosystems (Fageria et al. 2010). Therefore, the addition of nutrient-based fertilizers generates an immediate availability of nutrients and thus restores soil nutrient levels for a proper development of soil microbial communities and crops (Francis 1982). This is important because the law of the minimum indicates that the increase of crop yield per unit of limiting nutrient applied is directly proportional to the decrease from the maximum yield (Mitscherlich 1909).

The rhizosphere, zone of soil near to the root (1–2 mm), is essential to nutrient absorption (Hiltner 1904), which determines and modifies the nutrient bioavailability according to their degree of acidity or alkalinity by the roots (Hue et al. 1986; Kinraide and Parker 1987). Thus, positively charged nutrient elements such as K, S, Ca, and Mg exhibit lower availability at acid pH environment around the rhizosphere, depending on the degree of cation exchange complex saturation (Besoain 1985; Mora et al. 2002). Soil exchange capacity is a measure of its ability to hold and to release cations that are adsorbed onto a negatively charged clay particles (Kinraide and Parker 1987; Pansu and Gautheyrou 2006). Acidic pH soils affect the cation exchangeable complexes inducing substantial replacement of Ca^{2+}, Mg^{2+}, and K^{+} for H^{+}, which are more prone to loss by leaching, thus decreasing nutrient availability to plants (Besoain 1985; Kinraide et al. 1994).

Although most of the nutrient(s) uptake mechanisms are well known (Lambers et al. 2008), the effects of soil acidity on phytohormones are poorly understood, mainly because of the scarcity of experimental data. It is widely accepted that phytohormones are implicated in the majority of processes connecting with growth and nutritional status of plants (Barceló and Poschenrieder 2002). For example, a study conducted with durum wheat (*Triticum durum* Desf.) plants, grown at nutritional deficit (1 % of Hoagland solution), showed a decrease in shoot dry biomass accumulation, whereas the root dry biomass accumulation remained unchanged (Vysotskaya et al. 2008). These changes in shoot and root dry biomasses were associated with increases in ABA levels in leaves, apical root area (3–4 mm), xylem, and phloem; in contrast, ABA levels decreased in response to nutritional deficit in whole-root tissue (Vysotskaya et al. 2008). Exogenous application of ABA biosynthetic inhibitor, fluridone, to wheat plants subjected to a nutritional deficiency caused a further decrease in shoot biomass as well as a decrease in root biomass, but shoot-to-root biomass ratio was not affected by fluridone (Vysotskaya et al. 2008). This growth inhibitory effect of a decrease in ABA biosynthesis and thus endogenous accumulation by exogenous fluridone was associated with decreased stomatal conductance and CO_2 assimilation (Vysotskaya et al. 2008). This suggests a key role of ABA in plant response to nutrient deficiency. Limitation of proper nutrient supply causes a transport of newly synthesized and/or accumulated and stored ABA from roots to leaves via both xylem and phloem. Once in the leaf tissues, ABA likely protects plant from severe growth arrest by maintaining CO_2 assimilation at a level which is lower from optimal, but high enough to maintain physiological processes at reduced capacity. The key role of ABA in root-to-shoot and shoot-to-root signaling for plants subjected to N deficiency has been recently reviewed by Kiba et al. (2011). The

interactions of ABA with other phytohormones and secondary metabolites with growth-regulating activity in plants subjected to mineral deficiency are not well elucidated. However, it is likely that these interactions are similar to ABA interactions, which are better understood for plants subjected to abiotic stresses. Thus, similar to abiotic stress, nutrient deficiency-mediated increase in ABA biosynthesis causes a decrease in shoot growth via increase in the catabolism of growth-active GAs (Kurepin et al. 2013b). Furthermore, similar to abiotic stress-mediated increase in ABA levels, which maintains CO_2 assimilation via increases in the biosynthesis of glycinebetaine (Kurepin et al. 2015a), the nutrient deficiency may cause similar ABA-glycinebetaine-photosynthesis interactions.

Nitrogen forms such as ammonia (NH_4^+) and nitrate (NO_3^-) are the most studied nutrients related to the phytohormonal responses (Garnica et al. 2009, 2010; Krouk et al. 2011; Iqbal et al. 2013). The effect of N deficiency is probably the most studied nutrient limitation in plants because of the high significance of N for the biosynthesis of multiple plant organic molecules. There is a great interest in agriculture to use N-based fertilizers co-applied with phytohormones or other growth-promoting chemicals (Zaman et al. 2015). Thus, there is increased interest in researching the N-phytohormone interactions (Kiba et al. 2011; Zaman et al. 2015). A strong coordination has been proposed among ABA, auxin, and CKs in controlling the demand and acquisition of nitrogen in plants (Kiba et al. 2011; Zaman et al. 2015). Microarray analyses performed in *Arabidopsis* have demonstrated that exogenous application of CK represses two *AtNRT2* genes (*AtNRT2.1* and *AtNRT2.3*), three ammonium transporter genes, three amino acid transporter genes, and a urea transporter gene, suggesting a negative regulation of nitrogen uptake-related genes by CKs (Kiba et al. 2011). This repression was not observed with other phytohormones, indicating that the effect is CK specific. Likewise, a negative regulation by CKs on other nutrient acquisition-related genes in *Arabidopsis* and rice, such as high-affinity phosphate transporter genes (Pht1;2 and Pht1;4), high-affinity sulfate transporter genes (SULTR1;2 and SULTR1;4), and iron deprivation-inducible genes (IRT1, FRO2, and FIT), have been reported (reviewed by Kiba et al. 2011).

Applied N was shown to stimulate ethylene production by wheat roots at the first 24 h, but there was a subsequent decline after 24 h period (Garnica et al. 2009). Furthermore, ethylene production was higher when N was applied as NO_3^- than when applied as NH_4^+ (Garnica et al. 2009). Cytokinins biosynthesis, forms, and distribution also vary between plant organs and tissues depending on the form of supplied N source and the duration of a plant exposure to N (Garnica et al. 2009; Kiba et al. 2011). Garnica et al. (2010) showed that fertilization with NH_4^+ as a N source increased CK levels in wheat roots by about threefold, compared with NO_3^- as N source. In this report was also reported that different CK forms distribute differently in shoot and roots. Thus, it is observed that zeatin (Z) and *cis*-zeatin riboside (*c*ZR) levels increased by about 20 % in shoots and roots after 24 h period, following fertilization with NH_4^+. However, fertilization with NO_3^- caused no changes in either Z or *c*ZR levels (Garnica et al. 2010). Furthermore, the same authors reported that shoot ABA levels increased more wheat plants supplemented with NH_4^+ than NO_3^-, whereas IAA were not affected by either of the two N sources (Garnica et al.

2010). Hence, independently of nitrogen source (NH_4^+ or NO_3^-), at the first 24 hours, an increase of CK in shoots occurs, suggesting that the interaction between N and CK is related to biomass increment.

A study carried with tobacco transgenic plants overexpressing isopentenyltransferase (IPT), enzyme which catalyzes the limiting step in CK biosynthesis, demonstrated the link between oxidative stress and CK biosynthesis during N deficiency (Rubio-Wilhelmi et al. 2011). These tobacco plants subjected to an N deficiency did not increase leaf $O_2^{\cdot-}$, H_2O_2, or LP levels. However, the foliar biomass of tobacco transgenic plants was still higher than wild tobacco plants (Rubio-Wilhelmi et al. 2011). On the other hand, Wei et al. (2009), using urea ($CO(NH_2)_2$) as N source supplied to barley (*Hordeum vulgare* L.) plants, showed increases in the endogenous ABA, IAA, and ZR levels during the grain filling stage at 10 days post anthesis. Another study with urea as N source showed an increase in the number of female flowers per fruit spur in Chinese chestnut (*Castanea mollissima* BL.) trees treated with growth-active GA_3 (Qiguang et al. 1985).

Since there is an apparent involvement of multiple phytohormones in nitrogen signaling, the future challenge will be to understand the interaction between multiple phytohormones and the nitrogen signal. On the other hand, understanding the interaction between transport of both micro- and macronutrients and phytohormones in acidic soils is also an important future research direction.

6.4 Phytohormonal Responses to Aluminum Toxicity

The majority of acid soils are located in the tropical and subtropical regions. The primary limiting factor for crop productivity in these soils are the Al and manganese (Mn) toxicities (Kochian 1995; Raman et al. 2002; Tang et al. 2002). However, a low pH in soils per se is also stress factor limiting nutrient availability and affecting the development and growth of agronomically important crops (Mora et al. 2006). The most available studies about Al toxicity are referred to annuals species, particularly cereals (Mora et al. 2006; Kollmeier et al. 2001). Acid soils typically have high organic matter concentration (OM) (Mora et al. 2002), stimulating the decrease in soil pH due to the release of H^+ ions (Hede et al. 2001). These acid soils are characterized by a granular structure, amorphous clays (allophane or imogolite) (Besoain 1985; Shoji et al. 1993), low bulk density, high phosphorus fixation (Wada 1985), high stability of aggregates, and low quantity exchangeable bases such as Na^+, K^+, Ca^{2+}, and Mg^{2+} (Shoji et al. 1993). Aluminum is the third most abundant element in the Earth's crust. It is typically found as oxides and aluminosilicate complexes in soil (Wada 1985; Shoji et al. 1993; Delhaize and Ryan 1995; Barceló and Poschenrieder 2002; Hede et al. 2001, 2003). In acid soils, the Al dynamic plays an important role in low pH exclusively, leaving available Al forms such as phytotoxic Al^{1+}, Al^{2+}, and Al^{3+}, potentiating both stresses. The latter form causes the highest Al toxicity in plants under acidic conditions (Kinraide 1991; Delhaize and Ryan 1995; Mora et al. 2006; Wang et al. 2006).

Plants exposed to Al stress modify their physiological, biochemical, and morphological characteristics, depending on species or genotype, severity of Al toxicity, and the degree of Al tolerance (Delhaize and Ryan 1995; Reyes-Díaz et al. 2010). The inherited resistance mechanisms to Al toxicity in plants are not fully elucidated, although there is consensus that at least two major mechanisms, i.e., external exclusion and internal tolerance, might be involved. The main difference among them is the site of Al detoxification. The external exclusion mechanism involves preventing the toxic aluminum to enter in the apoplasm or symplasm (Hue et al. 1986; Kochian 1995; Kochian et al. 2004; Li et al. 2002; Kollmeier et al. 2001; Piñeros et al. 2005; Wang et al. 2006; Inostroza-Blancheteau et al. 2011, 2012). The proposed exclusion mechanism involves excretion of organic compounds (malate, citrate, and oxalate) from the roots that form stable complexes with the Al^{3+} ions in the soil solution, which are less phytotoxic than free Al^{3+} ions, thus avoiding the toxic Al species to enter the sensitive intracellular sites of the symplasm (Ma 2000; Li et al. 2002; Piñeros et al. 2005). The internal tolerance mechanism immobilizes, compartmentalizes, or detoxifies the toxic Al^{3+} in the symplasm or apoplasm (internal). As in other stresses, the degree of plant resistance to Al toxicity depends on the species, variety, or cultivar considered (Barceló and Poschenrieder 2002). Native species that grow naturally in acid soils are very resistant to Al stress as *Miconia ferruginata*, *Miconia pohliana*, *Palicourea rigida*, *Qualea grandiflora*, *Qualea multiflora*, *Qualea parviflora*, *Vochysia elliptica*, and *Vochysia thyrsoidea* (Haridasan 1982; Kochian 1995; Geoghegan and Sprent 1996), and some of them accumulate high concentrations of this metal in leaves, whereas others as *Oryza sativa* in a lesser amount (Li et al. 2002). Commercially important species such as *Medicago sativa*, *Hordeum vulgare*, *Lycopersicon esculentum*, and *Brassica napus* are considered Al-sensitive species. However, *Triticum aestivum* and *Zea mays* showed a wide range of sensitivity among its genotypes (Kochian 1995; Piñeros et al. 2005).

The major visible symptoms of plants exposed to Al toxicity are inhibition of root growth, chlorosis of leaf tissues, and stunted shoot growth (Matsumoto 2000; Mora et al. 2005; Rengel 2006; Reyes-Díaz et al. 2009, 2010). The inhibition of cell division in the root meristem zone is the typical response of cereal plants to Al exposure (Kochian et al. 2015). It was reported that more than 95 % of the Al associated to roots is located in the cell wall, suggesting that Al is responsible for the rapid displacement of Ca from the cell wall and the loss of elasticity (Kinraide et al. 1994; Rengel 2006). These changes affect also the nutrients and water absorption from the soil, thus affecting the metabolic processes of the whole plant (Quinteiro et al. 2013).

Another important Al stress response in plants is the exacerbation of the production of reactive oxygen species (ROS) causing oxidative stress in cells, cell membranes, cellular organelles, and organic molecules, which can result in cell death (Yakimova et al. 2007). It is also reported that Al can alter chloroplast and mitochondrial functions, which leads to ROS production and cell death via a ROS-activated signal transduction pathway (Kochian 1995; Kochian et al. 2004; 2015; Hede et al. 2001; Kopittke et al. 2015). Differential expression of ROS-related genes has been reported under Al stress conditions (Kumari et al. 2008;

Inostroza-Blancheteau et al. 2011; Reyna-Llorens et al. 2014). Plants are able to activate antioxidant enzymes such as superoxide dismutase (SOD), peroxidase, catalase, ascorbate peroxidase, and other nonenzymatic ROS-scavenging systems/molecules as ascorbic acid, α-tocopherol, flavonoids, and carotenoids, to counteract and decrease/tolerate the phytotoxic effect of Al-induced and decrease oxidative stress (Ma et al. 2007).

Biochemical, physiological, and morphological alterations are due to the high Al accumulation in the cell (Matsumoto 2000; Kochian et al. 2004), affecting phytohormone metabolism and action (Yakimova et al. 2007; Aloni et al. 2010; He et al. 2012; Puga-Freitas and Blouin 2015). A direct relationship between Al toxicity at acidic conditions and phytohormonal metabolism in root tissues has been reported (Shen et al. 2004; Garay-Arroyo et al. 2013; Reyna-Llorens et al. 2014), where Al stress-induced inhibition of root cell elongation also altered ethylene and auxin biosynthesis and accumulation in soybean (*Glycine max* L.) (Kopittke et al. 2015). Among the phytohormones affected by Al toxicity at acidic conditions are ABA, IAA, CKs, ethylene, GAs, and JA, whereas the involvement of SA, which is affected by multiple other environmental stresses (Kurepin et al. 2010, 2013c), is presently unknown.

The ABA plays an important role in the response to Al toxicity. The increases in ABA biosynthesis and signaling transduction were shown in maize (*Zea mays* L.), soybean, barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), and buckwheat (*Fagopyrum esculentum* Moench) (Shen et al. 2004; Hou et al. 2010; Reyna-Llorens et al. 2014; Fig. 6.1). This increase in ABA biosynthesis is directly related to an

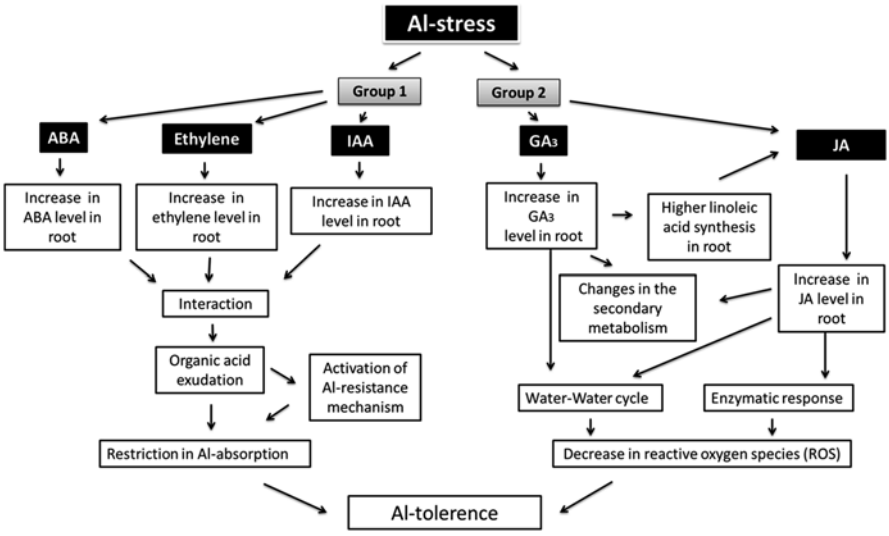

Fig. 6.1 Involvement of phytohormones in plant tolerance to Al toxicity. Two groups of phytohormones are involved in plant tolerance to Al toxicity. Group 1: exudation of organic acid regulation mediated by ABA, ethylene, and IAA. Group 2: decrease of ROS by secondary metabolism activation regulated by GA_3 and JA

increase in Al toxicity available at low pH, as was shown concomitant with increases in Al doses and endogenous ABA levels in the root apex of soybean plants (Shen et al. 2004; Hou et al. 2010; Reyna-Llorens et al. 2014). The Al toxicity and ABA biosynthesis link was further demonstrated with the use of a carotenoid and ABA biosynthesis inhibitor fluridone ([1-methyl-3-phenyl-5-(3-(trifluoromethyl)phenyl)-4(1H)-pyridinone]) (Gamble and Mullet 1986). Application of fluridone in plants exposed to Al toxicity reduced ABA accumulation and root growth, suggesting that the ABA inhibition increased the negative effect at lower Al (30 μM) concentration in soybean root (Hou et al. 2010).

Hou et al. (2010) also investigated the ABA transport and redistribution in plants subjected to Al toxicity by using a split-root experiment, where soybean roots were divided into part A and part B, with Al stress and without Al stress, respectively (Fig. 6.2). Using the [^{3}H]-ABA radioisotope technique, Hou et al. (2010) showed that more than 16 % of [^{3}H]-ABA fed to soybean roots exposed to Al toxicity (Fig. 6.2, inset 1) was detected 5 minutes later in stem and leaf tissues (Fig. 6.2, inset 2). Therefore, this author concluded that Al toxicity may accelerate ABA transport from roots to leaves (Fig. 6.2). Furthermore, [^{3}H]-ABA was also detected in part B root tissues, i.e., a part which was not exposed to Al toxicity (Fig. 6.2, inset 3). The ABA transport and accumulation in different plant organs may be possible due to recently discovered transporters that belong to ATP-binding cassette (ABC) transporter family and encoded by AtABCG40 (Kang et al. 2010), AtABCG25 (Kuromori et al. 2010), and AtABCG22 (Kuromori et al. 2011) genes in *Arabidopsis*. These

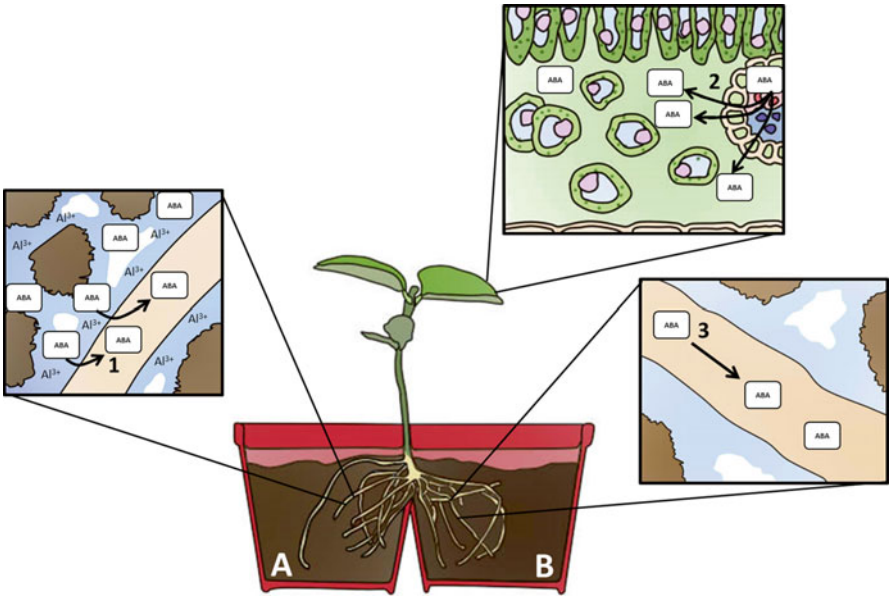

Fig. 6.2 Redistribution of ABA in soybean roots exposed to Al toxicity. The [^{3}H]-ABA fed in part A (Al toxicity) [1] is transported from roots to leaves [2] and then redistributed to roots in part B (not exposed to Al toxicity) [3] (Adopted from Hou et al. 2010)

transporters have now been detected in roots, vascular tissues, leaves, and flowers (Kuromori et al. 2010, 2011). There is likely another functional aspect of Al toxicity-mediated increase in plant endogenous ABA levels. Increase in soil Al concentrations was shown to decrease plant stomatal conductance (Quinteiro et al. 2013). Abscisic acid is well known as regulator of stomatal closure in response to osmotic stress or exogenous ABA application (Dodd and Davies 2010). Therefore, the redistribution of ABA from root to leaf tissues following the exposure of a plant to Al toxicity could be responsible of the decrease in stomatal conductance. Finally, Hou et al. (2010) also suggested that ABA could be involved in increasing plant tolerance to Al toxicity. However, the present knowledge on ABA downstream signaling action in high Al-stressed plants has to be further expanded.

Pretreatment of soybean plants with exogenous ABA followed by exposure to Al toxicity stimulated both root elongation and citrate efflux (Shen et al. 2004). Evidence has shown that Al induces the efflux of organic acids such as citrate, malate, and oxalate from roots (Ma 2000). This malate exudation has been linked to Al resistance gene in wheat as ALMT1 and MATE (*m*ultidrug *a*nd *t*oxic compound *e*xtrusion) family that Al-activated the malate transporter as an important Al resistance mechanism (Delhaize et al. 2004; Sasaki et al. 2004). However, pretreatment with K-252a, an inhibitor of broad range of protein kinases (Hossain et al. 2011), inhibited root elongation and citrate efflux in plants treated with ABA and Al toxicity (Shen et al. 2004). Similar results were reported by Reyna-Llorens et al. (2014), i.e., the variation in the relative expression of an *A*TP-*b*inding *c*assette (ABC)-like transporter gene (FeALS3) was correlated with organic acid exudation in Al-tolerant buckwheat plants. Furthermore, Reyna-Llorens et al. (2014) demonstrated that exudation of the malic, citric (at 12 h), and oxalic (at 48–72 h) acids can be stimulated in different times by Al toxicity. Thus, both ABA and Al can regulate organic acid exudation from mitochondria in tricarboxylic acid cycle (Fig. 6.1). The ABA long-distance transport from the site of ABA biosynthesis to the site of its biological action is well known (reviewed by Leng et al. 2014). However, recently, Kang et al. (2010) reported the first ABA transporter involved in both ABA transport and signaling. Kang et al. (2010) identified a plasma membrane ABC transporter called ABCG40, which is an ABA uptake transporter necessary for stomatal closure and regulation of lateral root development. This ABA uptake transporter is located in the guard cells in leaf tissues and endodermis cells in root tissues. Additionally, Kuromori et al. (2010) identified another ABA transporter, ABCG25. This ATP-dependent ABA transporter was expressed mainly in the vascular tissues and acts as an exporter of ABA. Thus, the endogenous increase in root ABA levels alters Al toxicity; likely leads to a long-distance transport of the newly synthesized ABA to leaves, where its signals permit stomata closure; and therefore minimizes further Al uptake via transpiration. This is supported by Perfus-Barbeoch et al. (2002) study, where exogenous ABA application reduced the root-to-shoot translocation of heavy metals, such as cadmium (Cd^{2+}), inducing the stomatal closure. It has been proposed that the increase in ABA accumulation in response to a heavy metal stress may increase plant tolerance via two different pathways (Kang et al. 2010). First, the

reduction of metal uptake and translocation from root to shoot, which is due to stomatal closure (Kang et al. 2010). Second, modification of gene expression that contributes to heavy metal tolerance (Talanova et al. 2000; Perfus-Barbeoch et al. 2002). Thus, ABA is an important factor for increasing plant tolerance to Al toxicity via Al-induced organic acid production. Moreover, we suggest that ABA mobility is a key factor in determining the degree of plant tolerance to Al toxicity (Fig. 6.1).

In plants exposed to Al toxicity, the role of auxin is mainly in regulation of cell division and root elongation (Ma et al. 2007; Garay-Arroyo et al. 2013). Early reports have shown that supplying IAA at 0.1 μM to the root elongation zone was able to diminish the inhibition of elongation of primary roots caused by Al toxicity in the distal transition zone of maize ATP-Y (Al-resistant) and Lixis (Al-sensitive) cultivars (Kollmeier et al. 2000). Furthermore, it was shown that Al-sensitive cultivar diminished the inhibition of root elongation more than Al-resistant cultivar when both were treated with exogenous IAA. One possible explication for this IAA action is the direct and proportional relationship between negative effects of Al toxicity and significant inhibition of apical IAA transport in *Arabidopsis* root tips (Sun et al. 2010). For example, in wheat plants exposed to Al toxicity, the blocked IAA transport in roots increased the local IAA accumulation (Yang et al. 2011). The increase in local IAA accumulation can lead to lateral or adventitious root formation (Kurepin et al. 2011a). The IAA transport is regulated by PIN2 proteins and it appears that Al toxicity inhibits the transport of PIN2 vesicles from plasma membranes to endosomes (Shen et al. 2008; Yang et al. 2011; Fig. 6.1). Moreover, the effect of Al toxicity on IAA distribution into apex of primary roots of the maize Al-resistant and Al-sensitive plants was observed (Kollmeier et al. 2000). Thus, there was a buildup in IAA accumulation in the root meristematic zone of Al-resistant maize (Fig. 6.1), whereas in Al-sensitive maize, there was a decrease in IAA accumulation in root meristematic and elongation zones due to a high accumulation of Al (Kollmeier et al. 2000). Aluminum can also inhibit IAA transport from shoot-to-root apex, causing an alteration of IAA distribution by a major retention of IAA in the root elongation zone of plants exposed to Al toxicity (Zhou et al. 2014). However, it is also possible that IAA could be involved in Al-induced efflux of malic acid, which regulates transport protein IAA output and increases the concentration in wheat root tissues (Yang et al. 2011; Fig. 6.1). Therefore, the increase in IAA levels may suggest a resistance mechanism which is linked to the increase in plant Al concentration with different tolerance levels. An increase of IAA accumulation could be restricted to the absorption of Al and its toxic effects on plants.

At molecular level, Mattiello et al. (2010) reported that genes involved in IAA biosynthesis, such as IAA amidohydrolase and anthranilate phosphoribosyl transferase, were upregulated in Al-sensitive genotype of maize exposed to acidic soil (Table 6.1). Meanwhile, indole-3-acetate beta-glucosyltransferase gene which encodes an enzyme responsible for auxin degradation was downregulated (Normanly et al. 2010; Table 6.1). On the other hand, the ACC oxidase gene was upregulated in response to soil acidity (Table 6.1). Therefore, the upregulation of genes involved in auxin synthesis could lead to higher IAA levels, and it is known that higher IAA

Table 6.1 Expression of phytohormone-related genes as influenced by Al stress

Genes	Functional annotations	Involved in	Response to Al stress	References
IAA3 and AnPRT	*Indole-3-acetic acid amidohydrolase and anthranilate phosphoribosyl transferase*	Auxin synthesis	Upregulated	Mattiello et al. (2010)
AUX1	*Auxin transporter-like protein*	Auxin transport	Upregulated	Zhou et al. (2014)
PIN7	*Auxin efflux carrier component 7*	Auxin transport	Downregulated	Kumari et al. (2008)
PIN1	*Auxin efflux carrier component 1*	Auxin transport	No changes	Zhou et al. (2014)
IAGLU	*Indole-3-acetate beta-glucosyltransferase*	Auxin degradation	Downregulated	Mattiello et al. (2010)
ACC oxidase	*1-Aminocyclopropane-1-carboxylic acid oxidase*	Ethylene synthesis	Upregulated	Sun et al. (2007), Chandran et al. (2008), and Mattiello et al. (2010)
ACC synthase	*1-Aminocyclopropane-1-carboxylic acid synthase*	Ethylene synthesis	Upregulated	Sun et al. (2007)

levels inhibit root elongation, but increase lateral and adventitious root formation (Kurepin et al. 2011a). However, a transcriptomic study performed in *Arabidopsis* revealed that transcripts putatively related to auxin transport (PIN7) were downregulated in root tissues in plants exposed to Al toxicity (Kumari et al. 2008; Table 6.1). Thus, it seems reasonable to suggest that reduction in auxin transport in roots could likely be involved in triggering the characteristic phenotype associated with soil acidity.

In plants exposed to Al toxicity, an increase in endogenous CK levels, plant hormone which regulates different aspects of shoot and root growth (Kurepin et al. 2008, 2012c), may induce ethylene production in root tissues. This was shown in root tissues of bean (*Phaseolus vulgaris* L. cv. Strike) plants exposed to Al toxicity: a rapid increase in endogenous CK levels, particularly bioactive zeatin and dihydrozeatin, levels contributed to the inhibition of root growth and increase in root ethylene evolution (Galuszka et al. 2001). However, increase in the activity of CK dehydrogenase, a CK-degrading enzyme in plants exposed to Al toxicity, caused increases in IAA levels and ethylene production (Massot et al. 2002; Fig. 6.1). This is not surprising since CKs act antagonistically to auxins and ethylene in root growth and development (Kurepin et al. 2011a).

No change in ethylene production has been observed in root tips of Al-sensitive or Al-tolerant maize cultivars 24 h after exposure to Al toxicity (Gunse et al. 2000). However, Sun et al. (2007) observed a rapid increase in ACC synthase and ACC oxidase activities, ethylene biosynthetic enzymes (Abeles et al. 1992), as well as upregulated gene expression of ACC oxidase enzymatic activity and thus in ethylene

production following plant exposure to Al toxicity. Furthermore, application of ethylene biosynthetic inhibitor aminoethoxyvinylglycine (AVG) or perception inhibitor Co^{2+} (Abeles et al. 1992) resulted in a decrease in ethylene production or activity, respectively, as well as eliminating the root elongation inhibition caused by Al toxicity after 12 h (Sun et al. 2007). Recently, Tian et al. (2014) demonstrated that the increase in ethylene production in root apex and the inhibition of root elongation in wheat plants exposed to Al toxicity were both ameliorated by a pretreatment of Al-stressed plants with 2-aminoisobutyric acid (AIB), an inhibitor of both ACC synthase and oxidase activities (Abeles et al. 1992, Fig. 6.1). Besides, Al toxicity-mediated changes in plant ethylene production have been observed to cause changes in malate anion concentrations (Tian et al. 2014; Fig. 6.1). Malate concentrations decreased with the application of AVG or AIB and increased when AVG and/or AIB were co-applied with Al stress (Tian et al. 2014). In addition, the application of ethylene or its biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) application can generate a lower malate efflux from root apex in response to Al toxicity (Abeles et al. 1992; Fig. 6.1).

Chandran et al. (2008) reported that gene involved in ethylene biosynthesis was upregulated in *Medicago truncatula* in response to plant exposure to Al toxicity. The release of organic acids from root tissues into the rhizosphere is an important response to soil acidity (Hue et al. 1986; Piñeros et al. 2005). In this context, Kobayashi et al. (2013) showed that increase in IAA in response to soil acidity is essential for the expression of an organic acid transporter called AtALMT1 (Table 6.1). Therefore, phytohormones such as auxin and ethylene may play an important role during the plant adaptation to acidic soil and Al toxicity. Thus, the next challenge is to identify how the use of exogenously applied phytohormones could facilitate an increased plant tolerance to acid soils without compromising the crop yield in the face of an uncertain, climate-related future.

The application of GA and GA_3, a potent shoot dry biomass accumulation and stem elongation promoters (Park et al. 2013; Zaman et al. 2014; Kurepin et al. 2015d), has been shown to decrease oxidative stress of plants exposed to Al toxicity (Khan et al. 2015). This is achieved via activation of two pathways: (1) increased levels of reduced glutathione and superoxide anion which are generated in the membrane surface and (2) increased rate of linoleic acid biosynthesis, a precursor of JA (Khan et al. 2015) (Fig. 6.1). Furthermore, exogenous GA_3 application to plants exposed to Al toxicity increases chlorophyll content and stems biomass (Khan et al. 2015). Application of GA_3 to Al-tolerant tomato (*Solanum lycopersicum* L. cv. LA2710) plants exposed to Al toxicity also increased endogenous SA levels (Khan et al. 2015). Consequently, higher levels of SA enhanced the efficiency of antioxidant systems in stressed plants (Hayat et al. 2008), such as ascorbate peroxidases, CAT, guaiacol peroxidase, glutathione reductase, and superoxide dismutase, convert H_2O_2 to water, thus completing what has been called the water-water cycle (Panda and Patra 2007). This suggests a direct role of GA_3 and the activation of water-water cycle in alleviating Al-induced oxidative stress (Fig. 6.1).

Jasmonates are phytohormones involved in antioxidant responses to different stresses (Keramat et al. 2009). Application of volatile, methylated form of JA

(MeJA) to *Brugmansia x candida* plants exposed to Al toxicity increased H_2O_2 accumulation and thus peroxidase activity, lignin accumulation, and NADH activity (Spollansky 2000). A similar response was also reported for roots of *Cassia tora* plants exposed to Al toxicity: decreased root elongation and stimulated oxidative damage measured as Tbars content were observed after applying increased concentrations of MeJA (0, 1, 5, 10, and 15 μM) (Xue et al. 2008). Moreover, expressions of coenzyme A ligase (4CL, EC 6.2.1.12), lipoxygenases (LOX), and L-phenylalanine ammonialyase (PAL, EC 4.3.1.5) were upregulated by exposure to either Al toxicity or exogenous MeJA (Fig. 6.1). Thus, a possible role of MeJA in activating responses associated with jasmonate signal transduction defense mechanism(s) and secondary metabolism, which may decrease the Al toxicity effects in root tissues, could be suggested (Fig. 6.1).

Therefore, two main mechanisms explaining the role(s) phytohormones may play when plants are exposed to Al toxicity can be proposed: (1) the role played by ABA, ethylene, and IAA in the mechanism of Al resistance via exudation of organic acids and (2) the secondary metabolism activation by the application of exogenous GA_3 and MeJA (Fig. 6.1). There is so far no evidence of a direct link between these groups.

6.5 Conclusions and Future Perspectives

The role of individual phytohormones and their cross-interaction with acidity and Al toxicity has been discussed in this chapter. Metabolism of different phytohormones is modified under acid soil conditions. However, further studies are needed to extend our knowledge and establish the mechanisms involved in phytohormonal responses to low pH stress. Most studies show separately the effect of hormones or soil pH, and the interaction between them is only showed in a few researches. Further studies would be critical in addressing the role of phytohormones in improving plant tolerance to acid soils and thereby increase the crop yield. Therefore, the integration of molecular and biochemical studies are necessary to know phytohormone mechanism under acid soils.

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Chapter 7
Use of Phytohormones for Strengthening Metal(loid) Phytoextraction: Limitations and a Case Study

Meri Barbafieri

Abstract Phytoextraction is a branch of phytoremediation technology in which plants are used to remove polluting substances from the environment. Among inorganic contaminants, metal(loid)s are an important problem. Of the abiotic stresses that strongly impact plant growth and healthy food, metal(loid)s in soil are an important and challenging area for scientists working on agricultural and environmental issues. Phytoextraction is basically a modified "agronomic" practice where plants are used not for food production but for "cleaning" and/or rendering contaminated media less harmful. Many factors, such as the survival of plants at high-contaminant concentrations, low soil fertility conditions, and unfavorable pH values, diminish the potential of this technique. Another factor to consider is the long period of time it can take to reduce contaminants to the level of natural content or to the limit established by the laws and regulations of each country. Very recently, phytohormones were studied to resolve several phytoremediation limitations. Interesting results (presented as a case study in the second part of the chapter) were obtained from our laboratory at ISE-CNR in Pisa on the use of phytohormones as an exogenous treatment for different phytoextraction mechanisms. It was shown to counteract difficulties plants have in combating phytotoxicity effects and to improve the technology's efficiency.

Keywords Induced/natural phytoextraction • Phytohormones • Phytoremediation • Phytotoxicity • Soil contamination

7.1 Introduction

Environmental contamination has spurred the scientific world to seek new techniques for rehabilitating contaminated areas and restoring them for social use in clean and/or profitable ways. When phytoremediation – an environmentally friendly

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and economically feasible technique – was conceived, it attracted great scientific and technical interest. Phytoremediation technologies offer a broad range of applications, and can be used for many kinds of contaminants (organic and inorganic) and media (soil, sediment, water, waste, etc.). The technology attracted the attention of stakeholders and legislators due to its low environmental impact and low cost; thus, this sustainable technology has received great attention from the green economy.

Many studies on phytoextraction, the use of plants to "extract" metals from contaminated soil, have focused on overcoming two important drawbacks: the survival of plants in unfavorable environmental conditions (contaminant toxicity, low fertility, etc.) and the often lengthy time it takes to reduce contaminants to the desired level.

The investigations integrating soil chemistry with plant biology expertise (on different experimental scales, from microcosm to field pilot trials) have shown that specific soil treatments combined with plant treatments led to impressive advances in the technology's performance.

Phytoremediation is basically a modified "agronomic" practice. It is not used for food production but for "cleaning" and/or rendering contaminated media less harmful. Consequently, different plants and plant abilities are needed. Moreover, different agronomic inputs to increase and maximize phytoremediation efficiency must be established and applied.

We speak of "assisted phytoremediation" when more than one action is used to "push" plants to increase their cleaning performance.

Here we describe and discuss the use of phytohormones as a recent alternative strategy that has attracted the attention of researchers for assisted phytoextraction. Phytohormones play a major role in cell division and cell differentiation. They can stimulate shoot initiation, bud formation, the growth of lateral buds, leaf expansion, and chlorophyll synthesis. They can also delay leaf senescence; enhance resistance to salinity, low temperature, and drought; and induce stomatal opening in some species (Letham et al. 1978; Pospisilova et al. 2000; Werner et al. 2001).

Application of exogenous phytohormones is examined as a viable technique for increasing the efficiency of metal extraction from contaminated soils by plants (Fuentes et al. 2000; Barbafieri and Tassi 2011; Barbafieri et al. 2012; Piotrowska et al. 2011; Bulak et al. 2014). Results also suggest that the chemical regulation of stomatal behavior by phytohormones can improve water and contaminant uptake in order to control and contain contaminant migration in subsurface water. However, further experiments are needed to increase our knowledge of the dynamics of the transport mechanism involving metal uptake, since this mechanism is dependent on plant characteristics and environmental parameters.

This chapter aims to discuss: (1) phytoextraction methods for metal-contaminated soil, and (2) the potential use of phytohormones as assistants to improve phytoextraction efficiency. Moreover, a case study is presented that involves the developed use of cytokinin on boron-assisted phytoextraction by a patented modulated application of nitrogen and phytohormone to a crop of *Helianthus annuus* grown on boron-contaminated soil.

7.2 Phytoextraction of Metal(loid)s from Contaminated Soil

7.2.1 The Technology

Contamination by heavy metals continues to pose a serious challenge for the remediation of polluted soils, as they are not degradable and have to be removed physically. Most currently used technologies for removing heavy metals from the soil strongly affect the biogeochemical characteristics of the soil. In many cases, the soil cannot even be considered a useful and productive soil resource anymore, and the treated soil has to be disposed of in landfills.

Phytoremediation is a broad term that comprises several technologies using plant properties as a tool to clean up water and soil.

Phytoextraction refers to the translocation of metal contaminants from soil up to the above-ground tissues by the root system (Baker and Books 1989; McGrath and Zhao 2003). After plants have grown for a certain period, they are harvested and may be incinerated to recycle the metals. This procedure, repeated several times, brings soil contaminant levels down to below legally acceptable limits. The time required for remediation depends on the type and extent of heavy contamination, duration of the growing season, the amount and characteristics of the produced biomass, and the plants' natural ability to accumulate heavy metals. Generally, phytoextraction is only applicable for sites containing low-to-moderate levels of metal contamination or involving treatment of only the mobile fraction of the contaminants.

Two different phytoextraction methods can be distinguished: *natural phytoextraction* – using natural metal hyperaccumulator plants that absorb, translocate, and accumulate an enormous amount of metals during their entire life period without visible toxicity symptoms; *induced phytoextraction* – using crop plants, usually at high biomass production, in which the accumulation process is induced by increasing contaminant bioavailability in soil via the addition of chemicals (Fig. 7.1). Synthetic amendments such as chelates (e.g., EDTA, EDDS, NTA), organic acids (e.g., citric acid), or ion competitors such as phosphate (Tassi et al. 2004) added to the soil enhance metal bioavailability, although the soil microbial community is usually neglected and there is a potential risk of leaching of metals to groundwater (Dickinson et al. 2009). The term *assisted phytoextraction* is used when more than one action is used to increase the technology's efficiency (Barbafieri et al. 2013). Several strategies to achieve higher heavy metal removal efficiency have been recently developed, such as enhancing the concentration of soluble heavy metals in the soil by adding synthetic chelate agents (e.g., EDTA). This then leads to an increase in the metal uptake of high biomass crop plants (e.g., *Brassica juncea*, *Helianthus annuus*, *Zea mays*, *Nicotiana tabacum*, etc.) (Meers et al. 2005; Di Gregorio et al. 2006). Moreover, bacteria inoculations (Ma et al. 2015) and setting up ad hoc agronomic practices (Giansoldati et al. 2012; Barbafieri et al. 2013; Bani et al. 2015) have been proposed to assist phytoextraction processes (Pedron et al. 2009; Pedron et al. 2014; Petruzzelli et al. 2014; Cassina et al. 2012).

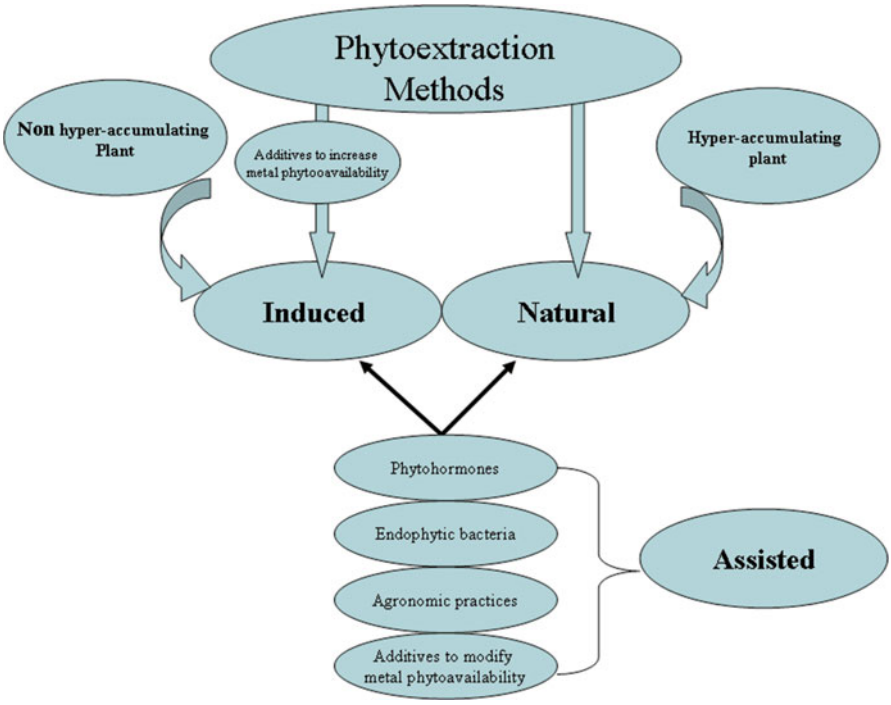

Fig. 7.1 Phytoextraction methods and "assistants" to improve the technology's efficiency. Induced phytoextraction: the use of non-hyperaccumulator plants requires additives to increase metal availability in soil. Natural phytoextraction: the use of hyperaccumulator plants. Each mechanism can be assisted to increase efficiency by using phytohormones, endophytic bacteria, agronomic practices, and/or additives to modify metal availability in soil

Effective phytoextraction requires both plant genetic ability and optimal soil and crop management practices (Prasad 2004; Di Gregorio et al. 2006; Tassi et al. 2008; Pedron et al. 2009). *Thlaspi caerulescens* (Cd and Zn hyperaccumulator) and *Brassica juncea* (heavy metal accumulator) are examples of species that well represent the two phytoextraction strategies described above. Metals such as Ni, Zn, Cu, and As are the best candidates for removal by phytoextraction, although Cd, Pb, etc. have been extensively studied as well. Genetic engineering studies have been performed to manipulate plant accumulation by means of the overexpression or knockdown of membrane transporter proteins (Rogers et al. 2000).

The hazardous plant biomass must be disposed of, in order to minimize environmental risk. The waste volume can be reduced by thermal, microbial, physical, or chemical means such as composting, compaction, or thermochemical conversion processes (combustion, gasification, and pyrolysis). Recycling the biomass from phytoextraction for fuel and other uses cuts down on the need for landfills and provides the contaminated site with an economic value. Added value to the phytoextraction process could be obtained by combining the biomass produced as an energy

source, resulting in an ore after incinerating the residual biomass. This would be possible in the case of phytomining, a particular example of phytoextraction. Phytomining involves the exploitation of subeconomic ore bodies using hyperaccumulator plants. For instance, the species *Alyssum bertolonii* and *Berkheya coddii* have a high potential for extracting Ni, due to their high biomass and a Ni concentration of 1 % in the dry matter (Robinson et al. 2003). Other metals such as gold, thallium, and cobalt have been extracted from tailings or other residues of low commercial value (LaCoste et al. 2001; Keeling et al. 2003). Heavy metal phytoextraction refers to the use of plants that can remove contaminants from soil and accumulate them in a harvestable part in a process alongside water and nutrient absorption by roots. Therefore, plant biomass production and the metal concentration in the biomass are fundamental success factors for the practical efficiency of phytoextraction (McGrath and Zhao 2003).

Among tolerant plants, three general classes of vegetation have been defined and recognized as growing in contaminated and metalliferous soils (Barbafieri et al. 2011). These comprise *metal excluders* (plants that avoid the translocation to upper parts over a broad range of metal concentrations in the soil, but can have large amounts in their roots), *metal indicators* (plants that accumulate metals in their above-ground tissues where the metal levels in the plant reflect metal levels in the soil), and *metal accumulators* (usually referring to hyperaccumulators that concentrate metals in the above-ground tissues to levels higher than 1 % of Zn, Mn; 0.1 % of Ni, Co, Cu, Cr, Pb, Al; 0.01 % of Cd Se; or 0.001 % of Hg on a dry-weight basis irrespective of the metal in the soil) (Baker and Books 1989).

7.2.2 *Mechanisms Associated with the Phytoextraction of Inorganic Contaminants*

Generally, it is agreed that the uptake of ions by plant roots consists of two phases: adsorption and accumulation.

Adsorption is predominantly related to cations in soil solution and is a non-metabolic process, whereas accumulation is an active process with specific carrier transporters. More specifically, the availability to plants of metal contaminants is governed by a variety of reactions that include complexation with organic and inorganic ligands, ion exchange and adsorption, precipitation and dissolution of solids, and acid-base equilibria. Thus, the extraction of metals by plants is usually limited by their availability in the soil.

The rates of redistribution of metals among the solid and liquid phases and their binding intensity are affected by the metal species, loading levels, aging, and soil properties.

The more bioavailable fractions for plant uptake are generally those with higher water solubility, i.e., metal fractions following the order: exchangeable > carbonate specifically adsorbed > Fe-Mn oxide > organic sulfide > residual (Barbafieri et al. 1996; Li and Thornton 2001). These fractions can be estimated by various extrac-

tion procedures (generally sequential extraction schemes are used to isolate specific fractions). Soft extractants such as non-buffered salt solutions, diluted acids, and complexing agents better correlate with the fraction for plant uptake (Lindsay and Norwell 1978). Neutral salts mainly dissolve the cation exchangeable fraction. Diluted acids partially dissolve trace elements associated with different fractions such as exchangeable carbonates, iron and manganese oxides, and organic matter. Complexing agents dissolve not only exchangeable element fractions but also the element fraction forming organic matter complexes and the element fraction fixed on soil hydroxides (Rauret 1998; Barbafieri 2000).

As a general rule, readily bioavailable inorganics for plant uptake include As, Cd, Cu, Ni, Se, and Zn. Moderately bioavailable metals are Co, Fe, and Mn, whereas Cr, Pb, and U are not very bioavailable (EPA 2009). Petruzzelli et al. (2015) demonstrated the importance of metal bioavailability in the context of phytoextraction technology.

Inorganic contaminants may be localized differently in roots and shoots, depending on the type of plant (sensitive or tolerant, i.e., metal excluder, indicator, or accumulator species). Recent studies have identified potential cellular/molecular mechanisms involved in the resistance and tolerance of plants to high concentrations of inorganic contaminants in the environment. Generally, the plants avoid metal accumulation in the cytosol to prevent toxicity symptoms. Plants present various mechanisms, and most of them are shared by several common metal-tolerant plants. However, it seems that specific mechanisms are employed for specific metals in particular species (Hall 2002; Clemens 2006; Hanikenne et al. 2008). For example, nickel hyperaccumulation was found in some specialized flora such as the *Alyssum murale* that has colonized Ni-rich serpentine soils. It was also shown that more than one mechanism may be involved in reducing the toxicity of a particular metal (Salt et al. 2000; Hartley-Whitaker et al. 2001). Inorganic contaminants (usually ions) are taken up by biological processes via membrane transporter proteins. The transporter proteins have unique properties (transport rate, substrate affinity, substrate specificity, etc.) that are regulated by the level of specific metabolites or regulatory proteins. The abundance of each transporter also depends on the tissue type and on environmental conditions (Pilon-Smits 2005).

The different mechanisms for the detoxification of metals and thus for increased tolerance to metal stress can be subdivided into mechanisms at the extracellular level and those at the cellular level.

Extracellular strategies include roles for mycorrhizas, the cell wall, and extracellular exudates. They can also involve the plasma membrane, which reduces the uptake or stimulates the efflux out of the cytosol.

Mycorrhizas and particularly ectomycorrhizas can be effective in reducing the effects of metal toxicity on host plants (Jentschke and Godbold 2000), but elucidation of their mechanisms is difficult, due to the high specificity of metals and fungal species. These mechanisms consist of various exclusion processes (reduced access to the apoplast due to the hydrophobicity of fungal sheath, chelation by fungal exudates, and adsorption onto the external mycelium) that restrict either the metal's movement to the host roots or the absorption of metals.

Metals in the soil solution are in direct contact with the root cell wall, but its adsorption has a limited effect on metal activity at the surface of the plasma membrane. However, root exudates can play a role in tolerance to metals. For example, Salt et al. (2000) showed that root exudates of non-hyperaccumulating plants (histidine and citrate) promote the accumulation of Ni-chelating compounds in roots, and state that exudates could have a role in a Ni-detoxification strategy. Ma et al. (2001) showed that buckwheat plants secrete oxalic acid from the roots in response to Al stress, and a nontoxic Al-oxalate compound was found in its leaves. Phytosiderophores (biosynthesized from nicotianamine) are mucigenic acids released by roots, and together with the proper nicotianamine, the organic acids (citrate, malate, histidine) and the thiol-rich peptides (glutathione [GSH] and phytochelatins [PCs]) have the ability to chelate metals at the root level. Chelated metals in roots may be stored in vacuoles or exported to the shoot via the xylem.

Heavy metals could affect the plasma membrane, and its damage would produce increased leakage of solutes from cells. The cell membrane of metal-tolerant plants may play an important role in preventing or reducing entry into the cell or through efflux mechanisms. One example of reduced uptake as an adapted tolerance mechanism to arsenic toxicity was found in *Holcus lanatus*. Phosphate and arsenate appear to be taken up by the same system (Tassi et al. 2004). The arsenate-tolerant genotype showed a much lower uptake for both anions than the non-tolerant genotype. Moreover, it was suggested that arsenate tolerance in *H. lanatus* is due to both this adaptive suppression transport system and constitutive phytochelatin production, since arsenate can still accumulate to high levels in tolerant plants (Meharg and Macnair 1992; Hartley-Whitaker et al. 2001).

The efflux of metal ions is another strategy for controlling extracellular metal levels at the plasma membrane. Membrane transporters are responsible for the uptake, efflux, translocation, and sequestration of essential and non-essential mineral nutrients. Although there is little direct evidence of plasma membrane efflux transporters in metal tolerance in plants, recent research has revealed that several classes of metal transporters could play a key role in tolerance. These include the Zn-regulated transporter (ZRT), Fe-regulated transporter (IRT)-like proteins (ZIP), natural resistance-associated macrophage proteins (Nramp), and cation diffusion facilitator (CDF) (Kim et al. 2004; Lee et al. 2007).

Root-to-shoot translocation requires an intermediate step of membrane transport. Membrane transporter proteins are needed to promote the movement of inorganics from the root endodermis to the root xylem. It is an area under study and is still unclear via which transporter proteins most metals are exported to the root xylem. The bulk flow in the xylem from root to shoot is driven by the transpiration stream from the shoot, where negative pressure pulls up water, solutes, and the bioavailable inorganic contaminants that have access through the root membrane. Chelators such as organic acids (histidine, malate, citrate), nicotianamine, and thiol-rich peptides can bind metals during xylem transport. Another membrane transport step is required to import metals into the leaf cells from the leaf xylem. Again, specific membrane transporter proteins are needed. Once inside the leaf symplast, the metal may be kept away from metabolic processes by compartmentation at the tissue level (epidermis or trichomes) or at the cellular level (vacuoles or cell wall).

At the *intracellular level*, other potential mechanisms exist for the repair of stress-damaged proteins (heat shock proteins or metallothioneins); for the chelation of metals in the cytosol by organic acids, amino acids, or peptides; and for transport away from metabolic processes with compartmentation in the vacuole. At the tissue level, metals were sequestered by chelators to form conjugates and aid in the detoxification process. Chelators such as the tripeptide GSH (glu-cys-gly) and the PCs can bind metals, and by the action of active transporter molecules, the metal-chelate complex can be transported to the vacuole where it can be further complexed, for example, by sulfide on PC-Cd complexes (Hall 2002; Pilon-Smits 2005; Memon and Schroder 2009).

The induction of metal-chelating proteins such as metallothioneins and phytochelatins increased the level of cell tolerance to an excess of metal ions by the sequestration of metals and accumulation in plant cells. Moreover, plants with high transporter activities from cytosol to vacuole can be more efficient at storing toxic inorganic contaminants. Thus, the overexpression or knockdown of membrane transporter proteins or the alteration of plant chelator production by genetic engineering allows us to manipulate plant accumulation (Memon and Schroder 2009). Selected or engineered plants that have high levels of transporters involved in the uptake of metals from the xylem into the leaf symplast are recommended for phytoextraction, while selected or engineered plants with higher production of chelators or conjugates that confer an enhanced sequestration and tolerance could be useful for phytostabilization.

Figure 7.2 schematically shows the main mechanisms regarding inorganic detoxification.

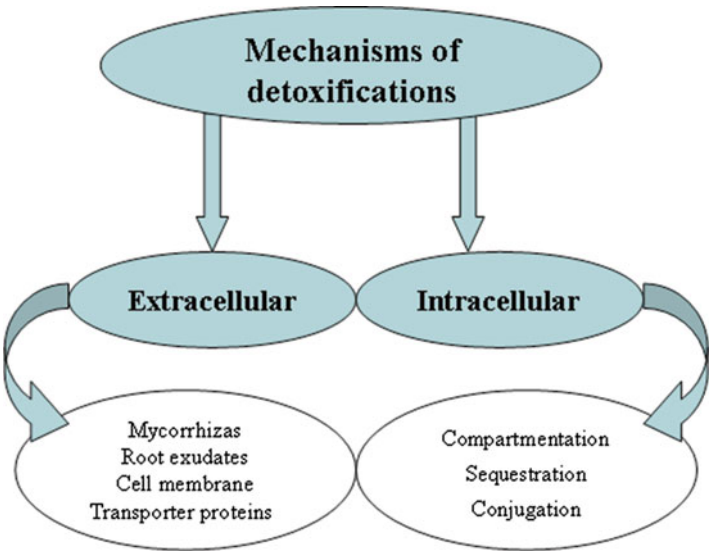

Fig. 7.2 Mechanisms of detoxification of inorganics in plants: extracellular mechanisms and intracellular mechanisms

7.3 Phytohormones and Their Help with Phytoextraction Technology

Phytohormones regulate and control virtually every aspect of plant growth and development (Ahammed et al. 2015). Phytohormone research is a crucially important area of plant science; phytohormones are one of the key systems that integrate metabolic and developmental events in the entire plant and the response of plants to external factors. Thus, they influence the yield and quality of crops.

As agriculture becomes more mechanized and science increases the possibilities for using various inputs to enhance production, the role of plant growth regulators (PGRs) becomes vital. PGRs in agriculture and horticulture provide agriculture professionals and researchers with the information needed to effectively tap these versatile resources to enhance crop production and quality. Phytoremediation can also obtain profitable benefits from the application of PGRs used in classic agricultural practices, but with special PGR and application protocols. Thus, phytoremediation that is aided by phytohormone treatment can be called "assisted phytoremediation by plant growth factor (phytohormone)" (Barbafieri et al. 2012).

Plant growth regulators could benefit phytoremediation in various ways, increasing the efficiency of a selected "depolluting plant." As an example, it is deliverable for a "depolluting plant":

1. To increase survival and development in stressful conditions; contaminated sites are often quite poor in fertility regarding soil structure and nutrient content.
2. To increase tolerance to toxic effects of different contaminants (organics and/or inorganics) at the level of concentration present in contaminated media.
3. To increase the rate of contaminant treatment (increasing degradation of organics, or increasing biomass production and metal uptake, and accumulation in the upper plant in case of inorganics).

The way in which phytohormones could be used to manipulate plants is shown schematically in Fig. 7.3 (Barbafieri et al. 2012).

7.3.1 Some Evidence Regarding Phytohormones Application in "Induced" Phytoextraction

Few studies have been conducted to verify the possible use of phytohormones for phytoremediation purposes (summarized in Table 7.1). Most of them focus on increasing stress resistance to metal toxicity and its uptake for phytoextraction as an alternative strategy for increasing the efficiency of "induced phytoextraction" (Ouzounidou and Ilias 2005; Tassi et al. 2008).

Some studies have examined the combined effects of phytohormones and heavy metals, most performed in hydroponics (Ouzounidou and Ilias 2005) or in spiked soil (Sayed 1997; Khan and Chaudhry 2006). Only a few publications (Fuentes

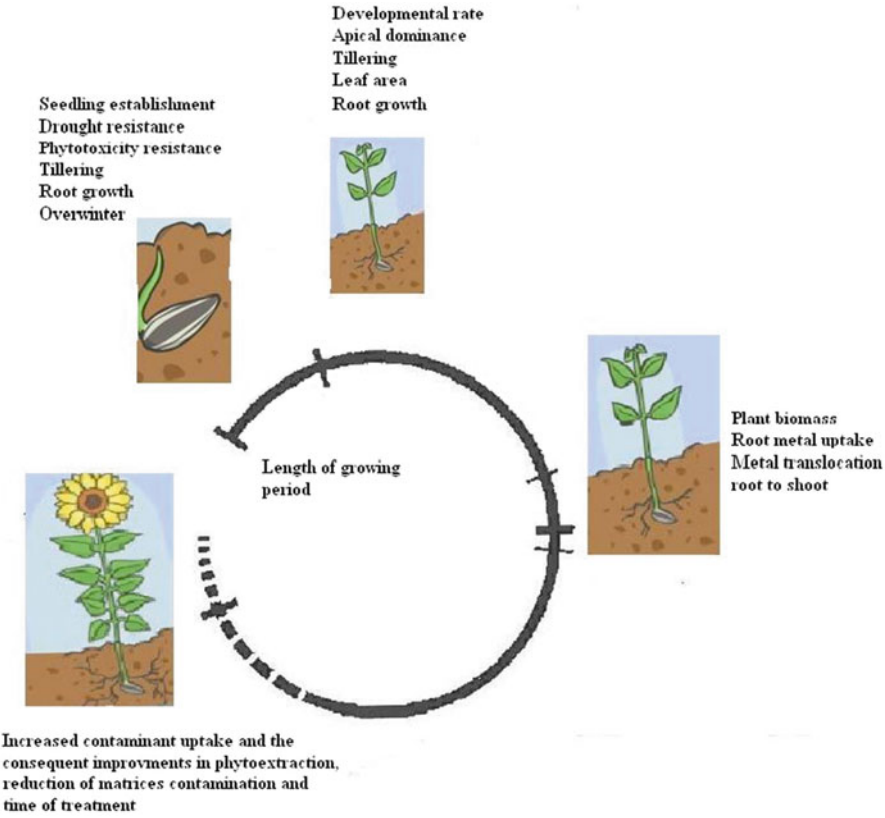

Fig. 7.3 Possible targets for manipulating plants for phytoextraction purposes by using phytohormones (From Barbafieri et al. 2012)

et al. 2000; Liphadzi et al. 2006; Tassi et al. 2008) have focused on the application of phytohormones in crop plants grown in highly contaminated soils, to improve phytoextraction. Fuentes et al. (2000) treated corn (*Zea mays*) by spraying either indolebutyric acid (IBA) or naphthylacetic acid (NAA), which resulted in a 41.2 % increase in Pb uptake using IBA and a 127.4 % increase in Pb and a 59.5 % increase in Zn uptake using NAA. Nevertheless, these results led to a high mortality (up to 45 % of the treated plants), a huge PGR concentration (NAA sprayed at 1000 mg kg^{-1}), and a decrease in overall biomass. Liphadzi et al. (2006) used IAA to increase root growth in sunflower plants and observed an increase in Cd and Pb accumulation in leaves with and without EDTA treatment in soil.

Cytokinins (CKs) could prove to be interesting phytohormones for phytoremediation. They are plant growth regulators that play a major role in cell division and cell differentiation. CKs can stimulate shoot initiation, bud formation, the growth of lateral buds, leaf expansion, and chlorophyll synthesis. CKs can also delay leaf senescence; enhance resistance to salinity, low temperatures, and drought; and

Table 7.1 Phytohormones used for phytoextraction in metal-contaminated soil or spiked solutions: (A) induced phytoextraction, (B) natural phytoextraction

Phytohormone	Concentration	Application form	Amendment	Plant species	Substrate	Main result	References
A) *Induced phytoextraction*							
IBA	10	Dip and spray	EDTA at 10 or 20 g/L	Corn	Mine site soil high in Pb and Fe	NAA increased 21% accumulation of Pb and Fe compared to IBA	Fuentes (2001)
NAA	100						
	1000 µg/mL						
NAA	700 µg/mL	Spray	50 or 100 mL/kg soil of NPK at a ratio of 5.6:1:3.1	Corn	1 kg of mine site soil in pots	100 mL nutrients increased Cu, Pb, and Fe accumulation by 108, 150, and 174%	Fuentes (2001)
IAA	3 and 6 mg/L	Applied to soil and spray to leaves	EDTA 1 g/kg soil	Sunflower	Soil sludge or composted soil	With/without EDTA, in composted soil IAA, increased Cd and Pb in leaves	Liphadzi et al. (2006)
						Without EDTA in soil sludge IAA increased Mn and Ni in leaves	
IAA, GA, kinetin	1, 10, and 100 μM	Added to nutrient solution	EDTA 0.2 mM	Alfalfa	Hydroponics	Kinetin plus EDTA increased Pb in leaves 17-, 43-, and 67-fold, respectively, compared to leaves of plants treated with Pb alone and 2-, 5-,and 8-fold compared to plants treated with Pb/EDTA	Lopez et al. (2007a)
IAA-kinetin	100 μM each	Added to soil	EDTA 0.8 mM	Alfalfa	Soil spiked with Pb at 80 mg/kg	Plants treated with EDTA plus IAA-kinetin had significantly more Pb in stems and leaves compared to Pb alone and Pb/EDTA	Lopez et al. (2009)

(continued)

Table 7.1 (continued)

Phytohormone	Concentration	Application form	Amendment	Plant species	Substrate	Main result	References
Kinetin	100 μM	Added to soil	CDTA 2.5 mM cystein 0.5 mM NTA 5 mM	*Prosopis* sp.	Soil spiked with As(III) 30 mg/kg and As(V) at 50 mg/kg	Kinetin plus cysteine increased total As in roots by 36 % from As(III) and 65 % from As(V). While kinetin plus CDTA increased total As in roots by 20 % from As(III) and by 100 % from As(V)	Lopez et al. (2008)
IAA	250 and 500 μM	Added to hydroponic solution		Corn	Hydroponic solution containing Pb at 20 μM	IAA at 500 μM increased Pb in roots by 144 % 5 days after treatment application; however, Pb in shoots decreased by 47 %	Wang et al. (2007)
Cytokin® (several cytokinin)	20 mg/L	Foliar spray at 22nd, 28th, and 33rd days after germination	EDTA 2 mM/kg soil in four diluted doses (one per dose) starting on day 23 after germination)	Sunflower	50 g of contaminated soil plus 150 g of inert silica pellets	EDTA plus PGRs	Tassi et al. (2008)
Phytagro (mixture of phytohormones)						Increased biomass production	
Kinetin						Cytokin plus EDTA increased Pb accumulation > 600 % in leaves and > 300 % in stems compared to control	
						Accumulation of Zn in leaves increased > 170 %	

IAA and NAA	1, 10, and 100 µM	Solution applied to 15-day-old plants in nutrient solution	EDTA 100 mg/L	Rattlebush (*S. drummondii*)	Half strength Hoagland solution spiked with Pb at 500 mg/L	Compared to control, NAA increased Pb in shoots by 654 % and IAA by 415 %	Israr and Sahi (2008)
						NAA plus EDTA increased Pb in shots 1349 % and IAA plus EDTA by 1252 %	
IAA	10^{-10} M	Applied to 6-day-old plants in nutrient solution	EDDS 500 µM	Sunflower	10 % Hoagland solution spiked with Zn (15 µM) or Pb (2.5 µM)	IAA plus EDDS increased Zn in shoots	Fässler at al. (2010)
						Pb was not detected	
B) *Natural phytoextraction*							
Cytokin®	10 ml per pot of solution A (15 mg/kg)	Foliar application 20 days after transplant. Solution A	None	*Alyssum murale*	4 kg of soil containing Ni at 1521 mg/kg	Foliar treatment increased shoot biomass by 53 %, soil treatment by 41 %, and the combined soil+leaves by 75 %	Cassina et al. (2011)
	50 ml/per pot of solution B (3 mg/kg)	Soil application 20 days after transplants. Solution B				Foliar plus soil treatment increased Ni phytoextraction by 75 %	
		Combined treatment foliar + soil					
Kinetin	250 µM	Solution applied to soil 15 days after germination	None	Mexican palo verde	1800 g of soil contaminated with Cr(III) at 60 mg/kg and Cr(VI) at 10 mg/kg	Compared to controls, kinetin increased total Cr accumulation by 45, 103, and 72 % in root, stem, and leaf of Cr(III)-treated plants and 53, 129, and 168 % in Cr(VI)-treated plants, respectively. Kinetin increased ascorbate peroxidase enzyme activity in Cr(VI)-treated plants	Zhao et al. (2011)

(continued)

Table 7.1 (continued)

Phytohormone	Concentration	Application form	Amendment	Plant species	Substrate	Main result	References
28-homo brassinolide	10^{-10} to 10^{-6} M	Seeds were soaked in the HBL solution for 8 h	None	Indian mustard	Acid-washed sand spiked with Cu at 50, 100, 150 mg/kg	HBL-treated plants had roots and shoots ~62 % and ~93 longer, respectively; 47 % more leaf area and 93 % higher dry mass compared to untreated plants	Fariduddin et al. (2009)
Cytokin, Promalin	15 mgl/L, 60 mg l/L and 60 mg l/L	Only spray foliar application three treatments at 5-day intervals	None	*A. corsicum*, *A. malacitanum*, *A. murale*, and *A. pintoda-silvae*	In pot 15 cm diameter with 1.5 kg of soil with total Ni 4707 mg/kg	No clear effect on biomass production and on Ni phytoextraction	Cabello-Conejo et al. (2013)
Four different PGR products commercially available with different phytohormones composition of: GA, CK, and IAA	0.1, 1, 10 mg/L; 1, 5, 10 mg/L; 0.01, 0.05, 0.1 mg/L; 5, 30, 50 mg/L	Only spray foliar application, three treatments at 2-week intervals		*Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum mural*, *Noccaea goesingense*	In 12.5-cm-diameter pot with 0.7 kg of soil with total Ni 2092 mg/kg	Variable effect depending on phytohormone and plant species. IAA most effective in increasing phytoextraction	Cabello-Conejo et al. (2014)

Revised from Barbafieri et al. (2012)

induce stomatal opening in some species (Letham et al. 1978; Pospisilova et al. 2000; Werner et al. 2001). Transpiration in plants is driven by a combination of abiotic (climate, soil water availability, ground water depth, etc.) and biotic (leaf area, stomatal functions, root amount and distribution, hormone synthesis, etc.) conditions (Letham et al. 1978). The rate of transpiration is also directly related to whether the stomata are open or closed, and it accounts for the movement of water from roots to shoots by subsequent water loss as vapor through the stomata. An increase in transpiration rate has been observed from 20 to 40 % when stomata are wide open (Vose et al. 2003). The chemical regulation of stomatal behavior is one strategy for improving water and contaminant uptake, since water absorbed at the roots by osmosis carries any dissolved mineral nutrients and/or soluble contaminants through the xylem. Thus, transpiration is considered a key process for the success of phytoremediation in soil and groundwater, since the vegetation must transpire enough water to control or take up contaminants (Rock 2003). The application of exogenous CKs can increase the transpiration rate and consequently the absorption of contaminants present in the soil solution. An increased transpiration rate in the excised leaves of *Brassica*, *Helianthus*, *Anthephora*, *Avena*, *Hordeum*, *Triticum*, and *Vigna* was found after the addition of exogenous CKs (kinetin, N6-benzyladenine, and zeatin) (Pharmavati et al. 1998; Pospisilova 2003). Kinetin also markedly increases the stomatal aperture in *Tradescantia* and *Paphiopedilum tonsum* leaves (Irving et al. 1992).

Results reported in Tassi et al. (2008) indicate that in treated sunflower plants, the exogenous application of a mixture of CKs had a positive effect on the aerial biomass and phytoextraction efficiency and increased the transpiration rate. CK treatment showed high Pb phytoextraction efficiency in leaf and shoot (about 120 % and 50 %, respectively) when compared to the control plants. Again, the treatment also showed a high increase for Zn, which was about 100 % in leaves and 20 % in shoots, compared to the control plants. When the EDTA treatment was added to increase metal bioavailability in the soil, CK treatment increased the transpiration rate by about 50 %, but the treatment did not produce a meaningful increase in the overall aerial biomass, but induced a higher increase in the biomass of leaves (about 30 %), which are the aerial tissues with the greatest Pb accumulation. The most positive result in phytoextraction efficiency was achieved when both CKs and EDTA were used. This result supports the hypothesis that phytoextraction can be improved by increasing both the dry mass and (to a lesser extent) the metal accumulation in the upper parts of the plants. The regulation of stomatal opening is also an important effect of CKs. This in turn can increase the transpiration rate and consequently the flux (via xylem sap) of water-soluble soil components or contaminants to the upper parts of plants. Results show that the application increased the transpiration rate of *H. annuus*. The increased transpiration rate and aerial biomass of plants treated with PGRs indicate that water and micronutrients are efficiently absorbed from the soil and translocated to the parts of the plant that are above-ground, but were only able to remove a limited fraction of soluble metals. This suggests that phytohormones can increase the metal translocation of an already absorbed metal fraction, as demonstrated in combined treatments with PGRs and EDTA.

Increasing plant transpiration alone does not lead directly to an increase in metal uptake. Many indubitably confirm the fact that metal absorbing processes are strictly regulated by complex metal homeostasis in the plant (Clemens 2001; Clemens et al. 2002; Verbruggen et al. 2009) as previously described in section 2.4 (mechanisms associated with the phytoremediation of inorganic contaminants).

The combined effects of EDTA and the growth-promoting 3-indoleacetic acid (IAA) on Pb uptake in *Medicago sativa* plants have been studied in hydroponics. Increased Pb content in leaves of plants exposed to Pb and EDTA/IAA is about 2800 % compared to those treated with Pb alone and about 600 % compared to those treated with Pb and EDTA (Lopez et al. 2005).

The results, published by Cassina et al. 2012, are really the first that investigated Hg phytoextraction in combination with plant growth factor (cytokinin, CK) and thioligand (TS) treatments as a viable technique to increase the efficiency of plant Hg removal. Hg is highly toxic and phytotoxic, so the study takes into consideration both the Hg transfer from soil to plant tissues and the effects on plant growth. The investigation provides new insights into exploring a new strategy to effectively increase Hg uptake by crop plants, with possible applications for phytoremediation technology. The obtained data have high scientific impact, since Hg is one of most difficult elements to approach when no hyperaccumulator plant is known in current plant-based technology. Results show that the combination of CK and TS had synergistic effects on Hg phytoextraction (increased up to 450 %). In one growing cycle, the plants subject to the combined treatment significantly reduced labile-Hg pools by about 40 %.

7.3.2 Some Evidence Regarding Phytohormones Application in "Natural" Phytoextraction

Phytohormones have a potential role in improving "natural" phytoextraction. An initial trial and results were reported on *Alyssum murale*, a well-known Ni hyperaccumulator (Cassina et al. 2011). In the study, 70-day-old *A. murale* plants were transplanted to plastic pots containing 4 kg of serpentine soil with 1521 mg/kg Ni (approximately 25 mg/kg diethylenetriaminepentaacetic acid (DPTA)-extractable). Twenty days after transplanting, plants were treated every 6 days and harvested after three foliar, in-soil, and combined soil and foliar applications of a mixture of cytokinins. The phytoextraction data indicated a significant tendency to raise higher values of Ni phytoextracted in the combined treatment (foliar + soil) where the amount of Ni phytoextracted increased by 75 %. No significant differences were found in Ni concentration in shoots, also due to the high data variability. However, significantly higher shoot biomass was found in all treatments compared to the controls; moreover, significant differences were observed among the different methods of cytokinin treatment. The foliar treatment increased the shoot biomass by 53 %, the soil treatment by 41 %, and the combined treatment (foliar + soil) by 75 %. The results suggest that *A. murale* is a plant species sensitive to cytokinin treatment and that cytokinin treatment is potentially useful for increasing phytoextraction capability by increasing biomass.

Other evidence has been reported on *Alyssum* plant species and various phytohormones (Cabello-Conejo et al. 2013, 2014). The most effective treatment was observed with cytokinin and IAA treatments, providing a significant increase in % Ni phytoextraction. In all cases the effects were due to the increased plant biomass. Currently, no increase in metal uptake has been observed. Hence, the development of an exact protocol for using plant hormones for hyperaccumulator plants remains open.

7.4 Case Study: Modular Application of Phytohormone and Nitrogen in the Phytoextraction of Boron by *Helianthus annuus*

The research reported here on phytoextraction focuses on overcoming two main drawbacks: the survival of plants in unfavorable environmental conditions (contaminant toxicity, low fertility, etc.) and the often lengthy time it takes to reduce contaminants to the desired level.

This study (patented by Barbafieri 2014) evaluated the effect of exogenous phytohormone (cytokinin) in promoting growth and alleviating stress in sunflower plants exposed to boron (B)-contaminated sediments. It is necessary to point out that B phytoextraction can be approached without hyperaccumulators or any chemical addition to increase B bioavailability, as it is readily available in soil and effectively translocated and accumulated to the upper part of the plants (Tassi et al. 2011; Giansoldati et al. 2012). However, its effectiveness is strongly affected by the high B phytotoxicity that severely compromises plant growth. Experiments were carried out at lysimeter scale where nitrogen fertilization was also evaluated. The treatments, alone or combined, were tested in plants that were allowed to grow until the end of the vegetative phase (70 days). This study aimed to test the hypothesis that an exogenous phytohormone supply, in particular CKs, alone or in combination with N fertilization, could help to overcome the stress caused by B contamination and to improve phytoextraction in the sediments from the Cecina river basin. Large-scale tests (lysimeters) were used to evaluate the effects of different treatments. To date, this research appears to be the first test carried out to evaluate the effects of phytohormone on B phytotoxicity and on the phytoextraction efficiency of *Helianthus annuus*.

7.4.1 Effect of Different Treatments on Plant Morphological Characteristics

The effect on total dry biomass, leaf area, and stem length (Table 7.2) in D is greater than the sum of the effects of each treatment taken into account individually (B and C), producing a synergistic effect with respect to the untreated lysimeter (A): 516 % versus (171 % + 123 %), 411 % versus (261 % + 58 %), 83 % versus (11 % + 34 %),

Table 7.2 Effect of different treatments on plant morphological characteristics as influenced by treatments in lysimeter experiment

	Leaves			Stems		Roots	Total Biomass
Lysimeter	Surface area (cm^2)	FW (g)	DW (g)	Length (cm)	DW (g)	DW (g)	DW (g)
Ctr	10.5 c	454 d	71.0 d	92.5 d	98.0 c	11.0 b	180 d
A	5.2 a	79.0 a	13.6 a	42.2 a	17.9 a	3.7 a	35.2 a
B	18.8 d	277 c	35.4 c	46.8 ab	50.0 b	10.2 b	95.2 c
C	8.2 b	156 b	24.0 b	56.5 b	45.0 b	9.4 b	78.4 b
D	26.6 d	595 e	73.0 d	77.1 c	138 d	22.0 c	233 e

Treatments: *Ctr* control (uncontaminated and untreated sediment), *A* contaminated sediment, untreated, *B* contaminated sediment, urea 200 kg ha^{-1}, *C* contaminated sediment, cytokinins (CKs) 3 mg kg^{-1}, *D* contaminated sediment, urea + CKs. Means with different letters in the same column differ significantly from each other ($P < 0.05$)
FW fresh weight, *DW* dry weight

respectively. Thus, the combined N fertilization and CK treatment helped plants to overcome the stress induced by high boron concentration, proved by a higher production of biomass (DW) than plants grown in uncontaminated sediment (Ctr lysimeter) which had 3 and 25 times lower total and available B content, respectively.

7.4.2 *Effect of Treatments on B Phytoextraction by* H. annuus

No significant difference in B content in the same tissue was found among all the treatments in contaminated sediments (A-D), meaning that fertilization and cytokinin treatment, both separate and in combination, had no decisive influence on B uptake and translocation by plants (Table 7.3a).

B phytoextraction (Table 7.3b) was calculated by multiplying B concentration by the biomass produced in the respective tissue. Although no significant differences in B concentration were observed among treatments (A-D), the amount of B removed by *H. annuus* in the same tissue was very different and depended on the kind of treatment, which in turn influenced the production of biomass. Considering leaves, urea treatment (lysimeter B) gave rise to higher B phytoextraction than CK treatment (lysimeter C); however, the combined treatment (lysimeter D) showed a synergistic effect giving rise to 55 mg B phytoremoved per lysimeter. The amount of B removed by stems and roots was, respectively, 10 and 100 times lower than by leaves, although a remarkable B phytoextraction was also observed in these tissues when compared with the untreated contaminated sediment (lysimeter A). The total amount of B phytoextracted in only one growth cycle was about 60 mg t^{-1} of sediment using urea fertilization and CK combined treatments, which is 5 times higher than the amount of B phytoextracted by plants grown in unfertilized sediment.

Lysimeter experimentation could represent this realistic situation and permitted an evaluation of the growth parameters and phytoextraction ability of *H. annuus* plants

Table 7.3 Boron content in mg kg^{-1} (a) and boron phytoextracted in mg lys^{-1} (b) in leaves, stems, and roots of *H. annuus* grown in the different treatments

(a)

Lysimeter	Leaves	Stems	Roots
Ctr	77.2 a	11.1 a	18.2 a
A	819 b	33.7 b	29.7 b
B	758 b	31.1 b	27.1 b
C	762 b	27.6 b	23.4 b
D	751 b	33.7 b	22.8 b

(b)

Lysimeter	Leaves	Stems	Roots	Total B phytoremoved
Ctr	0.08 a	0.02 a	n.d.	0.1 a
A	11.1 b	0.6 b	0.12 a	11.82 b
B	26.8 d	1.5 c	0.36 b	28.16 c
C	18.3 c	1.2 c	0.28 b	20.3 c
D	55.1 e	4.6 d	0.53 c	60.23 d

Treatments: *Ctr* control (uncontaminated and untreated sediment), *A* contaminated sediment, untreated, *B* contaminated sediment, urea 200 kg ha^{-1}, *C* contaminated sediment, cytokinins (CKs) 3 mg kg^{-1}, *D* contaminated sediment, urea + CKs. Means with different letters in the same column are significantly different from each other ($P < 0.05$)
n.d. not detectable

naturally exposed to variations in light, temperature, and humidity (rain events) during the complete growth cycle or maturity of plants (floral stage). In this scenario, our results proved the potential of *H. annuus* plants to phytoextract B in contaminated sediment where the adverse effects of B toxicity on plant growth were managed by the amelioration of soil quality (urea fertilization) and plant growth (the application of an exogenous plant hormone). The combined treatment in soil produced a synergistic effect in the biomass produced and the amount of B phytoextracted.

7.5 Concluding Remarks

Phytohormones have the specific ability to increase and support plant physiology and are well known and applied in horticulture, floriculture, fruit farming, and other agricultural fields. The main processes of phytoremediation involve physiology within the plant and/or in its immediate surroundings (rhizosphere); thus, it can take advantage of any "assistance" that improves the efficiency of the physiological mechanisms to make the phytoextraction processes more efficient. Phytohormones can be easily applied and are cost-effective and environmentally friendly. Such compounds offer a versatile resource for enhancing crop production and contaminant treatment with no side effects on health and environment. Consequently, phytoextraction can also benefit from its use. Nonetheless, the most appropriate phytohormones for

phytoremediation as well as the correct application method and timing still need to be identified in order to reap the maximum benefit from phytoremediation.

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Chapter 8
Plant Responses to Light Stress: Oxidative Damages, Photoprotection, and Role of Phytohormones

Aditya Banerjee and Aryadeep Roychoudhury

Abstract Light stress is the most uncharacterized and less studied among the various types of abiotic stresses experienced by the plant systems. Plants, being sessile organisms, cannot escape from such stresses, and one of the mechanisms of adaptation under such hostile circumstances is mediated through the altered regulation of phytohormones. In this book chapter, we have presented an exhaustive literature-based study on the different kinds of light stress encompassing light quality and type, the basic mechanism of perception of UV-B (the most harmful) rays by the plant system, the general metabolites which get upregulated under stress, and then a detailed excerpt on the role of phytohormones like auxin, gibberellic acid, cytokinins, ethylene, and abscisic acid under such conditions. Based on this account, our chapter also aims at integrating the perception of light stress-signaling pathway with the phytohormone-signaling networks, thus providing the idea of a universal cross talk occurring in plant cells, exposed to a variety of light stresses.

Keywords Light quality • Light stress • UV-B rays • Plant hormone • Stress signaling

8.1 Introduction

The environmental or abiotic factors are the major regulators of plant growth and distribution across the globe. These abiotic factors include soil water content, salinity, heavy metal content, and even the quantity of the incoming light striking the plants. A range of tolerance against these abiotic factors is exhibited by each plant

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species. The moment the limit of tolerance is exceeded, the plant experiences stress and upregulates the stress-responsive pathways. This ultimately leads to the survival of the stress-tolerant species and susceptibility or death of the sensitive species in the area under stress. Hence, these factors cumulatively dictate the geography of plant distribution. Light stress is the chief atmospheric stress acting upon plants which mainly inhibit growth via disrupting the photosynthetic pathway (Greenberg et al. 1989; McKenzie et al. 2003; Seppelt 2005). Phytohormones are the diverse chemical molecules which play prime roles in activating stress-responsive pathways required to guard against the potential damages caused by light stress (Effendi et al. 2013; Hayes et al. 2014). In this chapter, we would highlight the various light stress responses that are activated by the series of phytohormones, which on a broader level do have the possibility to act in tandem through a cross talk in their pathways. The type and quality of light stress affecting plant growth, the major signaling cascade involved in their perception, and the major effectors to combat light stress have also been dealt with in this chapter. The major form of light stress is of course the ultraviolet B (UV-B) light of shorter wavelength (280–320 nm). Such short wavelengths are normally filtered by the ozone layer in the stratosphere. However, due to the alarming increase in pollutants like chlorofluorocarbons (CFCs), the ozone layer is getting rapidly depleted resulting in harmful UV rays reaching the earth surface and posing a threat to plant growth (Arróniz-Crespo et al. 2004). Though several plants (species and cultivars), depending on their location, remain unaffected by small increases in UV-B rays, the overall constitution of the ecosystem obviously gets altered. This has remained a phenomenon of immense concern than loss of agricultural crops that are more easily manipulated.

8.2 Types of Light Affecting Plant Growth

8.2.1 UV Rays and Their Effects on Plant Growth

Solar UV radiation reaching the earth's surface is composed of UV-A (320–400 nm) and part of UV-B (280–320 nm), while most of the UV-B and all UV-C (<280 nm) radiation are absorbed by the ozone layer (Fig. 8.1). Over the last decades, depletion in the ozone layer has increased the level of solar UV-B radiation reaching the earth, and now approximately 0.5% of the total solar radiation accounts from UV-B. Exposure to UV light has been stated as a major deterrent of evolution to land (Arróniz-Crespo et al. 2004). The major effects of such exposure can be chlorophyll degradation and photoinduced DNA damages. Lignin, which is absent in bryophytes, plays a role in protecting the cells against UV light. The shifting of radiation to shorter wavelengths increases its damaging potential, and the factor by which the biologically effective radiation is increased by this effect is termed the radiation amplification factor (RAF) (Bornman 1991). Plant species with habitats around the higher latitudes are more prone to UV-B stress. This is because in

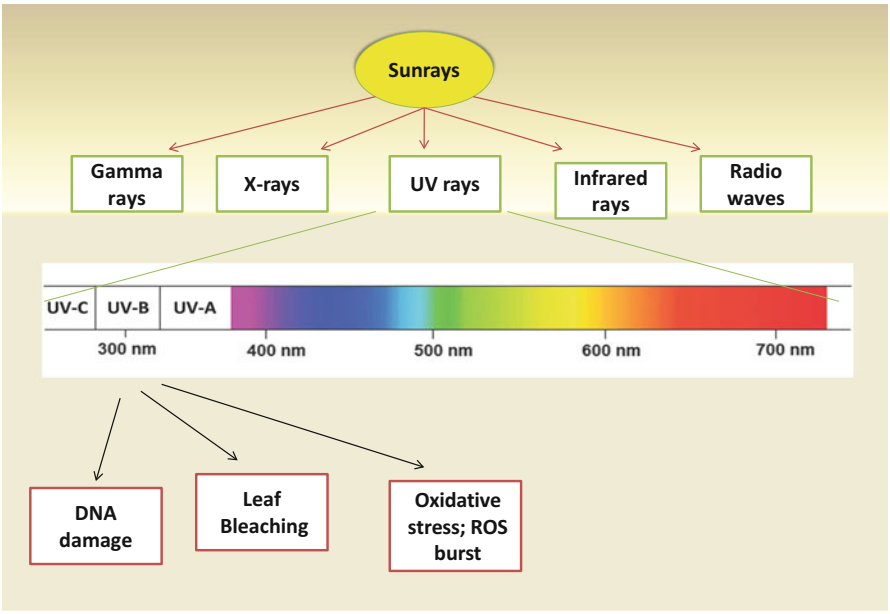

Fig. 8.1 Different types of light rays having varying wavelengths constitute the solar radiations. Out of these, the UV-B rays have the maximum potential to cause damages to both floral and faunal communities. The major photoinduced damages occurring in plants exposed to UV-B stress have been listed in the figure

comparison to the tropics, the temperate and higher latitude regions experience larger relative increase of effective UV-B on slight thinning of the ozone layer. Measurement of viability of species in varying latitudes has substantiated such inference (McKenzie et al. 2003).

Response to UV-B stress leads to the accumulation of UV-absorbing biomolecules like flavonoids, along with an increase in leaf thickness, lignin content, and surface reflectivity of the leaves. Such adaptations often are accompanied by stunted growth and reduced photosynthetic activity. Bleaching of leaves together with degraded cell membranes have also been documented as a result of UV-B exposure. Measurement of net and partial photosynthesis, flash-induced absorption changes, and fiber optic microprobes have been used to quantify the internal photosynthetically active radiation (PAR) of leaves, phytoluminography, and even to monitor chemiluminescence (Seppelt 2005). Different partial reactions of photosynthetic electron transport chain has led to the identification of UV-sensitive targets like the reaction center of photosystem II (PSII), the light-harvesting complex (LHC), the acceptor site of PSII, and the donor site of PSII. Experiments have proved that on exposure to UV-B radiation, the functional integrity between the water-splitting complex and P680 on the oxidizing side of PSII is lost (Gill et al. 2015). Greenberg et al. (1989) showed that the chloroplast protein D1 (Q_B) also gets rapidly degraded by UV-B rays. Thus, apart from being a prime target of atrazine herbicides, D1 is

also affected upon UV-B exposure, probably by the semiquinone anion radical. Since the Mn cluster of water oxidation is the most fragile component of the electron transport chain, protein matrix absorption of UV-B rays or other redox components have the ability to dissociate the Mn cluster and altogether inactivate photosynthesis.

Plants have often been observed to respond differently in greenhouses with suboptimal PAR in contrast to those growing under normal sunlight. The first example of such interaction between PAR levels and UV-B radiation was forwarded in *Phaseolus vulgaris* (Bornman 1991). Here, the bean plants were grown under three levels of visible light (230, 500, and 700 μmol m^{-2} s^{-1} referred to as low light (LL), medium light (ML), and high light (HL)), with or without enhanced UV-B radiation. Resistance to UV-B rays was observed in plants grown under HL conditions, while the LL conditions elicited increased response to UV stress. The ML plants exhibited intermediate response patterns. Significant increase in leaf thickness and pigment changes were observed in plants under all three conditions with least leaf thickness in the LL-grown plants. This was due to plant growth under suboptimal greenhouse conditions which did not completely mimic the natural environmental conditions. Hence, UV-B-exposed LL-grown plants did not show pronounced induction of UV-B absorbing compounds, and the leaf reflectivity was the highest in the blue and red regions of the spectrum, as derived by using bifurcated optical fibers. This indicates a phenomenon which decreases the penetration of PAR. In another experiment, the two species of *Brassica*, one from a northern latitude origin (*B. campestris*) and the other from southern latitude (*B. carinata*), were grown under high visible light (1800 μmol m^{-2} s^{-1}) supplemented with UV-B radiation. *B. campestris* showed maximum sensitivity to UV-B radiation with 45 % increase in leaf thickness and decrease in chlorophyll content. Though the internal PAR of the leaves changed with respect to the controls leading to attenuation of the transmitted light, the scattered light within the leaf in the palisade and spongy mesophyll tissue considerably increased (Berli et al. 2011). This observation shows that exposure to UV-B rays not only alters the anatomical features of the leaves but also their microenvironment thereby influencing photosynthesis.

Aromatic amino acids like phenylalanine, tyrosine, and tryptophan as well as histidine, cysteine, and cystine absorb in the UV-B region and hence can be direct targets of UV-B rays. Photooxidation of tyrosine to dityrosine and conversion of tryptophan to N-formyl kinurenine have been reported. The latter photoproduct so formed has the ability to interact with DNA after absorbing UV-A rays. Though cysteine is a poor absorber in the UV-B region, it can undergo photolysis at high quantum efficiency, leading to the splitting of the covalent disulfide bonds into reactive sulfhydryl groups. Hence, the tertiary structure of the entire protein is disturbed leading to aggregate formation. Characteristic examples include ribulose 1, 5-bisphosphate (Rubisco), ATPase, violaxanthin de-epoxidase, and protein subunits of PSI and PSII. Plant cell membranes are rich in phospholipids and glycolipids, consisting of unsaturated fatty acids (FAs) which are destroyed by UV-B radiation in the presence of oxygen (Hollosy 2002).

UV-B rays have the ability to destroy the genomic DNA by initiating the production of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidinone dimers (6–4 PPs). Formation of such adducts lead to the inability of the polymerases to access such bulky regions, resulting in disrupted replication, transcription, and finally cell death. To reverse such abrupt cellular failure, plants like *Arabidopsis* contain photolyases which have substrate specificity for either CPDs or 6–4 PPs and utilize UV-A and blue light to monomerize the pyrimidine dimers (Frohnmeyer and Staiger 2003; Gill et al. 2015). Cyanobacteria like *Anabaena, Nostoc*, and *Scytonema* contain a mycosporin-like amino acid called shironine. It has a cyclohexanone or cyclohexenimine chromophore conjugated with an amino acid or its imino alcohol accumulates in response to UV-B stress evidently during the daily light period (Sinha et al. 2001).

8.2.2 Gamma Rays and Their Effects on Plant Growth

Irradiation of plant cells with gamma rays resulted in dissolution of the pectin through increased activity of polygalacturonase and pectin methyl esterase. However, the cells in apple treated with calcium were less prone to such cell wall alterations, particularly due to the formation of stable cementing material calcium pectate. A very high radiation dose of 5 kGy finally resulted in a completely disintegrated middle lamella (Kovacs and Keresztes 2002). Gamma irradiation at exposures above 0.2 kGy in banana resulted in dilations between thylakoid membranes and a loss of granal stacking. Exposure to 0.2 kGy radiations retarded the fruit softening and yellowing, while higher gamma ray exposure values accelerated such changes. Marked decrease in sensitivity to exogenously applied ethylene was reported in banana under irradiation exposures between 0.6 and 1.0 kGy (Kovacs and Keresztes 2002). In another experiment, chlorophyll and carotene in acetone-ethanol solutions were irradiated with 60 krad of gamma rays and the optical absorption was measured. The results were expressed by an opacity term, which was derived from the ratio of the incident to the emergent light intensities. The persistent decrease of the opacity behavior of the chlorophyll and carotene solutions was recorded (Ramiarez-Nina et al. 1998). This indicates that following irradiation, fewer organic dye molecules remain to absorb light at a specific wavelength. Chlorophyll in silicon oxide matrix was also irradiated with 0, 30, and 60 krad, and surprisingly smaller changes were observed in irradiated gel samples than in solutions (Ramiarez-Nina et al. 1998). Such results were observed probably due to the presence of fewer free radicals in the gel structure in comparison to the large amount in the liquid sample solutions. Cia et al. (2007) reported the use of gamma irradiation to evade anthracnose which is the main post-harvest disease in papaya. It was perhaps a novel interpretation which showed the use of these harmful rays toward beneficial plant growth instead of generating stress in the plant. It was concluded that doses of 0.75 and 1 kGy of gamma irradiation reduced the lesion size caused by *Colletotrichum gloeosporioides* (causal organism of anthracnose) and anthracnose incidence in papaya fruit (Cia et al. 2007; Patrícia et al. 2007).

8.2.3 Low Light Intensity and Its Effects on Plant Growth

Low light intensity is about 40–50 % of natural light. Hence, it is about 40–60 % less than the light intensity during the dry season. The growth pattern and productivity of two indica rice genotypes of kharif and three of rabi were assessed under low light intensity. It was observed that growth of plants under shade affected the dry matter accumulation at all stages. Such effects were critical from primordial initiation onward. Within 26–9 °C range of mean maximum temperatures, the expression of the panicles appeared to be a function of the light intensity. As a result of exposure to low light intensity and shading from the primordial initiation stage, the grain and panicle yields in the rice plants were sufficiently reduced. Low light intensity was depicted to be a major constraint for higher yields during monsoons, as low yields of 3.2–4.4 tons ha^{-1} were recorded in rice cultivars which yielded 8–10 tons ha^{-1} under exposure to high light (Retkute et al. 2015). Thus, it seems that low light intensity causes stress by insufficient supply of energy-rich photons which fuel the photosystems and regulate photosynthesis. Insufficient photosynthesis results in insufficient food production for the autotrophic plants leading to unusual retarded growth patterns and lowered productivity. This has led to the hypothetical possibility of the probable existence of a cross talk between the signaling cascade perceiving low light intensity and the quantitative trait loci encoding plant productivity. Boo et al. (2000) showed that the rice plants had similar oxidative stress profiles under drought, when incubated with 5-aminolevulinic acid (ALA) in dark and separately exposed to low light intensity. This has been accredited to the formation of singlet oxygen in the plants both under drought stress and after incubating with ALA.Treatment of the rice plants with ALA resulted in high accumulation of protochlorophyllide which under low light intensity photodynamically generated singlet oxygen (Boo et al. 2000).

Vantuyl et al. (1985) reported the incidence of flower bud abortion in Asiatic hybrid lilies exposed to low light intensity. It was found that these lilies required additional light to prevent flower bud abscission and flower bud blasting during the winter season when natural light intensity is quite low. Among the cultivars, Connecticut King and Enchantment appeared to be the most sensitive to low light conditions. Low light intensity was also reported to upregulate ethylene biosynthesis especially during the critical stage. This is because ethylene is the essential hormone which initiates abscission and hence abortion of the flower buds. According to Durieux et al. (1983), a short period with very low light intensity at the critical stage for bud abscission should be sufficient for a disproportionate increase in percentage of flower bud abortion. Artetxe et al. (2002) however reported an unusual result where low light intensity levied the oxidative stress in duckweeds (*Lemna minor*) under cadmium (Cd) and zinc (Zn) stress and rendered them tolerant to these heavy metals. The low light (LL)-treated plants exhibited reduced symptoms of Cd- and Zn-induced cytotoxicity with no effect on the relative chlorophyll contents. Such surprising results were unexpected as we have discussed in the previous instances that the LL plants are less protected toward oxidative stresses. Along with

increasing the phytochelatin content, LL plants showed enhanced Glu accumulation particularly to be channelized for glutathione biosynthesis. Large increases in total ascorbate, tocopherol, and xanthophylls-cycle pigments were also observed which altogether launched an antioxidative response (Artetxe et al. 2002).

8.2.4 High Light Intensity and Its Effects on Plant Growth

Severe photoinactivation and photodamage of photosynthetic apparatus and degradation of photosynthetic proteins have been documented in plants exposed to light intensity which is abnormally higher than what is utilized in photochemistry. Degradation of D1 protein of PSII, large subunit (LSU) of the Rubisco, and decreased levels of PSI polypeptides like PsaA, PsaB, and PsaC are the major detrimental effects of high light intensity (Jiao et al. 2004). Higher plants which are often exposed to such detrimental doses of high light intensity during summer have evolved photoacclimation strategies. A series of dynamic alterations in the chloroplast occurs involving a reduction in the number and size of light-harvesting complexes and accumulation of D1 proteins. Light stress response thus includes the modification of the stoichiometry, content, and activity of PS complexes like chlorophyll levels, activity of the electron transport chain, plastocyanin content, ATP synthase activities, Rubisco LSU synthesis, and transcriptional regulation of PSII/PSI ratio (Jiao et al. 2004; Pfannschmidt et al. 1999). Energy transition between PSII and PSI is also controlled by regulating the synthesis and activity of the light-harvesting complex II (LHCII) (Allen 2003). Jiao et al. (2004) reported high light stress-induced synthesis of the β-isoform of the chloroplast ATP synthase coupling factor (CF1) as a positive response during chloroplast photoacclimation in *Brassica rapa*. The gametophytes and sporophytes of *Laminaria saccharina* exposed to high light stress (500 μmol m^{-2} s^{-1} for two hours) exhibited photoinhibition of photosynthesis with fast kinetics (Hanelt et al. 1997). Maximum photodamage was recorded in the younger sporophytes, some of which did not recover fully even after 12 h of exposure to lowered light intensity. However, the kinetics of recovery in the old sporophytes and gametophytes showed a fast and a slow phase. Hanelt et al. (1997) inferred that the fast phase was indicative of a decline in the photoprotective processes following stress, and the slow phase corresponded to steady recovery from stress. It was also investigated and seen that the resistance of the older sporophytes and gametophytes was not only due to an increase in the light absorbing pigments like xanthophylls but also due to a rapid change in the thallus structure. *Laminaria* sporophytes became multilayered when their size exceeded one centimeter along with thickening of the blades. In this way, by a mechanism of self-shading, the plants have the ability to protect their chloroplasts by rearranging them into a low-absorbing position in the cytosol. Similar cases have also been reported in *Porphyra purpurea* where the effect of photoinhibition decreased due to self-shading of overlapping thalli (Hanelt et al. 1997). The effect of high proton flux density (PFD) was studied in the desert resurrection plant *Selaginella lepidophylla* to analyze the

chlorophyll fluorescence under such extreme conditions (Eickmeier et al. 1993). Significant reductions in the intrinsic fluorescence yield and photochemical efficiency of PSII were observed in hydrated physiologically competent stems under extremely high intensity of light (2000 μmol m^{-2} s^{-1}). However, the recovery of the resurrection plants under low PFD was rapid. *Borya nitida* are poikilohydrous angiosperms known for their tolerance to extremes of temperatures and also to intense visible radiation. Exposure to two hours of high light intensity (650 μmol m^{-2} s^{-1}) resulted in no photoinhibition in *Borya*, while 70 % photoinhibition occurred under exposure to low light intensity (120 μmol m^{-2} s^{-1}) (Retkute et al. 2015). Thus, it was inferred that *Borya* flourish best under harsh extreme habitat probably by maintaining a high basal level of antioxidative front to tackle oxidative stress. Morphological adaptations like compact rounded leaves and their orientations to afford maximum reflectance also aid *Borya* in tackling high light stress. Funk et al. (2010) reported that *Brassica nigra* (invasive) and *Encelia californica* (native) were tolerant to high light stress than *Ricinus*, *Salvia*, *Artemisia californica* (native), or *Nicotiana glauca* (invasive).

Such inferences could be drawn from the observations that *Brassica* and *Encelia* both were resistant to photoinhibition in response to high light stress.

8.3 Quality of Light Affecting Plant Growth

8.3.1 Blue Light Affecting Plant Growth

The quality of light is one of the most crucial variables affecting photosynthetic parameters and concentrations of the phytochemicals in plants (Whitelam and Halliday 2007). High pressure sodium (HPS) lamps are often used to produce supplementary blue light for the plants. In case of the greenhouses, the light-emitting diodes (LEDs) are procured for service (Paradiso et al. 2011). Blue light (having a bandwidth 420–460 nm) in a cross talk with phytochrome signaling regulates stomatal opening, mainly via potassium and chloride uptake and malate biosynthesis. Blue light activates the proton pumping ATPase which aids in ion uptake. The C-terminal phosphorylation by a Ser/Thr kinase activates the ATPase. An action spectrum was exhibited by the blue light-specific stomatal opening with a maximum peak at 450 nm and two minor peaks at 420 and 470 nm (Talbott et al. 2003). The role of phytochrome in blue light-mediated stomatal opening was hypothesized from the fact that the absorption spectrum of phytochrome extends into the blue region of the spectrum. In the orchid genus *Paphiopedilum*, phytochrome plays a direct role in stomatal opening (Talbott et al. 2002). Photosynthesis-dependent stomatal opening is absent in this genus as the leaves have minute amounts of chlorophyll. The chloroplastic carotenoid and zeaxanthin have been identified as the putative photoreceptors of blue light in the guard cells. Hence, the *Arabidopsis* mutants *npq1*, which failed to accumulate zeaxanthin due to defective violaxanthin de-epoxidase, lacked blue light-specific responses (Talbott et al. 2003).

Kinoshita et al. (2001) reported that the double mutants of *phot1* and *phot2* exhibited more impaired blue light response than the single mutants alone. It was also suggested that the stomata from the double mutant would show blue light response at higher fluence rates of blue light (Kinoshita et al. 2001).

In another instance, it was seen that predawn and high light intensity treatment supplemented with blue light decreased the quantum yield of PSII and enhanced the accumulation of phenolics, flavonoids, and pigments in *Lactuca sativa* (Ouzounis et al. 2015). Here two cultivars of lettuce, viz., Batavia (green) and Lollo Rossa (red), were subjected to variable light intensities with or without supplemented blue light. Though the total fresh and dry weights were not affected in the plants under blue light, these were more compact in growth and development. Difference in varietal response to blue light was also observed as the red cultivar under blue light showed reduced quantum yield of PSII with an increase in non-photochemical quenching. However, the green lettuce variety exhibited no such difference except an increase in the stomatal conductance. These results obviously indicate the fact that high light levels not only trigger photoprotective heat dissipation in the plant system but also the specific spectral composition of the light itself at low intensities (Ouzounis et al. 2015).

8.3.2 Red and Far-Red Light Affecting Plant Growth

Red light has a bandwidth of 620–640 nm and often acts as a pivotal regulator in plant physiological functions. Alyabyev et al. (2002) showed that at high temperature, the rate of oxygen consumption of summer wheat seedlings exposed to blue light decreased by 40–45 % when compared with the control plants under similar conditions. The heat production rate of wheat seedlings exposed to blue light was also higher than those grown under red light. This was the first report of a comparative analysis between the red and blue light-mediated responses which aided in understanding the contrasting signaling mediated by them. Depending on the quality of light exposure, differential rates of respiration were observed in plants growing in optimum temperature. The excised roots of plants exposed to blue and white light exhibited slightly lowered rate of respiration in comparison to those under red light. However, high temperature treatment reduced the respiration rate in all plants with the lowest inhibition in the blue light-treated plants. Such results possibly indicate greater stability achieved by the plants under blue light at higher temperatures (Alyabyev et al. 2002). This observation also corresponds to the fact that the rate of oxygen uptake of roots is dependent on the spectral composition of the light it receives. Such correlation might be because the plant tissues are highly light conducting and photosensitive (Kim et al. 2007). Electron transport and activation of enzymes related to respiratory metabolism and membrane stabilization are often regulated by blue light which can be accredited to such correlation with respiratory rate.

Far-red (FR) light generally has an emission peak at 740 nm and half bandwidth of 25 nm. It has a correlation with the plant photoreceptors. The chief plant

photoreceptors are phytochromes and cryptochromes which regulate the circadian clock in plants. In *Arabidopsis*, five phytochromes (Phy A-E) have been identified and characterized, of which PhyA is predominant in etiolated seedlings and PhyB in the light-grown plants (Qin et al. 2010). Identification of SUB1, a calcium-binding protein, has strengthened the possibility of a cross talk between the phytochrome- and cryptochrome-mediated signaling networks (Guo et al. 2001). Qin et al. (2010) depicted the involvement of calcineurin B-like protein (CBL)-interacting protein kinase 14 (CIPK14) in the PhyA-mediated FR light inhibition of greening in *Arabidopsis* seedlings. CIPK14 was also found to affect the expression of *Protochlorophyllide Oxidoreductase* (*POR*) genes like *PORA*, *PORB*, and *PORC* in *Arabidopsis*. Greening was not initiated in the *cipk14* seedlings under FR light even after 15 h of exposure to white light. However, the phyA seedlings showed greening within 0.5 h of white light exposure. Expression of *CIPK14* appeared to be regulated by both the circadian clock and PhyA, thus indicating the role of CIPK14 in the FR inhibition of seedling greening mediated by PhyA by negatively regulating the PhyA-dependent repression of the *POR* genes (Qin et al. 2010).

Far-red elongated hypocotyls 3 (FHY3), also known as chloroplast division 45 (CPD45), acts as a crucial factor in FR light-signaling pathway in *Arabidopsis*. The FHY3/CPD45 mediates perception of FR light and regulates chloroplast division. *ARC5* is a nuclear gene encoding a dynamin-related protein involved in chloroplast division (Gao et al. 2003). The chloroplast division mutant *arc5-3* had no defect in FR light sensing, while constitutive overexpression of *ARC5* aided in recovering from chloroplast division defects but not from those in FR light signaling in the *cdp45* mutants (Chang et al. 2015). Constitutive overexpression of *FHY1* repaired the fallacies in FR signaling, but not in the chloroplast division mechanism in *cdp45* mutants. Chang et al. (2015) hence reported that FHY3/CDP45 regulates FR light signaling and chloroplast division together through parallel activation of *FHY1* and *ARC5* independently. Apart from these two pathways, CDP45 also acts as an important regulator of circadian rhythm and other important biological processes essential for proper plant growth and development. This fact was supported by the observation that CDP45 regulates the expression of *Early Flower4* (*ELF4*) and interacts in the *CCA1/LHY-TOC1* circadian clock feedback circuit via association with the *ELF4* promoter (Li et al. 2011). Thus, the link between photomorphogenesis, circadian rhythm, and FR signaling is probably through FHY3/CPD45 which acts as a crucial node in the entire gene regulatory pathway and even acts as a transcription factor (TF) to transcribe the downstream effector genes. Kegge et al. (2015) reported that the emission of total volatile organic compounds (VOCs) was reduced in *Hordeum vulgare* cv. Alva under low red and FR light conditions when compared with their control counterparts. The basic notion of this study was to investigate the effect of light quality on the emission of VOCs which act as crucial signaling molecules in plant-plant interactions. Thus, the altered VOC emission by Alva cultivars surprisingly affected the carbon (biological) allocation in the receiver plants of another *Hordeum* cultivar named Kara (Kegge et al. 2015).

Blue, red, and far-red light signaling visually does not seem to cause much stress like the UV-B rays because they do not harm the morphological queues of plants.

However, from the point of regulating plant growth patterns, they do trigger essential photomorphogenesis which may have common patterns under true light stress. In this section, we have tried to highlight only such phytoregulations mediated by the quality of light, reaching the plant absorptive surfaces.

8.4 Signaling Pathways Involved in UV-B Stress

Since light stress is more focused mainly on the UV-B mediated stress, in this section we would briefly discuss the receptive pathway related with UV-B-mediated response in plants (Fig. 8.2). The major UV-B photoreceptor is UVR8 which is a seven-bladed β-propeller protein first identified in the *Arabidopsis* mutants which showed hypersensitivity to UV-B light (Li et al. 2013). Apart from the sequence homology of this receptor with Regulator of Chromatin Condensation 1 (RCC1) found in humans, UVR8 is the prime regulator of multiple genes involved in UV damage repairs. Such results have been concluded after exhaustive transcriptome studies of UVR8 (Brown et al. 2005). An interesting phenomenon was identified that the UVR8 proteins are constitutively expressed irrespective of UV-B light stress. The UV-B rays stimulate the distribution of UVR8 in the cell mainly concentrating them in the nucleus (Kaiserli and Jenkins 2007). The UV-B stress was still required for the constitutively nuclear-localized proteins. Though the biological

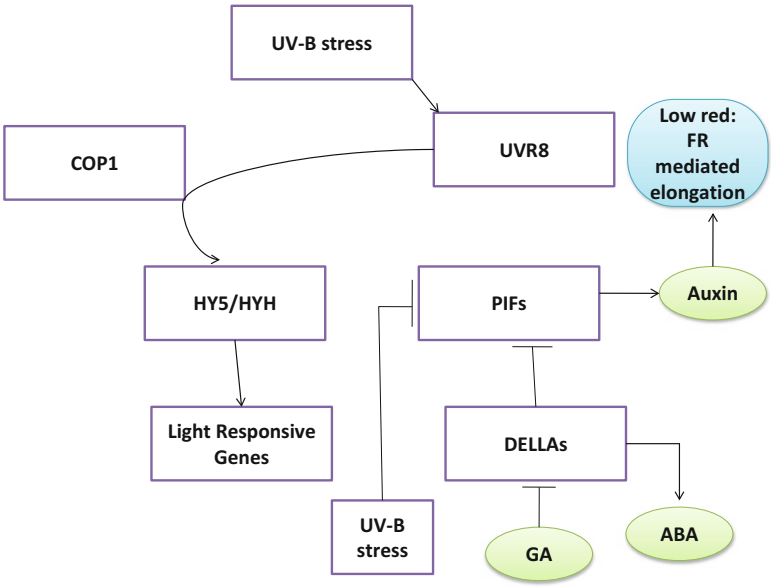

Fig. 8.2 The light-signaling pathway involved in UV-B perception and the potential cross talk among the major phytohormones, ABA, auxin, and GA with this light receptive pathway

significance of UVR8 still remains largely unknown, it has been depicted that this protein can even act as a potential TF by associating with the promoter region of *Elongated Hypocotyl 5* (*HY5*) (Cloix and Jenkins 2008). HY5 is a nuclear bZIP TF involved in the regulation of photomorphogenesis under a wide spectrum of wavelengths including blue, red, and FR lights. HY5 is involved in the upregulation and downregulation of light-responsive genes through association with the promoters of the annotated genes (Zhang et al. 2011). Oravecz et al. (2006) showed that HY5 is a positive effector in UV-B-mediated signaling because the *hy5* mutants displayed sensitivity to UV-B. New alleles like *cop1* and *uvr8* were identified by Favory et al. (2009) by using *HY5p: LUC* reporter construct. Involvement of other TFs like HY5 is also possible as a subset of UVR8, and Constitutive Photomorphogenic 1 (COP1)-dependent genes were not upregulated by HY5/HY5 Homolog (HYH) (Feher et al. 2011).

Favory et al. (2009) reported the importance of the physical interaction between UVR8 and COP1, an E3 ubiquitin ligase. The C-terminus of UVR8 consists of a 27 amino acid region, which, when digested, destroyed the UVR8 activity. Single amino acid changes in UVR8 and COP1 proteins resulted in an abrogated direct interaction between them, due to which the entire UV-B-mediated response pathway became aberrant (Favory et al. 2009; Li et al. 2013). COP1 is a conserved RING finger E3 ubiquitin ligase involved in plant development, mammalian cell survival, growth, and metabolism (Lau and Deng 2012). However, in this discussion we would deal mainly with the aspects of COP1 as a central repressor in light signaling. It functions as an E3 ligase, targeting different photomorphogenesis-promoting proteins like HY5 and HYH for degradation under visible light (Li et al. 2013). However, under UV-B stress, COP1 accumulates in the nucleus and forms protein complexes with four WD40-repeat proteins. Suppressor of PhyA-105 (SPA1), SPA2, SPA3, and SPA4 is not obligatory for proper UV-B-mediated stress response (Zhu et al. 2008). Structural stabilities and similar levels of UVR8 in the wild-type and *cop1-4* mutants depict that COP1 does not stabilize UVR8, as it does in case of other photoreceptors like phytochromes and cryptochromes (Favory et al. 2009; Jang et al. 2010).

Rizzini et al. (2011) reported that a UVR8 dimer is the major UV-B receptor and proposed that a number of conserved tryptophan residues like W285 have key roles as light sensors in UVR8. In vivo experiments showed that the mutation of either tryptophan (W233 or W285) residues to alanine totally abolished the photomorphogenic responses mediated by UVR8 in *Arabidopsis* mutants. This is because absorption of UV-B photons by these two tryptophans dissociates the salt bridges, resulting in destabilization of the dimeric form and signal initiation (Christie et al. 2012). Redimerization of UVR8 has been seen to be mediated by Repressor of UV-B Photomorphogenesis (RUP1) and RUP2. This dimerization negatively regulates the stress-responsive cascade as UVR8 and COP1 cannot mutually interact with each other (Heijde and Ulm 2013). RUP1 and RUP2 are members of the WD40-repeat protein superfamily. Members of this superfamily consist of at least one copy of a conserved motif (WD40 motif: 40 amino acids long and typically ending with tryptophan and aspartate residues). RUP1 and RUP2 have been reported to contain

seven WD40 repeats with no other apparent domains (Gruber et al. 2010). *RUP1* and *RUP2* have been reported to be upregulated under UV-B stress in a UVR8-, COP1-, and HY5-dependent manner (Gruber et al. 2010). Thus, if RUP1 and RUP2 are considered as negative regulators in the UV-B-mediated stress-responsive pathway, then the direct interaction of these proteins with UVR8 to promote its dimerization can be seen as a direct interaction of the negative regulators at the photoreceptor level (Li et al. 2013). It has been postulated that since COP1 is essential for the UV-B responsive expression of RUP1 and RUP2, there should be sufficiently high basal level of RUPs, if the RUP-mediated dimerization of UVR8 is COP1 independent, as hypothesized by some groups (Gruber et al. 2010; Heijde and Ulm 2013).

FR Elongated Hypocotyl 3 (FHY3) and its homologue FR Impaired Response 1 (FAR1) are transposase-derived TFs, which, apart from participating in PhyA-mediated signaling, are also involved in multiple development processes like circadian rhythm, chloroplast development, chlorophyll biosynthesis, and shoot branching (Stirnberg et al. 2012; Tang et al. 2012; Li et al. 2013). These processes are regulated by light in association of FHY3/FAR1, with PhyA in vivo. Other TFs which have been identified to work in tandem with FHY3/FAR1 are HY5, Phytochrome-Interacting Factor1 (PIF1), Circadian Clock Associated 1 (CCA1), and Late Elongated Hypocotyl (LHY) (Tang et al. 2012). The tandem functioning of these TFs with FHY3/FAR1 has presented a possibility of a cross talk existing in the light-signaling pathway, where FHY3/FAR1 may be the central node. Huang et al. (2012) reported that FHY3 and HY5 physically interact with their cis-elements in the COP1 promoter. This means that COP1 is inducible by UV-B light stress. FHY3 acts as a positive regulator in light stress-mediated signaling, as the *fhy3* mutants exhibited distorted UV-B-induced hypocotyl growth and high UV-B sensitivity. On the contrary, FAR1 is not obligatory for the UV-B photomorphogenetic pathway, because it was monitored that the *far1* mutants were devoid of any apparent impairments, while the *fhy3/far1* double mutants also showed no further impairment of the UV-B-induced hypocotyl growth (Huang et al. 2012). Li et al. (2013) have suggested that HY5 promotes *COP1* expression through a positive feedback loop. This is based on the fact that though FHY3 and HY5 both positively regulate *COP1* gene expression, the accumulation of HY5 under UV-B exposure requires COP1. Another emerging signaling intermediate of the UV-B photomorphogenetic pathway is the Salt Tolerance (STO also referred to as BBX24) which is a B-box Zn finger protein. STO is a negative regulator of the UV-B-responsive signaling cascade, interacting with COP1 and repressing the transcriptional activities of HY5 (Jiang et al. 2012). The UV-B stress-responsive pathway is still a major focus of research for several scientific groups as some schools of scientists find the emerging players in this signaling cascade as potent targets of developing transgenic plants with considerable tolerance toward UV-B stress.

8.5 Major Metabolites Which Confer Resistance Against Light Stress

8.5.1 Flavonoids

Phenolic compounds constitute one of the most important groups among the bioactive compounds found in plants since they have diverse functioning in signaling and defense pathways. Flavonoids and hydroxycinnamic acids are the major phenolic compounds found in fruits and berries determining the color, aroma, astringency, and antioxidant properties (He and Giusti 2010). The flavonols, anthocyanins, and proanthocyanidins (PA) constitute the major flavonoids found in flowers and fruits. These flavonols, apart from serving as visual signals for pollinators in flower and fruit dispersal, act as photoprotectants by scavenging the free radicals formed under UV-B exposure (Bogs et al. 2007). Suppressed flavonoid biosynthesis has been reported in fruits which have been shaded from sunlight. In *Litchi chinensis*, fruit bagging treatments resulted in lowered accumulation of anthocyanins as well as the anthocyanin biosynthetic genes like *chalcone synthase* (*LcCHS*), *chalcone isomerase* (*LcCHI*), *flavones 3-hydroxylase* (*LcF3H*), *dihydroflavonol 4-reductase* (*LcDFR*), *anthocyanidin synthase* (*LcANS*), and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*LcUFGT*). These genes were again reported to be upregulated on debagging and exposure of fruits to sunlight (Wei et al. 2011). The purpose is to inhibit chlorosis and bleaching on exposure to light, which can only be done if these genes are particularly upregulated. Light has also been reported to regulate flavonoid biosynthesis in strawberry (*Fragaria* X *ananassa*), peach/nectarine (*Prunus persica*), pear (*Pyrus pyrifolia*), and apple (*Malus X domestica*) (Zoratti et al. 2014; Sun et al. 2014). Agati et al. (2013) reported the significance of flavonoids in their ability to scavenge ROS and control the development of individual organs and the whole plant. The chloroplast-located flavonoids have been portrayed as hydrogen peroxide and singlet oxygen scavengers, so that they can prohibit programmed cell death under light stress. The vacuolar flavonoids, together with the antioxidant enzymes, like peroxidases and ascorbic acid, form a second antioxidant system. The flavonols have also been depicted as the key developmental regulators, as they have the ability to mediate auxin transport and catabolism. Thus, Agati et al. (2013) concluded that UV-B photoprotection is just one of the essential functions of flavonoids which have several other myriad roles in plant growth regulation.

However, the most important functions of flavonoids arise when the plants face UV-B stress. During such stress, there is a burst in reactive oxygen species (ROS) production, resulting in various damages which we have discussed before. Koyama et al. (2012) reported a decrease in the flavonol content in the skin of grape berries (under UV shield). However, the cinnamic acid and PA levels were much less affected. In Sauvignon blanc grape berries, substantial increase in flavonols like quercetin and kaempferol glycosides were detected by Liu et al. (2014) when the fruits were exposed to UV-B light stress. Of the four *Vitis vinifera* flavonol synthase (*VvFLS*) genes, two were found to be transcriptionally active and only one (VvFLS4) responded to UV-B mediated stress (Liu et al. 2014).

Peng et al. (2013) reported the expression of *MdMYBA* and the related anthocyanin pathway genes in apple skin in response to UV-B stress. Martinez-Luscher et al. (2014) showed that the anthocyanin and flavonol contents were high in red grapevine variety (cv. Tempranillo) exposed to UV-B stress. Qualitative differences were also found in the flavonol profiles in the UV-B treated fruits when compared to that of the untreated fruits (Martinez-Luscher et al. 2014). Accumulation of flavan-3-ol and high transcript levels of related genes was observed in the developmental stages (3–11 weeks after flowering) of Cabernet Sauvignon grapevine variety, exposed to UV-A irradiation (Zhang et al. 2013). However UV-B and UV-C irradiations gave rise to same results only in berries of 7–11 weeks of flowering, based on which Zhang et al. (2013) inferred that flavan-3-ol accumulation increased on exposure to UV light stress, only in berries which were still in their developmental phases, but not in those which were mature. Enhanced transcript levels of flavonoid biosynthesis genes like *DFR* and *UFGT* were observed in the skin of nectarines (cv. Stark Red Gold) on exposure to white light, supplemented with UV light for about 72 h (Ravaglia et al. 2013). Castagna et al. (2014) also reported flavonol accumulation in the flesh of tomato fruits at green mature stage, harvesting them following UV-B exposure. Namli et al. (2014) observed an increase in the total flavonoids, phenolics, and hypericin content in the in vitro cultures of *Hypericum retusum* var. Aucher exposed to UV radiations. When the cultured plantlets were exposed to UV-B rays for 15, 30, 45, and 60 min, the highest total phenolics, flavonoids, and hypericin accumulation (43.17 ± 0.8; 35.09 ± 0.8; 2.7 ± 0.05 mg g^{-1}, respectively) was achieved at 45 min of UV-B exposure, whereas the contents of these metabolites in the naturally grown plants were 23.33 ± 0.9, 18.62 ± 0.3, and 1.6 ± 0.01 mg g^{-1} respectively (Namli et al. 2014).

Scattino et al. (2014) observed the increased accumulation of flavonols and anthocyanidin glycosides in two cultivars of peach where the transcript levels of the structural phenylpropanoid and flavonoid pathway genes were consistent with the levels of the detected metabolites (Scattino et al. 2014). Crupi et al. (2013) reported that the accumulation of stilbene cis- and trans-piceid along with quercetin-3-O-galactoside and quercetin-3-O-glucoside was enhanced in grape berry skin under UV-C stress. The accumulation was higher by about three folds than the control berries which were not exposed to UV-C light stress. The radical-scavenging properties were found to be higher in the papaya fruits exposed to UV-C irradiation. Such antioxidative activity was found to be mediated by an enhanced level of flavonoids (Rivera-Pastrana et al. 2014). Thus, in the above excerpt, we have provided a brief discussion on the varied cases where flavonoid accumulation has been depicted as a result of mainly UV stress. From all these results, it is clearly implicated that flavonoids do have important roles as antioxidants considering generation of plant tolerance against light stress (Fig. 8.3).

8.5.2 Xanthophylls

The xanthophyll cycle consists of light-dependent conversions of three oxygenated carotenoids in a cyclic reaction which involves de-epoxidation of the diepoxide violaxanthin, via the monoepoxide antheraxanthin to the epoxide free form of

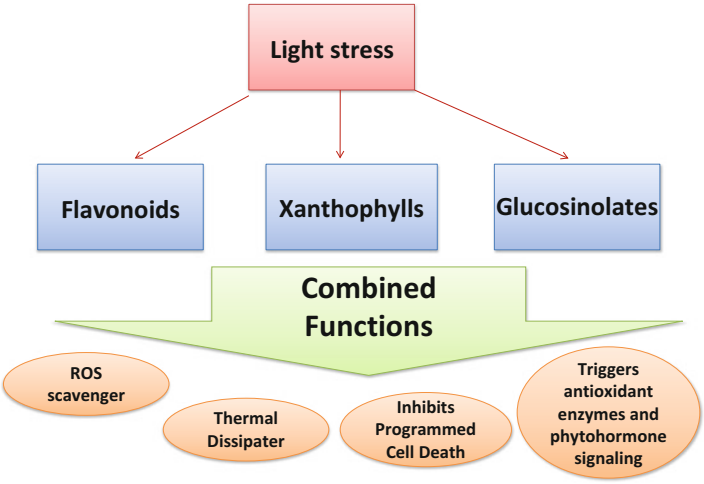

Fig. 8.3 The major metabolites which accumulate in plant tissues exposed to light stress and their major roles in generating plant tolerance against photodamages and light stress

zeaxanthin. This is followed by an epoxidation sequence in the reverse direction (Demmig-Adams and Adams 1992). It was found that this xanthophyll cycle acts as a thermal dissipator in plants when preventing overheating of particular tissues which are more exposed to light (Fig. 8.3). The leaves of *Euonymus kiautschovicus* Loesener experienced a wide degree of light stress mainly in response to different levels of incident photon flux densities at similar photosynthetic capacities among the leaves. The intrinsic PSII efficiency, non-photochemical fluorescence quenching and the levels of zeaxanthin along with antheraxanthin in leaves have been considered as functions of actual light stress (Demmig-Adams and Adams 1996). Thus, under a given degree of light stress, the same conversion state of the xanthophyll cycle, accompanied by constant level of energy dissipation, was found, irrespective of the usage of species or conditions, causing light stress. Xanthophyll-independent energy dissipation was not reported, since all increases in thermal dissipation were associated with simultaneous increases in the levels of zeaxanthin and antheraxanthin in these leaves (Demmig-Adams and Adams 1996).

8.5.3 Glucosinolates

Schreiner et al. (2014) recently reported the production of defense-related compounds like glucosinolates in members of Brassicaceae, on exposure to UV light stress (Fig. 8.3). The glucosinolates are sulfonated thioglycosides which share a common glycone moiety with a variable aglycone side chain. Based on this, the glucosinolates can be differentiated into aliphatic, indolyl, and aromatic glucosinolates (Schreiner et al. 2014). UV-B-mediated increased glucosinolate content has been reported in

Brassica oleracea var. *italica* (broccoli), *Arabidopsis thaliana*, and *Tropaeolum majus*. UV-B doses led to high accumulation of mainly aliphatic methylsulfinylalkyl glucosinolates and indole 4-methoxy-indol-3-ylmethyl glucosinolate in broccoli and *Arabidopsis* and an aromatic glucosinolate in *Nasturtium*. It was also reported that multiple exposures of high (up to 0.9 KJ m^{-2} d^{-1}) doses of UV-B did not elicit a stronger response in glucosinolate accumulation than that which occurred as a result of lower UV-B doses. UV-C irradiation in broccoli florets resulted in high accumulation of 4-methoxy-indol-3-ylmethyl glucosinolate, 4-hydroxy-indol-3-ylmethyl glucosinolate, and 4-methylsulfinylbutyl glucosinolate. Unripe *Nasturtium* green seeds exhibited sixfolds enhanced level of benzyl glucosinolate accumulation, whereas the mature leaves exhibited only threefolds increase in the same metabolite content (Schreiner et al. 2014).

8.6 Phytohormone-Mediating Plant Stress Tolerance Against Light Stress

Plants have been depicted to possess the intrinsic ability to tackle stress by altering its physiological growth parameters. Such alterations are often brought about by a dynamic change in the concentration of phytohormones. It has been reported that plants can regulate their physiological responses by changing the concentration of indole-3-acetic acid (IAA), cytokinin (CTK), abscisic acid (ABA), ethylene, and other minor phytohormones. In this section, we would mainly highlight the consequences of light stress, especially mediated by UV rays on the phytohormone levels and the resultant effects on the plant system. In Fig. 8.2, we have tried to reflect a possible model of cross talk among the plant signaling pathways involved in UV-B perception and three phytohormones, viz., auxin, gibberellic acid (GA), and ABA.

8.6.1 Auxin

Liu and Zhong (2009) emphasized the fact that UV radiation reduces the concentration and activity of IAA through photodegradation. This has a direct effect on the cell physiology as the cells can no longer metabolize and utilize IAA. Witztum and Keren (1978) observed the significant decrease in IAA levels in the fronds of *Spirodela oligorhiza* exposed to UV light stress. The daughter colonies of the stressed fronds exhibited a high percentage of abscission. Such physiological decay could be reversed if the stressed fronds were imbibed in appropriate IAA solutions. However, this resulted in a strong release of ethylene, which first provided the possibility of a cross talk between auxin- and ethylene-signaling pathways during UV stress. Yang et al. (1993) inferred that exogenous application of IAA of appropriate concentrations could result in growth of plants exposed to UV-B radiation. Liu and Zhong (2009) also reported that the IAA content in the leaves of UV-B stressed

Trichosanthes kirilowii was lower than that of the control plants. Another auxin with a much stable structure than IAA is the α-naphthalene acetic acid (NAA) which acts as an artificial plant growth regulator and is not degraded by IAA oxidase. NAA causes acidification of the cell wall medium and formation of proteins, thus promoting total plant growth (Wang 2000). In *T. kirilowii* exposed to UV-B rays, addition of NAA promoted an increase in height and leaf area. This data represents the fact that NAA replaces the decrease in the endogenous IAA levels under light stress. It has also been found that UV-B radiation alters the ability of the cells to utilize NAA, and so the alleviation effect of NAA on the damages caused by UV-B stress is limited. It was also seen that UV-B radiation decreased the gibberellic acid 1/3 (GA1/3) content in the leaves of *T. kirilowii*. Addition of NAA reduced the effect of UV-B on the endogenous content of GA, because it was observed that the GA content was higher in the T3 plants where NAA has been exogenously applied than in the T2 plants (Liu and Zhong 2009). UV-B also reduced the leaf area, and hence the production of less leaf area under such UV-B stress has been portrayed as a defensive mechanism against this abiotic stress. Reduction of cell size accompanied with conditional change in leaf structure, reduction in cell number through decreased cell division, and expansion have been accredited to such reduced leaf area under UV-B stress (Hofmann et al. 2001). Irina et al. (2004) observed that in *Pisum sativum*, the leaf area and biomass decreased after UV-B exposure and accounted the inactivation of CTKs to be responsible for such a phenomenon. However, Liu and Zhong (2009) found that zeatin riboside (ZR) levels increased in the leaves of *T. kirilowii* plants under UV-B stress after addition of NAA.

In previous sections, we have clearly discussed the signaling pattern involved in UV-B-mediated response in plants. Our main intention is to merge this signaling with those of the phytohormones which occur in tandem to tackle stress. Hayes et al. (2014) showed that the UV-B photoreceptor UVR8 provides an unambiguous sunlight signal that inhibits shade avoidance responses in *Arabidopsis* by antagonizing the phytohormones like auxin and GA. UV-B has also been reported to trigger the degradation of the TFs like phytochrome-interacting factor 4 (PIF4) and PIF5 and stabilization of the growth repressors like DELLA. Such UV-B-mediated responses block the auxin biosynthetic pathway, via a dual mechanism (Hayes et al. 2014). Leivar and Quail (2011) inferred that the PIF proteins act as potent signaling hubs, regulating the auxin and GA activity to control plant development and growth. However, the cross talk and mechanism of interaction of these pathways are not completely known, and hence further research is required to investigate the intricate happenings between the UV-B-mediated signaling cascade and the phytohormone biosynthetic pathways.

Hectors et al. (2012) reported that auxin is a component of the regulatory system that controls both UV-mediated accumulation of flavonoids and UV-induced photomorphogenesis. The leaf area of *Arabidopsis* Col-0 plants raised under low doses of UV radiation (0.56 KJ m^{-2} day^{-1}) decreased by about 23 % on average when compared with the control plants, and the level of free auxin also declined in the stressed young leaf tissues. The auxin influx mutant *axr4-1* and the auxin biosynthesis mutant *nit1-3* exhibited stronger morphogenetic responses than the control plants

with higher levels of decrement in the leaf area. It was also inferred that auxin mediated UV acclimation in the plants through the regulation of flavonoid concentration and flavonoid-glycosylation pattern and controlled the UV stress-induced photomorphogenesis (Hectors et al. 2012).

Lin et al. (2002) reported the relation between auxin and polyamine content in the leaves of Nancheum (NC) and Shan You63 (Sy63). The three major enzymes like arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and S-adenosylmethionine decarboxylase (SAMDC) were found to be increased in both the plants. However, in the leaves of IR65600-85, only the ADC and ODC activities increased by 115.93 % and 14.45 %, respectively, but the SAMDC activity decreased surprisingly by 33.01 % on exposure to UV-B radiation for one to two weeks. In late treatment time course (21–28 days), the activities of ADC and ODC substantially increased in the leaves of Sy63, NC, and IR65600-85 under UV-B stress. However, the SAMDC content was reduced by 40.06 %, 19.20 %, and 38.21 % in Sy63, NC, and IR65600-85, respectively, when all were exposed to UV-B stress. The activity of polyamine oxidase was also low in the plants which ultimately resulted in high accumulation of polyamines especially putrescine. Apart from these results, Lin et al. (2002) also reported that after exposure of the plants to UV-B stress for 7–28 days, the IAA and GA1/3 contents decreased by 58.92 % and 45.48 %, respectively, in the leaves of Sy63; by 43.31 % and 56.20 %, respectively, in the leaves of NC; and by 38.60 % and 47.33 %, respectively, in the leaves of IR65600-85. Another contradictory result was observed for CTKs, when it was found that the ZR levels reduced in the leaves of the three plants with UV-B treatment for 7–14 days, but peaked in the late courses of exposure (21–28 days). However, the production of the "universal stress hormone" ABA was quite high. So, a low ratio of IAA/ABA, GA1/3/ABA, and ZR/ABA led to the suppressed growth of the plants so that more resources could be channelized to tackle the damages caused by UV-B stress (Lin et al. 2002).

Zhang et al. (2014) showed that PhyB plays a major role as photoreceptor in sensing the ratio of red to FR light in order to mediate the shade avoidance response (SAR). Change in homeostasis of phytohormones like auxin and strigolactone has been partially held responsible for SAR, though the link between PhyB and hormonal homeostasis has not been unveiled. Constans-like 7 (COL7) plays a role in increasing the branching number under high red: FR, but not under low red: FR, from which it was inferred that COL7 can be involved in the PhyB-mediated SAR. COL7-induced branching proliferation was suppressed by mutating *PhyB* and it was also reported that COL7 inhibits auxin biosynthesis by elevating the mRNA expression of *Superroot2* (*SUR2*) which encodes a suppressor of auxin biosynthetic pathway. This suppression of auxin synthesis occurred only under high red: FR and not under low red: FR. Thus, it can be said that following photoexcitation, PhyB stabilizes COL7, which regulates the light perceived changes in auxin biosynthesis (Zhang et al. 2014). In another report, Effendi et al. (2013) found that the auxin receptor, auxin binding protein (ABP) is involved in the direct regulation of plasma membrane activities, including the number of PIN proteins and auxin efflux transport. A steady-state level of ABP1 and auxin-inducible growth capacity are maintained by the phytochromes under red light. The hypocotyl lengths were larger in

abp1-5 and *abp1/ABP1* mutants exposed to FR light, but not in the null mutant of Transport-Inhibitor-Response1 (tir1-1) auxin receptor. Auxin transport has been depicted to be an important condition for FR-induced elongation. So, naphthylphthalamic acid (NPA) which is an auxin transport inhibitor reduced elongation more strongly in the low red: FR light-enriched white light than in the high red: FR light-enriched white light condition (Effendi et al. 2013). It was also found that decreased phytochrome action occurred in conjunction with auxin transport in the *abp1* mutants, because after adding NPA, hypocotyl gravitropism was inhibited on exposure to both red and FR light. The *abp1-5* mutant lines exhibited a reduction in the transcription of FR light-induced genes, which included several genes regulated by auxin and shade. The same set of genes under the same conditions was found to be even lower in the *abp1/ABP1* mutants. In the *tir1-1* and *PhyA-211* mutants, the shade-induced gene expression was reported to be greatly attenuated. These data obviously support the fact that ABP1 directly or indirectly plays a role in auxin-mediated light signaling (Effendi et al. 2013). Zhao et al. (2013) reported that high-intensity blue light-induced hypocotyl phototropism is mediated by phototropins mainly through the regulation of the cytosolic calcium concentration in *Arabidopsis*. It was seen that the addition of an inhibitor of auxin efflux carrier resulted in the inhibition of phototropism and cytosolic calcium bursts, which obviously points to the role of polar auxin transport in high blue light-induced responses. Phytochrome kinase substrate (PKS1), the phototropin-related signaling element, has been depicted to interact physically with the phototropins, auxin efflux carrier PIN Formed 1 in vitro, and with calcium-binding protein, Calmodulin4 in vivo (Zhao et al. 2013).

Intact auxin signaling regulates proper brassinosteroid (BR)-mediated responses in plants, as has been ascertained from several genetic and physiological studies (Zhou et al. 2013). It was demonstrated that high BR concentration induced the differential growth of etiolated hypocotyls, resulting in variable morphological alterations. However, the dominant mutant of IAA19, *msg2*, and Auxin Responsive Factor7 (ARF7) mutant, *arf7*, were insensitive to the BR effect and could suppress enhanced BR-mediated downstream signaling. Reduced BR response in *msg2* was verified from systemic microarray analysis, from which Zhou et al. (2013) finally inferred that BR employs auxin signaling components IAA19 and ARF7 to trigger the downstream responses. Since the relation of light quality with hypocotyl growth has already been correlated in the previous sections, it can be predicted that BR, along with auxin, entail pivotal significance in the light-mediated responses. Tao et al. (2008) reported the existence of an aminotransferase named TAA1 which catalyzes the accumulation of indole-3-pyruvic acid (IPA) from L-tryptophan. This reaction leads to the rapid accumulation of auxin through a previously uncharacterized pathway. Such rapid accumulation of auxin is required to initiate the multiple alterations at the morphological level associated with shade avoidance (Tao et al. 2008). Among the early auxin-responsive genes, the *Aux/IAA* genes are the best characterized. These genes encode short-lived transcriptional repressors which regulate downstream responses. Singla et al. (2006) found 15 expressed sequence tags (ESTs) in wheat by screening available databases, and these ESTs exhibit high homology with the *Aux/IAA* homologues in the other related species. *TalAA1* is one

such *Aux/IAA* gene, which encodes a protein containing all the four conserved domains, characteristic of the Aux/IAA proteins. Expression of *TalAA1* is regulated by light intensities and is tissue specific and auxin inducible. *TalAA1* is upregulated in the presence of calcium ions and is also induced by BR, thus showing that the interplay and cross talk between hormones is essential for plant growth and development under light stress (Singla et al. 2006). Based on the fact that cryptochromes participate in several photomorphological responses in seed plants, Imaizumi et al. (2002) analyzed the functions of cryptochrome in the moss *Physcomitrella patens*. The reason of choosing this bryophyte has been accredited to the fact that this is a well-characterized species, where gene replacement occurs at a high frequency by homologous recombination. In *Physcomitrella*, two cryptochrome genes and single and double disruptants of these genes were generated. It was surprisingly found that the induction of side branching on protonema and gametophore induction and development are regulated via cytochrome-mediated signaling. The disruptants exhibited higher sensitivity to exogenous auxin than the wild type, accompanied with an altered expression pattern of the auxin-inducible genes, especially when the disruptant lines were exposed to blue light. These data from the reports of Imaizumi et al. (2002) indicate the potential of cytochrome-mediated light signaling in repressing the auxin-mediated downstream cascades in order to restructure the plant developmental pattern. The detailed excerpt of the various roles of auxin under light stress or variable light quality does illustrate the importance of these phytohormones in maintaining the plant metabolic homeostasis under aforesaid conditions. We have also tried to highlight the still uncharacterized versions of cross talks and interplay among the phytohormones like auxin, CTK, and BR which are even more crucial for the proper generation of plant responses against light stress and light quality.

8.6.2 Cytokinins (CTKs)

CTKs play crucial roles in regulating plant growth, metabolism, and development under both normal and stress conditions. It has been seen that during desiccation stress arising out of salinity, drought, variations of temperatures, and even under heavy metal toxicity, the levels of CTK alter rapidly (Atanasova et al. 2004). According to Buchanan-Wollaston et al. (2003), the plants experience senescence-like symptoms under stress conditions due to the burst in the production of ROS. The CTKs have often been portrayed as antioxidants due to their anti-senescence property. Vaseva et al. (2006) studied the changes in CTK oxidase/dehydrogenase, involved in CTK biosynthesis in plants exposed to UV-B stress. The authors chose two pea cultivars based on phenotypic differences; the cv. “Scinado” was taller and fast growing, whereas the cv. “Manuela” showed slow growth, accompanied by shorter stems and broader leaves. The initial level of CTK content in the leaves of both the varieties sufficiently differed from each other. The control Manuela leaves exhibited lower CTK concentration compared to Scinado. However, the CTK oxidase/dehydrogenase (CKX) behaved differently in the leaves of plants after

exposure to UV-B radiation. In Manuela, UV-B radiation completely inhibited CKX activity which led to lowered CTK content, except only the phosphorylated forms. On the contrary, the leaves of Scinado exhibited a completely opposite trend, leading to increased accumulation of CTK due to high CKX activity (Vaseva et al. 2006). The CTK ribosides, cis-zeatin (cisZ), and isopentyl adenosine riboside monophosphate (iPRP) levels were unaffected by irradiation in the Manuela roots, though a drastic decrease in the isopentyl adenine (iP) titer (more than 2.5-fold compared to the control) was recorded. The Scinado roots exhibited lowered CTK content with the exception of cisZ, accompanied with very low CKX activity (about tenfolds inhibition compared to the control) after exposure to UV-B stress. Thus Vaseva et al. (2006) reported the better adaptability of the Scinado cultivars under UV-B stress due its high CTK content, mainly because of the fact that CTKs have antioxidant and anti-senescence properties. It was found that the Manuela cultivars are more prone to UV-B stress. Thus, apart from inferring that a high CTK level is a prerequisite for better UV-B stress adaptability, it was also predicted that the different CKX responses in Scinado and Manuela could be due to the presence of different alleles controlling the studied processes in their genomes (Vaseva et al. 2006).

8.6.3 Ethylene

The response of the plants to tackle light stress or to adapt to the quality of light to which it is exposed depends on a complex network of interactions among multiple phytohormones. Weller et al. (2015) identified a mutant with greatly increased leaf expansion and delayed petal senescence by screening for pea mutants showing altered photomorphogenesis under red light. The mutant was quite insensitive to ethylene due to the occurrence of a nonsense mutation in the single pea orthologue of the ethylene-signaling gene *Ethylene Insensitive2* (*EIN2*). Phytochrome-deficient plants having *ein2* mutation had the ability to reverse the effects of ethylene overproduction. Increase in leaf expansion under monochromatic red: FR or blue light was recorded in the *ein2* mutant seedlings. That *ein2* enhances both phytochrome- and cytochrome-dependent responses in a LONG1-dependent manner was confirmed from the interaction of ein2 with PhyA and PhyB and also from the *long1* mutants (Weller et al. 2015). However, it was inferred that ethylene was not the limiting factor for the development of seedlings in darkness or under high-irradiance white light. It was also concluded that ethylene signaling was responsible for the constrained leaf expansion during de-etiolation in pea and that the downregulation of ethylene biosynthesis might be linked with the photomorphogenetic development of the plant mediated by phytochromes and cryptochromes (Weller et al. 2015). UV-B mediates stomatal closure via production of hydrogen peroxide and also affects ethylene biosynthesis. He et al. (2011) linked UV-B stress response and ethylene activity, based on the fact that both induce stomatal closure through the production of hydrogen peroxide. So, it is possible that the UV-B-mediated stomatal closure actually occurs via ethylene-mediated hydrogen peroxide production. This

probability was investigated in *Vicia faba* by epidermal strip bioassay, laser scanning confocal microscopy, and assays of ethylene production (He et al. 2011). The report became interesting when the experiments showed that ethylene might be epistatic to UV-B radiation in stomatal movement, because stomatal closure, induced by UV-B stress, was inhibited when the ethylene biosynthesis and signaling cascade was interfered. It was also observed that on exposure to UV-B stress, the ethylene accumulation preceded the hydrogen peroxide production which also supported the hypothesis of auxin-mediated stomatal regulation on exposure to UV-B radiation. He et al. (2011) also suggested that such stomatal closure occurs via a peroxidase-dependent hydrogen peroxide production, mediated by auxin because the inhibitors for peroxidase, but not for NADPH oxidase, strongly inhibited hydrogen peroxide production upon UV-B radiation.

The following report by Becatti et al. (2009) showed the effect of UV-B shielding on ethylene production for the ripening in tomato fruits, along with the ethylene-mediated carotenoid accumulation in the ripened fruits, exposed to UV-B rays. The authors, therefore, selected *rin* and *nor* tomato mutants, which were unable to produce ethylene required for fruit ripening in the cv. Alisa Craig, which were cultivated under control and UV-B depleted conditions till fruit ripening. The requirement of functional *rin* and *nor* genes was declared essential after observing that following UV-B depletion, the ethylene production decreased in Alisa Craig. The carotenoid content in the ripened fruits was found to be controlled by UV-B-mediated plant responses, either in an ethylene-dependent or ethylene-independent manner (Becatti et al. 2009). In another finding, it was seen that PhyB-1-mediated ethylene production increased only with high photosynthetic photon flux density (PPFD) and high red: FR light ratio in *Sorghum* (Finlayson et al. 2007). Enhanced ethylene production promoted shade avoidance by reducing the leaf blade, leaf sheath elongation, though the total shoot elongation was hindered. Finlayson et al. (2007) also reported that PhyA could participate in the transduction of shade signals in light-grown *Sorghum* plants by triggering ethylene-mediated responses.

8.6.4 Abscisic Acid (ABA)

ABA is the universal stress hormone which has been reported to be upregulated under almost all forms of abiotic stress. It can be clearly deciphered that UV-B stress causes several morphological and molecular damages to the plant system, and hence like other abiotic stresses, ABA level is expected to enhance under such conditions. The downstream signaling cascade mediated by ABA has been depicted to be involved in triggering downstream stress-responsive genes which encode protein products having potential roles as stress relievers. One such product is the OsWRKY89 which is ABA inducible. This protein encoding gene *OsWRKY89* was identified in rice which has been depicted to regulate responses during UV-B stress. Increased wax deposition on the surfaces of leaves was reported in UV-B-stressed transgenic plants overexpressing *OsWRKY89*. The increased wax deposition

drastically reduced the percentage of UV-B transmittance through the leaves. Further researches are required to create a database on the involvement of multiple WRKY proteins regulating the responses induced by radiation stress (Banerjee and Roychoudhury 2015).

Liu et al. (2009) also suggested the accumulation of ABA in plants exposed to UV-B stress, and the authors also documented that ABA might be responsible for the corresponding decrease in the levels of IAA, GA, and CTKs. The NAA, IAA, GA3, and 6-benzyladenine (6-BA) have functions contradicting those of ABA and hence can decrease the total endogenous ABA levels. In *Trichosanthes kirilowii*, as explained earlier, UV-B stress leads to reduced accumulation of IAA and GA1/3, whereas the levels of ZR and ABA significantly increased. Thus, it was concluded by Liu et al. (2009) that growth regulation in *T. kirilowii* exposed to UV-B stress is not only mediated by altered IAA concentrations or activities but also by the integrated changes in concentrations and activities of GA and ZR which affected the endogenous levels of ABA. This observation was in line with those of Tossi et al. (2012) who inferred that an increase in endogenous ABA concentration is a universal response to UV-B stress. It was also proposed that the induction of common signaling components like ABA, nitric oxide, and calcium bursts in plant and animal cells exposed to UV-B radiation points toward the evolution of a general mechanism in divergent multicellular organisms to tackle the damages caused by high doses of UV-B stress (Tossi et al. 2012). In another experiment, it was observed that ABA concentration increased by 100 % in the UV-B-irradiated leaves of *Zea mays*, whereas the maize *viviparous14* (*vp14*) mutant, due to a defective ABA biosynthesis pathway, was hypersensitive to UV-B stress (Tossi et al. 2009). However, the UV-B-mediated damages were attenuated both in the wild-type and *vp14* mutants after exogenous ABA treatments. It was also suggested that UV-B perception induced an increase in the ABA concentration which stimulated NADPH oxidase activity, hydrogen peroxide generation, and also a nitric oxide synthase-like-dependent mechanism leading to increased nitric oxide production, required to maintain cellular homeostasis under stress (Tossi et al. 2009). Gil et al. (2012) investigated the terpene profiles as determined by gas chromatography with electron impact mass spectrometry (GC-EIMS) analysis of in vitro cultured plantlets of *Vitis vinifera* exposed to field-like UV-B dosage. In young leaves exposed to low UV-B dosage, notable increases were found in the levels of membrane-associated triterpenes like sitosterol, stigmasterol, and lupeol. On the other hand, antioxidants like diterpenes α and γ tocopherols, phytol, the sesquiterpene E-nerolidol and the monoterpenes carene, α-pinene and terpinolene cumulatively accumulated in the leaves under high dosages of UV-B stress. Along with the increase in the cellular concentration of these antioxidant terpenes, rise in the levels of the sesquiterpene phytohormone, ABA, was also recorded (Gil et al. 2012).

Berli et al. (2010) exposed one-year-old field-grown plants of *Vitis vinifera* to PAR along with weekly supplemental sprays of ABA. They observed that on exogenous ABA treatment, the levels of UV-B-absorbing flavonols, quercetin, and kaempferol significantly increased. Though the levels of two hydroxycinnamic acids named caffeic and ferulic acids remained unaffected on exposure to UV-B

stress, their levels increased on exogenous ABA treatments. Such UV-B-independent increased accumulation was also true for the cell membrane β-sitosterol, solely by exogenous ABA treatments. However, simultaneous treatments of both UV-B radiation and exogenous ABA were required to upregulate the activities of antioxidant enzymes like catalase, ascorbate peroxidase, and also carotenoids (Berli et al. 2010). Berli et al. (2011) reported that in the UV-B-exposed grapevine leaf tissues, the ABA levels increased sufficiently to trigger the biosynthesis of phenols that filter the harmful radiations and act as potential antioxidants. It was found that when the grapevine plantlets were exposed to both high and low dosages of UV-B irradiation and weekly exogenous supplements of 1 mM ABA (+ ABA) or water (− ABA), the reduction of UV-B delayed the development and maturation of berries, whereas the + UV-B and + ABA treatments hastened sugar and phenol accumulation. However, individual treatments of + UV-B or + ABA reduced berry growth and the sugar content per berry, without affecting the concentration of sugar at harvest (Berli et al. 2011). Assays performed also exhibited that the ABA levels in the berry skins were high in the + UV-B and + ABA combined treatment which led to hastened berry ripening, thus indicating toward the possibility of some role of ABA in regulating fruit ripening under stress conditions. Under such treatments, the berry skin phenols increased with a change in the anthocyanin and non-anthocyanin profiles, thus enhancing the proportion of phenols which display high antioxidant properties (Berli et al. 2011).

ABA produced by the vascular parenchyma cells has been depicted to regulate the bundle sheath cell (BSC)-specific expression of *Ascorbate Peroxidase2* (*APX2*) in the leaves of *Arabidopsis* exposed to high light stress. ABA mediates *APX2* expression by triggering the combined activation of Sucrose Non-fermenting-1-related protein kinase, SnRK2.6 (Open Stomata1 protein kinase), protein phosphatase2C ABA Insensitive2 (ABI2), and Gα (GPA1)-regulated signaling cascades (Gorecka et al. 2014; Galvez-Valdivieso et al. 2009). The degree of susceptibility of the BSCs to photoinhibition under high light stress is regulated by the ABA-activated signaling network through influential non-photochemical quenching. However, except the guard cell responses to initiate stomatal closure, no major ABA-mediated response in the transcriptome was detected in the whole leaves exposed to high light stress (Gorecka et al. 2014). Piskurewicz et al. (2009) reported that under the canopy, seed germination was slowed by FR light along with the inactivation of the photoreceptors. Such conditions elicited a decrease in the GA content and an increase in the ABA level. Seed germination is generally promoted by GA, via the proteasome-mediated degradation of the DELLA repressors, whereas ABA inhibits seed germination via stimulation of the ABI repressors. The link between phytochrome-mediated light responses and the GA/ABA concentration ratio has not yet been clearly deciphered. However, it has been depicted that exposure to FR light stabilizes the DELLA factors like GAI, RGA, and RGL2, which in turn stimulated ABA synthesis, thus inhibiting seed germination through the production of ABI proteins. Such transcription of *GAI* and *RGA* was mediated by the basic helix-loop-helix TF named PIL5. Under low GA concentration, high GAI and RGA levels inhibited seed germination. Interestingly under white light, GAI and RGA were expressed under the *RGL2* promoter and could

substitute RGL2 to trigger ABA synthesis. Testa rupture was inhibited by the DELLA proteins, while ABI3 blocked endosperm rupture thus prohibiting seed germination under low GA levels, induced by FR light (Piskurewicz et al. 2009). FR and red light perceived by phyA and phyB in tomato were found to function antagonistically in mediating cold tolerance. This antagonism was regulated by the ABA- and JA-related genes and the C-repeat binding factor (CBF) stress-signaling pathway (Wang et al. 2015). Chen et al. (2008) observed that HY5 mediated ABA responses during seed germination, early seedling growth, and root development in *Arabidopsis. ABI5* transcription is activated through the association of HY5 with the promoter of the *ABI5* gene. Translated ABI5 acts as a crucial TF to upregulate the *late embryogenesis abundant* (*lea*) genes in seeds. It was found, via chromatin immunoprecipitation assays, that ABA further enhances the binding of HY5 with the *ABI5* promoter and the overexpression of *ABI5* solely restored ABA sensitivity in the *hy5* mutants. Chen et al. (2008) identified a possible integration of light and ABA-signaling pathways which helped young seedlings to develop tolerance toward abiotic stresses. After the perception of light, the phytochromes promoted the expression of Light-Harvesting Chlorophyll a/b protein encoded by the PSII *LHCB* genes. Staneloni et al. (2008) fused the *LHCB* promoter to a reporter gene in etiolated *Arabidopsis* seedlings and exposed them to continuous FR light. This was done only to activate phytochromes and not photosynthesis. The seedlings were also treated with exogenous ABA. The authors found a motif containing the core CCAC sequence in the *LHCB* promoter which is required for ABI4 binding, thus facilitating the ABA-mediated downregulation of the associated gene. However, the ACGT sequence containing G-box was not found in the promoter sequence. Thus, Staneloni et al. (2008) proposed a model in which hydrogen peroxide, produced in the chloroplasts under high light conditions, is associated with the ABA-mediated signaling to altogether regulate *LHCB* expression. Thus, it could be clearly seen that ABA have tremendous roles to play in the plant system in order to mediate stress-responsive and stress-tolerance signals, which aid the plant to better tackle light-related abiotic stresses.

8.7 Conclusions

Several breakthrough outcomes have been recorded over the past years regarding plant tolerance toward abiotic stress. Among these, the most uncharacterized and less studied is the light-associated stress and their effects and responses in plants. Though sunlight is obligatory for plant growth and development via photosynthesis, it contains harmful rays which mainly fall within the UV-B range of the spectrum. The plant nuclear DNA is an inherently unstable biomacromolecule which can be spontaneously damaged metabolically or through the generation of several stress-responsive harmful factors. Light stress which includes high and low light intensity along with the ROS-generating UV-B rays have such disastrous effects, leading to the ultimate degeneration of genomic integrity. Phytohormones are the major chemical regulators which determine alterations in the patterns of plant morphogenesis at

molecular and physiological levels. Recent researches have emphasized the fact that such phytohormones, especially auxin and ABA, have vital roles to play in light stress-mediated responses. Our chapter has also covered the integrated signaling of UV-B perception of the plant and the downstream cross talks present among the auxin-, CTK-, GA-, ethylene-, and ABA-signaling cascades. However, a proper blueprint containing clean demarcations and accurate knowledge of the responsible factors participating in such cross-talk signaling has not yet been created. Thus, the future perspectives in this field are bright enough, since light stress cannot be regulated as such, along with increasing CFCs and ozone layer depletion. In spite of such constraints, crop development and large-scale production of food crops are required for the ever growing population, a large fraction of which suffer from "hidden hunger." The breeders cannot avoid and vanquish land stretches based only on the constraint that they experience high doses of light stress. This brings forth the need of transgenic technology which can create stress-tolerant varieties, viable under such stress conditions, thereby increasing the overall yield and crop production. Hence the inquisitive science community is on the verge to better understand the phytohormone responses in plants exposed to a variety of light stresses, so that the biosynthetic or downstream genes regulated by these hormones can be targeted for up- or downregulation, in order to create stress-tolerant transgenic cultivars for human consumption.

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Chapter 9
Involvement of Phytohormones in Plant Responses to Ozone

Elisa Pellegrini, Alice Trivellini, Lorenzo Cotrozzi, Paolo Vernieri, and Cristina Nali

Abstract Among various contaminants, ozone (O_3) is considered the most ubiquitous and phytotoxic atmospheric pollutant in industrialized and developing countries. It causes extensive risks for plant life, in terms of survival and productivity of wild and cultivated species. Plant response to O_3 resembles the biotic defense reactions and includes two steps: the first is a biphasic oxidative burst with a massive, rapid, and transient increase in apoplastic reactive oxygen species (ROS) production; the second is the induction of the hypersensitive response (HR) and systemic acquired resistance (SAR). In particular, the acute O_3 exposure (high concentrations for a few hours) results in the activation of programmed cell death (PCD) response that interacts with the synthesis of several hormones and other signaling molecules. The cross talk among all these molecules and their complex and interconnected signaling pathways are more important to determine (1) the initiation, propagation, and containment of O_3-induced cell death, (2) the degree of the sensitivity of plants to this contaminant, and (3) the regulatory potential that plants have to promptly respond to oxidative stress. The present chapter reviews the role of phytohormones (such as ethylene, abscisic acid, gibberellins, auxins, and cytokinins) and other signaling molecules (such as salicylic and jasmonic acids, proline, and brassinosteroids), as well as their synergistic and antagonistic effects, in the complex signaling pathway involved in plant responses to O_3 stress.

Keywords Hypersensitive response • Ozone • Plant hormone • Programmed cell death • Reactive oxygen species • Systemic acquired resistance

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9.1 Introduction

Ozone (O_3) is a secondary product formed by the pollution of the atmosphere, as it is generated when primary polluting substances [such as carbon monoxide (CO), hydrocarbons, nitrogen oxides (NO_x), and halogens] of automobile exhaust and industrial wastes are exposed to the solar light. If in the stratosphere O_3 is decreasing, its concentration in the ground-level layer of the atmosphere (called troposphere) is increasing with negative impacts on the (1) environment and (2) health of living organisms. O_3 is a greenhouse gas and especially is one of the most powerful oxidants known (with a redox potential of 2.07 V). It is recognized that O_3 sources in the continental boundary layer of the atmosphere include both transport from the stratosphere and in situ photochemical production. In the troposphere, O_3 is produced by O_2/O_3 equilibrium reactions involving nitrogen monoxide/dioxide (NO/NO_2) and light (reaction 9.1). In addition, photooxidation reactions of CO (reaction 9.2), unburned hydrocarbons such as methane (CH_4, reaction 9.3), formaldehyde, and other non-methane organic compounds (reaction 9.4) also contribute to tropospheric O_3 (Rao et al. 2000). Further volatile organic compounds from plants take part in photochemical reactions that lead to O_3 formation as well (Pinto et al. 2010):

$$NO_2 + O_2 + h\nu \leftrightarrow NO + O_3 \tag{9.1}$$

$$CO + 2O_2 + h\nu \rightarrow CO_2 + O_3 \tag{9.2}$$

$$CH_4 + 4O_2 + 2h\nu \rightarrow HCHO + H_2O + 2O_3 \tag{9.3}$$

$$RH + 4O_2 + 2h\nu \rightarrow R'CHO + H_2O + 2O_3 \tag{9.4}$$

Due to these reactions, known as "photochemical smog mechanism'', the concentration of tropospheric O_3 has increased considerably since preindustrial times (The Royal Society 2008). Ground-level O_3 is increasing at a rate of approximately 0.5–2 % per year over mid-latitudes of the Northern Hemisphere owing to a rapid industrialization and urbanization during the last three decades (IPCC 2013). Episodes of high concentrations of surface O_3 over large parts of Europe and North America usually occur during the summer in dry and sunny weather conditions. Ambient O_3 concentrations in Europe, Japan, and the United States usually range between 20 and 60 ppb (for O_3, 1 ppb is 1.96 μg m^{-3} at 20 °C and 101.325 kPa) during high light intensity seasons, but acute O_3 peaks exceeding 200 ppb are frequently reached around large cities (Tamaoki 2008) or during heat wave episodes (Pellegrini et al. 2007; Lorenzini et al. 2014a, b). High O_3 concentrations are no longer limited to local hot spots or urban regions. Elevated O_3 concentrations in rural areas are caused by the transport of O_3 and its precursors from urban and industrialized regions over distances of hundreds of kilometers (Stockwell et al. 1997). Moreover, as a result of the increased emission of O_3 precursors, tropospheric O_3 is expected to increase 20–25 % by 2050 and as much as 40–60 % by 2100, in

developing countries such as China and India (Meehl et al. 2007). For these reasons, as reported by the Royal Society (2008), O_3 can no longer be considered a mere local air quality issue, but it is a global problem requiring a global solution.

Tropospheric O_3 is considered the major air pollutant causing negative effects on plant growth and productivity because of its toxicity and widespread occurrence (Long and Naidu 2002; Wilkinson et al. 2012). The O_3 impact on natural ecosystems and crop productivity (quality and quantity) is relevant (The Royal Society 2008). Exposure to O_3 can result in foliar lesions such as chlorosis and necrosis, but the appearance and the severity of these leaf injuries differ among plant species and varieties (Tamaoki 2008). However, it is well known that O_3 may produce functional alterations even without, before the onset of, or in addition to visible effects on a plant (Gottardini et al. 2014). O_3 reacts with plants in a (1) solid (e.g., with the leaf cuticle), (2) gas (e.g., with hydrocarbons emitted by plants), and (3) liquid phase. In the last case, O_3 has the biggest effect on plants, which includes the dissolution of O_3 in aqueous media followed by its reaction with lipids, proteins, and other cellular components (Rao et al. 2000). Nonetheless, plants are able to respond to air pollutants in a similar manner as to other stress factors. Mechanisms include the (1) exclusion, (2) tolerance, (3) compensation, and (4) repair (Levitt 1980). Effects of O_3 on plants depend upon the concentration of the pollutant and the duration of the exposure. Two types of exposure are defined: (1) acute, high concentrations, above 120 ppb for a few hours, as may occur at most polluted sites and (2) chronic, an elevated background concentration with daily peak concentrations in the range of 40–120 ppb over several days in the growing season (Long and Naidu 2002). Chronic exposures to low levels of O_3 result in a (1) decline in photosynthesis, (2) growth inhibition, and (3) premature senescence, normally even without visible damage (Krupa 2003; Leisner and Ainsworth 2012; Vainonen and Kangasjärvi 2015). Conversely, acute exposures, which can occur several times throughout the growing season, lead to the induction of cell death and visible leaf injuries in sensitive plants (Rao et al. 2000).

In this chapter, we focus mainly on processes induced in plants by acute O_3 exposures. The reader interested in the effect of chronic O_3 exposure on plant performance is referred to several field studies (Booker et al. 2009; Gottardini et al. 2014; Hoshika et al. 2015).

The acute O_3 exposure results in the activation of programmed cell death (PCD) response resembling the hypersensitive response (HR) observed in plant-pathogen interactions (Baier et al. 2005; Kangasjärvi et al. 2005). HR, which often precedes systemic acquired resistance (SAR), is characterized by the induction of (1) various cellular protectant genes, (2) the host cell death, and (3) the restriction of pathogen growth and spread. PCD is a physiological process that selectively targets and eliminates unwanted cells, and it plays a role in influencing plant responses to a variety of biotic and abiotic stimuli (Rao et al. 2000; Pellegrini et al. 2013; Vainonen and Kangasjärvi 2015). Kangasjärvi et al. (2005) proposed this mechanism of plant O_3 sensitivity as defined in the lesion formation: (1) regulation of O_3 flux to leaves, (2) O_3 degradation to reactive oxygen species (ROS, normally produced by the plant cellular metabolism) and detoxification [mainly by ascorbate (AsA)] in the apoplast,

(3) O_3 sensing/perception, (4) O_3-induced active production of ROS, (5) early activation by O_3 of mitogen-activated protein kinases (MAPK), and (6) hormonal regulation of the O_3-induced lesion formation. A common element seen with O_3 and other abiotic and biotic stresses is the active production of ROS in the apoplast. Therefore, O_3 may also be used as a non-invasive tool to mimic and to study signaling pathways triggered by active apoplastic ROS formation (Pellegrini et al. 2013; Vainonen and Kangasjärvi 2015).

Like CO_2, O_3 enters leaves through open stomata by the diffusion according to ambient concentration gradients. Therefore, stomatal apertures control and restrict the O_3 flux into leaves and in this way form the "first line of defense" (Vainonen and Kangasjärvi 2015) in order to reach the acclimation (Hoshika et al. 2013). O_3 generally decreases the stomatal conductance few hours after the beginning of exposure (also restricting the CO_2 flow into leaves), and this continues for several hours. Furthermore, O_3 produces a quick (within 1 min) transient decrease in the conductivity followed by a recovery before the longer, final decrease in stomatal conductance (Vahisalu et al. 2010). These results suggest that the sensitivity to external stimuli is an important factor in the regulation of stomatal movements and, consequently, in overall sensitivity/tolerance to O_3. The O_3-induced control of the stomatal aperture via ethylene (ET) can either promote their opening/closure, depending on (1) if plants have experienced an additional stress and (2) how its intensity was (Wilkinson et al. 2011). Also the enhancement of calcium (Ca^{2+}) level in the cytosol determines stomata closure via the regulation of the ion channel activity (Vainonen and Kangasjärvi 2015). In addition, stomatal movements in response to O_3 in the guard cell are regulated by other anion channels, such as *open stomata* 1 (OST1) and *slow anion channel* 1 (SLAC1) (Vahisalu et al. 2010). OST1 is involved in abscisic acid (ABA)-dependent stomatal responses regulating the activity of several ion channels in guard cells. Similarly, the activation of SLAC1 is essential in stomatal closure triggered by O_3 and by other signals including ABA, Ca^{2+}, NO, and light/dark transitions (Negi et al. 2008). However, it is still poorly understood how O_3 impacts the stomatal functionality. Kangasjärvi et al. (2005) proposed that (1) changes in the photosynthesis, (2) hormone signal-mediated active ROS production in guard cells, and (3) Ca fluxes and anion channels could be possible mechanisms that impact the stomata regulation. The stomatal closure has been frequently observed in both trees and herbaceous species exposed to O_3. However, differences in the O_3 tolerance cannot always only be attributed to changes in the stomatal conductance, as plant responses to O_3 also include protective and repair processes (Castagna and Ranieri 2009).

O_3 does not penetrate deeper into intercellular spaces but rather decomposes at the cell wall and the plasma membrane. After O_3 enters the apoplast, it is immediately degraded into several ROS (Vainonen and Kangasjärvi 2015). According to studies by Weiss, O_3 dissociates in aqueous solutions as a function of the endogenous hydroxyl ion concentration, generating ROS such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in the apoplastic and the intercellular space within plant cells (Heath 2008; Sandermann 2008). While O_3 reacts with lipid molecules generating stoichiometric amounts of aldehydes and H_2O_2 – a process referred to as

ozonolysis – both $O_2^{\bullet-}$ and H_2O_2 react with transition metals (e.g., through the Haber-Weiss mechanism or the Fenton reaction) to generate hydroxyl radicals ($OH^{\bullet}$), which are the most reactive chemical species in the biological world. Furthermore, O_3 can react with thiol groups, amines, and/or phenolic compounds, intensifying the production of $OH^{\bullet}$ and singlet oxygen ($^1O_2$). $O_2^{\bullet-}$ can also react with another very influential signaling free radical species, $NO^{\bullet}$, to give off peroxynitrite (Rao et al. 2000; Gill and Tuteja 2010). These ROS are highly reactive and toxic, and they cause damage to (1) proteins, (2) lipids, (3) carbohydrates, and (4) DNA, which ultimately may result in cell death. To protect themselves against ROS, plant cells and organelles such as chloroplasts, mitochondria, and peroxisomes deploy antioxidant defense systems. To remove and detoxify excess ROS, plants have evolved antioxidant defenses as both enzymatic [e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)] and nonenzymatic (e.g., phenylpropanoids, proline, α-tocopherol, and carotenoids) which have been the subject of extensive research and the focus of recent reviews (e.g., Møller et al. 2007; Castagna and Ranieri 2009; Gill and Tuteja 2010; Farmer and Mueller 2013). The first antioxidant defense against O_3-derived ROS takes place in the apoplast, where AsA is believed to provide an important protection from the oxidative injury. The ROS detoxification formed from O_3 in the apoplast can therefore be considered as the next line of defense (Vainonen and Kangasjärvi 2015). The antioxidant role played by AsA is strictly dependent on the cell ability to maintain it in a reduced state. This is usually enacted by the so-called Halliwell-Asada cycle, which takes place in the chloroplast and cytosol. This mechanism regenerates the pool of reduced AsA at the expense of reduced glutathione (GSH) by monodehydroascorbate reductase (MDHAR) or by dehydroascorbate reductase (DHA). Glutathione is a powerful ROS scavenger, but, like AsA, only its reduced form has antioxidant properties. GSH is cyclically regenerated by GR at the expense of NADPH oxidation (Foyer and Noctor 2011). Other low-molecular-weight compounds (e.g., phenylpropanoids) present in the apoplast may also play a role in the cell defense. When the antioxidant capacity of the apoplast is exceeded, the ROS accumulation in the extracellular space further propagates inside cells (Kangasjärvi et al. 2005).

Based on the assumption that ROS are highly reactive and lead to the cellular and organelle dysfunction, their generation is frequently considered deleterious and harmful. However, several studies indicate that ROS may be important components of signal transduction pathways that influence plant defense responses and PCD for a wide variety of stimuli, including pathogens such as fungi, bacteria, and viruses (Lamb and Dixon 1997; Jabs 1999). The production of secondary ROS due to the O_3 degradation in the apoplast induces an endogenous, enzymatic ROS generation or "oxidative burst" similar to that seen in the HR-mediated cell death (Baier et al. 2005) triggering a series of signaling cascades that work simultaneously, alternatively, or in series (Vaultier and Jolivet 2015) by amplifying the ROS wave in different subcellular compartments (Fig. 9.1). Similarly to pathogen response, the O_3-induced ROS production is biphasic: an early sharp peak detectable within 1 h of O_3 exposure and a late broad peak developing at 9–12 h after the onset of exposure (Jones and Dangl 2006). A heterotrimeric G protein has been suggested to mediate

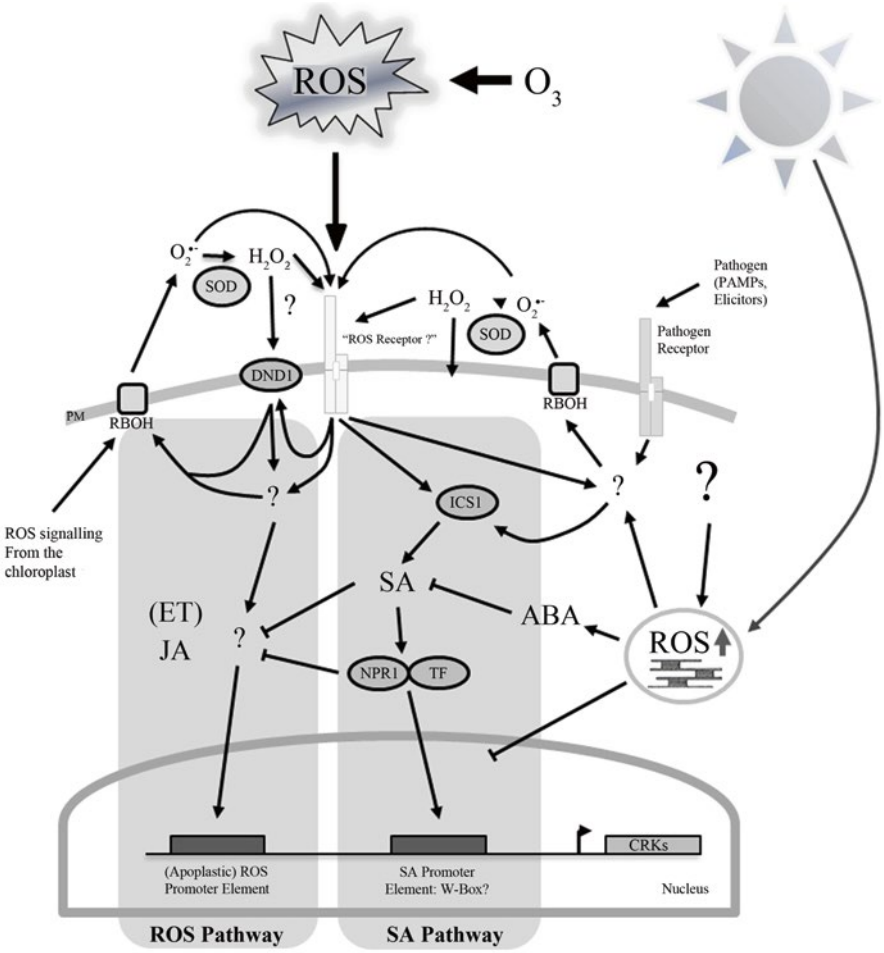

Fig. 9.1 Reactive oxygen species (ROS) and hormonal pathways induced by ozone (O_3). O_3 enters leaves through stomata reacting with components of the cell wall and generating ROS. O_3 and ROS [hydrogen peroxide (H_2O_2) and anion superoxide ($O_2^{\bullet-}$)] induce an active production of ROS in the apoplast which is at least partly dependent on membrane bound NADPH oxidase (RBOH) that produces $O_2^{\bullet-}$. Similar ROS production in the apoplast takes place after infection of a plant with a pathogen or treatments with pathogen-derived elicitors (PAMPs). Inside the cell, the signaling pathway is splitted into two pathways. In the ROS pathway, defense no death 1 (DND1) mediates a required step of the signaling pathway. Jasmonic acid (JA) and ethylene (ET) act as positive regulators; by contrast, salicylic acid (SA) and *nonexpressor of pathogenesis-related gene* 1 (NPR1) are negative regulators. In the SA pathway, ROS or pathogens activate SA biosynthesis via *isochorismate synthase* 1 (ICS1) where NPR1 is a required component. Since NPR1 is a positive regulator of the SA pathway and a negative regulator of the ROS pathway, this implies that the separate signaling pathway use different transcription factors and promoter elements to regulate cysteine-rich receptor-like kinase (CRK) expression. Increased ROS production in the chloroplast activates separate signaling pathway(s) leading to repression of CRK expression. One of these pathways could involve abscisic acid (ABA) and negative cross talk with the SA pathway (Modified and redrawn from Wrzaczek et al. 2010. Abbreviations: *SOD* superoxide dismutase)

the biphasic ROS production in response to O_3 (Joo et al. 2005; Booker et al. 2012). In any event, the apoplastic ROS generated by the O_3 degradation or by the oxidative burst in response to O_3 needs to be (1) perceived by cells and (2) elaborated and delivered to nucleus in order to ensure an appropriate response by the plant. Several potential mechanisms may work simultaneously to create a complex signaling network for the O_3 response. These include (1) changes in the cellular redox homeostasis, (2) perception by apoplastic proteins, (3) oxidative damage to membranes, and (4) transport of apoplastic H_2O_2 through aquaporins across plasma membranes. Additionally, the ROS production in chloroplasts has been detected for a short time after O_3 exposure, suggesting a strong connection between the apoplast and the chloroplast in ROS signaling. Guard cell chloroplasts represent the main source of ROS under high O_3 stress, but this stress also propagates the ROS signal at the cell wall via peroxidases and at the plasma membrane via NADPH oxidases (RBOH) activated by Ca, OST1, and Ca-dependent protein kinases (CPKs) (Vahisalu et al. 2010) playing a critical role in amplifying the ROS signal (Vainonen and Kangasjärvi 2015). As such, ROS alone may not be sufficient to induce PCD at the entire-plant level, but they act in concert with other signaling components to trigger the cell death (Rao et al. 2000). Hence, O_3 exposure activates several intercellular signaling pathways that lead to changes in the hormonal homeostasis and biochemical and transcriptional responses. The constitutive MAPK activation has been shown to play a key role in the HR-like cell death, suggesting that the MAPK signaling is a part of the ROS-induced PCD pathway. Ahlfors et al. (2004) documented that MPK3 and MPK6 were rapidly induced in response to a transient exposure of O_3 (between 30 min to 2 h) in *Arabidopsis thaliana* plants. Then MPKs translocate to the nucleus, where they activate target transcription factors and regulate gene expression. Typically, MPK3/MPK6 substrates involved enzymes linked to ET biosynthesis (Meng et al. 2013). Previous studies have shown that MPK3 and MPK6 can be activated by different MKKs and participate in specific signaling pathways. For example, MKK4/MKK5/MKK9 activates MPK3/MPK6 to promote ET (Liu et al. 2008), and MKK9 activates MPK3/MPK6 to regulate leaf senescence (Zhou et al. 2009) and ET signaling (Yoo et al. 2008).

Another early event triggered by the O_3 exposure is the increase of the concentration of free cytosolic calcium ion (Ca^{2+}) in guard cells (Evans et al. 2005) which acts as a second messenger regulating a signaling network involved in the response of a different range of developmental and environmental stimuli (Tamaoki 2008; Kudla et al. 2010; Short et al. 2012).

Collectively, plant hormones regulate every aspect of the plant growth and development, as well as their response to biotic and abiotic stresses (included chronic and acute O_3). Plant growth regulators include classical phytohormones ET, ABA, auxins (IAAs), cytokinins (CKs), and gibberellins (GAs), as well as other signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), proline (Pro), and brassinosteroids (BRs). Moreover, the participation of several phytohormones in response to acute O_3 exposure, following the production of ROS, has been established. Recent studies have demonstrated that these signaling pathways do not act independently and that the degree of the lesion formation induced by O_3 is influenced by a cross

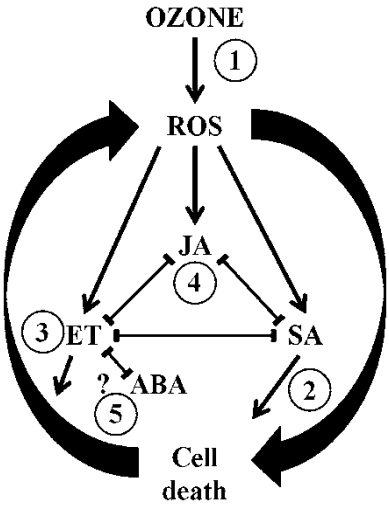

Fig. 9.2 Oxidative cell death cycle induced by ozone. Ozone stress causes the accumulation of reactive oxygen species (ROS) and results in salicylic acid (SA) accumulation (*1*) and programmed cell death (*2*). Cell death activates ethylene (ET) production, which is required for the continuing ROS production and, consequently, for the propagation of cell death (*3*). Jasmonic acid (JA) counteracts the progression of the cycle by antagonizing the cell death promoting SA and ET function (*4*). Abscisic acid (ABA) can antagonize ET function and might also have this role in ozone-induced cell death (*5*) (Modified and redrawn from Kangasjärvi et al. 2005)

talk between these signal pathways (Tuominen et al. 2004; Gomi et al. 2005; Yaeno et al. 2006; Ludwików et al. 2009; Tamaoki 2008). An early O_3 response is the biosynthesis of ET followed by the production of SA, JA, and ABA. ET and SA signaling promote an enhanced ROS production and PCD, which all together form a feedback loop (Fig. 9.2). JA attenuates this cycle by reducing the ROS production followed by (1) ET biosynthesis and (2) cell death. ABA is especially important as the regulator of stomatal closure and O_3 entry. Recently, connections between the oxidative stress and classical plant hormones IAAs, CKs, and GAs have garnered attention (Blomster et al. 2011; Peleg and Blumwald 2011; Tognetti et al. 2012).

The present chapter reviews the role of phytohormones and other signaling molecules, as well as their synergistic and antagonistic effects, in the complex signaling pathway involved in plant responses to O_3 stress.

9.2 Roles of Hormones in Ozone Response

9.2.1 Ethylene (ET)

Ethylene (C_2H_4) is essential in modulating plant responses to both biotic and abiotic stresses, in addition to its key roles in the regulation of growth and senescence (Lin et al. 2009). In plants exposed to stresses such as wounding, flooding, drought,

osmotic shock, O_3, and pathogen/insect invasion, the rate of ET production increases rapidly (Wang et al. 2002; van Loon et al. 2006) leading the activation of cellular responses through ET signaling and interactions with other hormone signaling pathways (Overmyer et al. 2008; Wang et al. 2002; Trivellini et al. 2011).

ET is synthesized by two enzymes encoded by small gene families, 1-aminocyc lopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), respectively (Fig. 9.3). The two-step reactions are first catalyzed by ACS involving the conversion of S-adenosyl-L-methionine (SAM) to ACC and then ACO catalyzes the conversion of ACC to ET with the release of CO_2 and cyanide (Wang et al. 2002). ACS is the rate-limiting step in ET biosynthesis, and it controls the major

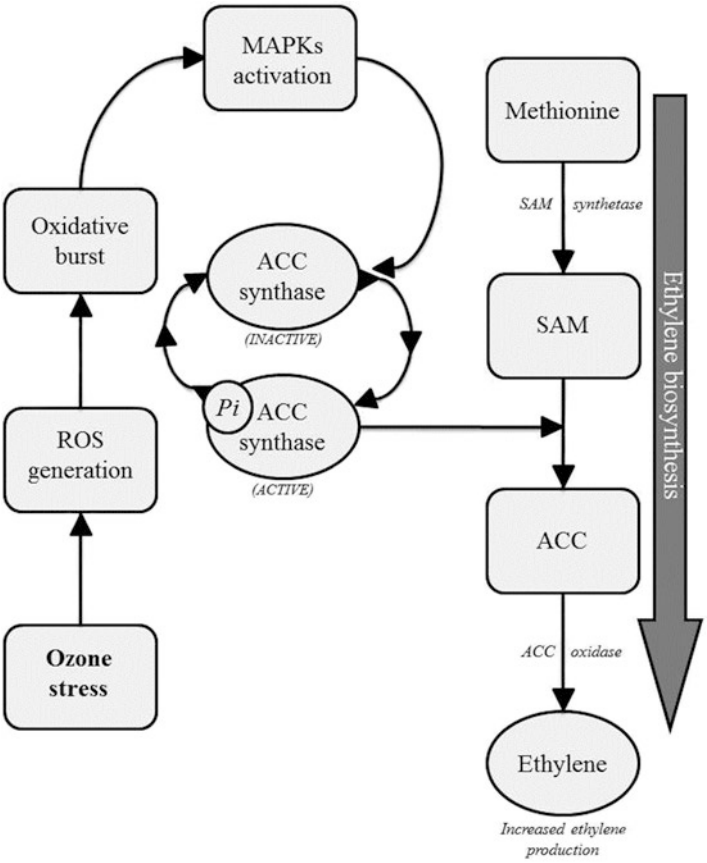

Fig. 9.3 Proposed pathways for ethylene biosynthesis in ozone-exposed plants. Ozone stress causes the accumulation of reactive oxygen species (ROS) and results in an oxidative burst inside plant cells. Mitogen-activated protein kinases (MAPKs) respond to the resulting oxidative burst and induce the activation/phosphorylation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. The phosphorylated ACC synthase becomes stabilized and subsequently enhances the ethylene production (Modified and redrawn from Iqbal et al. 2014. Abbreviations: *SAM* S-Adenosylmethionine)

regulatory step in stress-induced ET (Tsuchisaka et al. 2009). For this reason, ACO activity is constitutively present in most vegetative tissues. In fact, the activity of ACS in non-stressed tissues is low and does not induce a significant amount of ET, whereas ET synthesis induced by biotic/abiotic stresses is linked to a rapid enhancement in ACS activity (Tamaoki 2008).

The induction of ET biosynthesis is one of the fastest O_3-dependent biochemical responses observed in plants, occurring within few hours (Nakajima et al. 2001; Moeder et al. 2002; Pellegrini et al. 2013). In O_3-fumigated tomato plants, LE-ACS1A, LE-ACS2, and LE-ACS6 are induced, and the expression of ST-ACS4 and ST-ACS5 is increased in potato (Schlagnhaufer et al. 1997; Tuomainen et al. 1997). This response was similarly rapid in birch (Vahala et al. 2003) and in an O_3-sensitive poplar clone (Diara et al. 2005). In O_3-exposed beech plants (180–200 ppb, 8 h d^{-1}, for 6 consecutive months), the transcript levels of ACS1, ACS2, and ACO1 were significantly enhanced (Nunn et al. 2005; Betz et al. 2009).

ET production is strictly regulated at various levels. It is accepted that, in addition to the transcriptional regulation (Tsuchisaka and Theologis 2004; Li et al. 2012), posttranslational regulation is crucial for plant development and stress-induced ET production (Christians et al. 2009; Han et al. 2010; Lyzenga et al. 2012). The increase in ET production in response to O_3 occurs simultaneously or even precedes the induction of ET biosynthesis genes, thus pointing toward a tight post-translational control of enzymes involved in the biosynthesis of ET (Vainonen and Kangasjärvi 2015). ET biosynthesis is tightly regulated by controlling the stability of ACS protein post-transcriptionally. The involvement of ACS2 and ACS6 in O_3-induced ET biosynthesis by their transcriptional activation (Blomster et al. 2011) occurs through their regulation at the protein stability level by MPK3 and MPK6, two *Arabidopsis* pathogen-responsive MAPKs (Li et al. 2012). MPK3 and MPK6 not only control the stability of ACS2 and ACS6 proteins via direct protein phosphorylation, but these kinases also regulate the (1) expression of ACS2 and ACS6 genes by phosphorylating another MPK3/MPK6 substrate and (2) WKRY53 transcription factor, involved in the MPK3/MPK6-induced ACS2/ACS6 gene expression that play key role in supporting high ET levels. ET biosynthesis at the ACS stability level is also regulated by the protein degradation via proteasome-dependent pathway. The *ethylene overproducer* 1 (ETO1)/ETO1-*like* (Wang et al. 2004; Christians et al. 2009) interacts with ACSs by inhibiting their activity and targeting them to degradation (Yoshida et al. 2006). The O_3 sensitivity in terms of leaf injury is greater in ET-overproducing *Arabidopsis* mutants (Kangasjärvi et al. 2005) compared to ET-insensitive ones (Overmyer et al. 2008). The high accumulation of ET in ET-overproducer mutants upon acute O_3 exposure is proportional to the SA level in these mutants (Rao et al. 2002). Thus, SA and ET act in concert to regulate the O_3-induced cell death in *A. thaliana*. Instead, blocking the ET perception, *jasmonate resistant 1* (*jar1*) prevented the spread of cell death (Tuominen et al. 2004; Kazan 2015). The O_3-induced spreading cell death is stimulated by early, rapid accumulation of ET, which can suppress the protecting function of JA thereby allowing the cell death to proceed. All these evidences suggest that ET

acts synergistically or antagonistically with other signaling molecules to regulate the response to O_3.

At a morphological level, ET promotes a direct reduction of shoot and root cellular expansion via O_3-induced ET production, as proposed by Wilkinson and Davies (2010), since this hormone naturally controls the root growth (Gallie et al. 2009). O_3 caused the reduction and the abortion of bud and flowers (Black et al. 2007) and the decrease in fruit weight and fruit number in a significant manner (Leisner and Ainsworth 2012), and in these processes ET could be actively involved (Wilkinson et al. 2011).

9.2.2 Abscisic Acid (ABA)

ABA plays considerable roles, and it is involved in the complex network that controls plant responses to abiotic and biotic stresses, during growth and development as well as in the regulation of stomatal aperture. Generally, high salinity and drought stresses promote an enhancement of endogenous ABA content which (1) modulates the gene expression and (2) induces the closure of stomata (Shinozaki and Yamaguchi-Shinozaki 2007). Under stress conditions such as water deficit, ABA increases in guard cells, so stomata close and leaves retain water (Tardieu et al. 2010). The O_3-induced ET production alters the sensitivity of stomata to ABA in O_3-sensitive species (Wilkinson and Davies 2010), inducing stomatal pores to remain more open. Thus, O_3 – via ET – contributes to support the disruption of ABA-mediated stomatal signaling.

Evidence has been reported that ROS accumulation in the apoplast following a stress is involved in the induction of stomatal closure (An et al. 2008; Khokon et al. 2011) modulating the responsiveness of guard cells to ABA (Munemasa et al. 2013). ROS were expected to induce the accumulation of free ABA, resulting from a decreased degradation or its release from conjugated forms (Daszkowska-Golec and Szarejko 2013; Song et al. 2014). Mittler and Blumwald (2015) recently proposed that an elevation in apoplastic ROS levels could result in higher ABA accumulation in guard cells, whereas enhanced ABA levels could result in an enhancement of ROS production in guard cells, creating a positive loop to promote stomatal closure.

ABA biosynthetic pathway is known (Fig. 9.4), but the signaling and perception machinery remains largely ambiguous caused by the high number of components involved in its network complexity. ABA is perceived in cells by pyrabactin resistance protein 1 (PYR1) or PYR-like protein (PYR1/PYL) ABA receptors (Ma et al. 2009) that bind themselves to ABA leading to the suppression of the protein phosphatase 2Cs (PP2Cs). PP2Cs interact with a sucrose non-fermenting 1 (SNF1)-related protein kinase 2 family (SnRK2s), which inhibits the positive regulator of the ABA response and then facilitates the downstream ABA signaling.

Protein phosphatases have also been implicated in the MPK6 regulation (Brock et al. 2010), representing a key component in the regulation of ET production under stress (Schweighofer et al. 2007). ABI1 protein phosphatase 2C is a negative

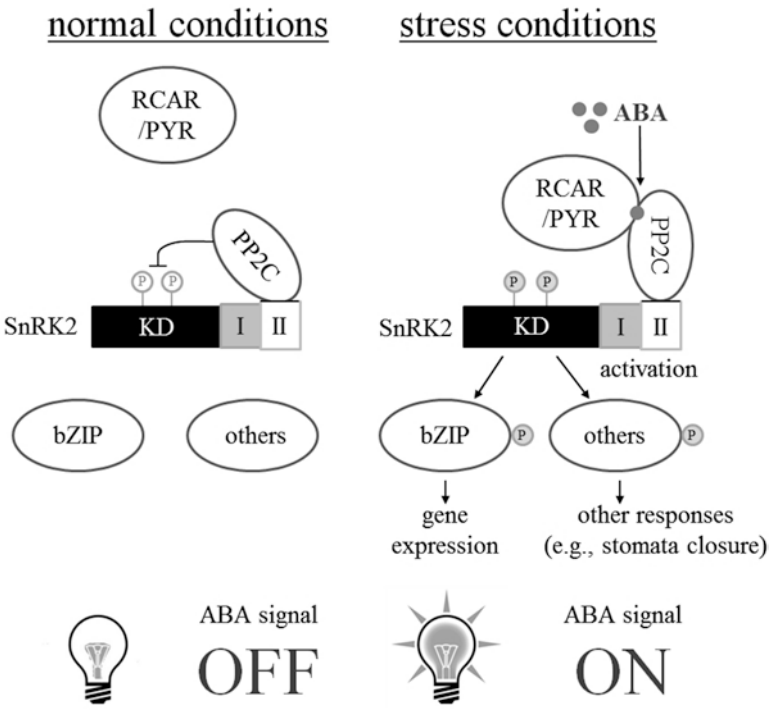

Fig. 9.4 Proposed pathways for abscisic acid biosynthesis in ozone-exposed plants. Under normal conditions, abscisic acid (ABA) level is low; type 2C protein phosphatases (PP2C) bind sucrose non-fermenting 1-related protein kinase subfamily 2 (SnRK2) and inhibit its kinase activity by dephosphorylating activation loop. Under ozone stress conditions, ABA level is increased; ABA receptors (regulatory components of ABA/receptors pyrabactin resistance, RCAR/PYR) interact with PP2C and activate SnRK2. The kinase activity phosphorylates target proteins such as bZIP-type transcription factors and turns on ABA response (Modified and redrawn from http://www.riken.jp)

regulator of ABA signaling (Gosti et al. 1999; Miyazono et al. 2009), which is known to inhibit the MPK6 activity (Leung et al. 2006). Ludwików et al. (2015) show that the ABI1 protein phosphatase, a negative regulator of ABA signaling, is involved in ET production in plants via interaction with ACS and MPK6 under O_3 exposure. O_3-induced ACC levels in *abi1* mutants increased in comparison to wild type, and the ACS6 activity was significantly higher in mutants leading to an increase in ET production in response to O_3. However, previously data reported that *abi1* mutants are O_3 tolerant (Ludwików et al. 2009). Interestingly, these mutants achieve the stress tolerance by switching on the regulation of cellular redox state, exhibiting a significantly reduced ion leakage (Ludwików et al. 2015). These results improve the current knowledge on ABA-ET cross talk where ABA may control ET synthesis and its own biosynthesis by controlling the ABI1 phosphatase activity and, at the same time, highlighting the striking role of ROS homeostasis and redox state in O_3-tolerance mechanisms. There are few studies reporting the effect of O_3

on ABA content despite its recent role in controlling ET evolution in response to O_3. ABA content was enhanced in O_3-fumigated *Melissa officinalis* plants (200 ppb, 5 h) preceding the secondary ROS production peak (Pellegrini et al. 2013). However, in some species, O_3 has no effect on its endogenous content (Döring et al. 2014). Jiang and Zhang (2002) documented a significant increase in ABA levels which preceded that of ROS in leaves of detached maize plants exposed to water stress. Using global transcriptional profiling, the expression of ABA biosynthetic gene 9-cis-epoxycarotenoid dioxygenase (NCED) is positively regulated by the late O_3 stress (350 ppb, 6 h) suggesting a key role of NCED3 enzyme in triggering ABA accumulation in *Arabidopsis* (Ludwików et al. 2009).

9.2.3 Gibberellins (GAs), Auxins (IAAs), and Cytokinins (CKs)

GA is a pentacyclic diterpene which influences the (1) cell elongation, (2) dormancy, (3) seed germination, (4) senescence, and (5) tolerance against environmental stresses (Rodrigues et al. 2011). Actually, no data on the action of exogenously applying GA to plants neither the effect of O_3 on GA biosynthesis and signaling pathway are found in literature despite its prominent role in the mitigation of environmental stresses. In fact, exogenous treatments with GA improve the tolerance of *Catharanthus roseus* to heavy metals (Pandey et al. 2007). Moreover, adverse effects of salt stress were alleviated by GA3, restoring the normal growth and development in soybean plants (Hamayun et al. 2010). Also, the leaf senescence was inhibited by applying exogenous GA, whereas paclobutrazol, an inhibitor of GA synthesis, accelerated PCD (Li et al. 2010). Interestingly, a mutation in the negative regulator of GA signaling, SPINDLY, is responsible of the improvement of the tolerance to drought and to salt (Qin et al. 2011). Jibran et al. (2013) suggested that GAs may work antagonizing the effect of ABA, making intriguingly this scenario in a putative condition of O_3-tolerance mechanisms.

The phytohormone auxin regulates almost every aspect of plant development and has a well-known role in the cell growth and development, and its role in organ architecture has been clarified (Woodward and Bartel 2005; Kim et al. 2011).

Recently, IAAs have also been linked to oxidative stress. Malfunctions in the antioxidative capacity of a thioredoxin and GSH mutant resulted in altered auxin homeostasis and development in *Arabidopsis* (Bashandy et al. 2010). Iglesias et al. (2010) have shown that an auxin receptor mutant showed an increased tolerance to methyl viologen, H_2O_2, and salinity stress, reporting evidences on the contribution of auxin signaling pathway to adaptive responses against abiotic stress in *Arabidopsis*. Moreover, many phytopathogens (*Erwinia herbicola* pv. *gypsophilae*; *Rhodococcus fascians*) can interfere with the normal growth and development of plant processes by modulating the auxin host biosynthesis or producing ex novo hormones (Manulis et al. 1998; Vandeputte et al. 2005). In fact, when the auxin signaling was suppressed, this condition mediates the pathogen tolerance via SA-auxin antagonism (Wang et al. 2007a, b). Additionally, the functional analysis

of oxidative stress by H_2O_2 activated MAPK cascade in plants that downregulated auxin-responsive genes (Kovtun et al. 2000). Plants, exposed to mild chronic stress, manifested a clear reduction in cell division leading to a more compact growth with lateral growth enhanced (Potters et al. 2009), and this physiological response is called "stress-induced morphogenic response" (SIMR). Blomster et al. (2011) used O_3 as a model of ROS inducer in *Arabidopsis* and by the analysis of transcriptomic data reported that several aspects of auxin homeostasis and signaling were modified by apoplastic ROS and the change in gene expression was not completely overlapped with (1) abiotic stress, (2) pathogen responses, and (3) SA signaling. Thus, this evidence indicates that ROS signaling regulates auxin response using a different and likely novel pathway. The alteration of auxin biosynthesis and signaling is a logical consequence that allows plants to cope with environmental stresses, by changing their growth and morphology.

CKs are adenine- or phenylurea-based compounds that regulate the plant growth and development via a complex network of CK signaling (Perilli et al. 2010) and have a well-established senescence-retarding role (Gan and Amasino 1995). As already reported, various phytohormones regulate the mechanisms of the plant response to abiotic and biotic stresses, and also CKs have a function in the adaptation of stress responses (Ha et al. 2012). For example, elevated CK content in ipt transgenic creeping bentgrass (*Agrostis palustris*) promotes drought tolerance through increasing the activity of antioxidant enzymes such as SOD, peroxidase (POD), and CAT (Merewitz et al. 2012). Also, in transgenic tobacco, the overexpression of isopentenyltransferase (IPT) gene prevents the degradation of photosynthetic protein complexes during drought and promotes drought tolerance (Rivero et al. 2010). Nishiyama et al. (2011) explored the role of CKs in stress responses using low-CK-content mutants on the relative adaptation of plants to stresses. A decrease in CK content was reported to determine the hypersensitivity to ABA and the upregulation of stress- and/or ABA-responsive genes, resulting in an increased stress tolerance of CK-deficient and CK-signaling mutants. Thus, the higher stress tolerance of CK-deficient plants could be attributed to their repression of CK signaling that reacts faster to ABA and stresses.

Very few studies examined the role of CKs in response to oxidative stress induced by O_3. Protective effects of exogenous application of synthetic CKs were assessed on some sensitive crops exposed to high O_3 doses. The application of kinetin on leaves retarded their yellowing, inhibited the loss of free sterols, and suppressed O_3-induced foliar necrosis in bean plants (Tomlinson and Rich 1973). Pauls and Thompson (1982) showed that the application of a zeatin solution protected the integrity of cell membranes from O_3 damage. Another study reported the effect of long-term exposure to non-necrotic levels of O_3 on cytokinin levels in beech trees (Winwood et al. 2007). The authors documented that the O_3-induced increase in root development was the most likely cause of increased CK export to the shoot. Thus, the O_3-induced reduction of CKs in leaves led to declining re-translocation from leaves via the phloem to the root system – a change known to relieve inhibition of fine-root production (Riefler et al. 2006). All these data suggest a protective effect of CKs in response to O_3 stress by a chemical interaction between the compounds and O_3.

9.3 Roles of Other Signaling Molecules in Ozone Tolerance/Resistance

9.3.1 Salicylic Acid (SA)

SA is a plant phenolic compound (*o*-hydroxybenzoic acid) considered as a hormone-like endogenous regulator (Iqbal et al. 2014). Among signaling molecules, SA has received particular attention in relation to its ability to regulate various aspects of plant responses to biotic and abiotic stresses through extensive signaling cross talk with other growth substances (Horváth et al. 2007). In higher plants, two distinct SA synthesis pathways have been identified: (1) phenylalanine ammonia-lyase (PAL) and (2) isochorismate (Fig. 9.5). Nonetheless, *Arabidopsis* is actually the only plant in which SA is known to be synthesized via the isochorismate pathway (Ogawa et al. 2007). The first pathway occurs in the cytoplasm, and SA is synthesized only from the phenylalanine by the action of PAL via *t*-cinnamic acid and benzoic acid. The isochorismate pathway takes place in chloroplasts, and SA is produced from

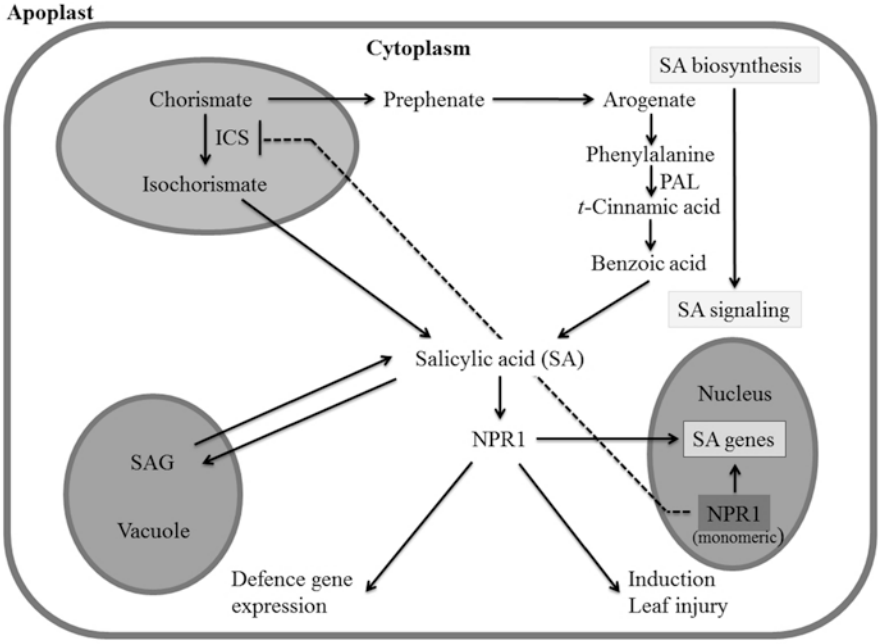

Fig. 9.5 Proposed pathways for salicylic acid biosynthesis and signaling in ozone-exposed plants. There are two pathways for salicylic acid (SA) biosynthesis in plants. In *Arabidopsis*, SA is produced from chorismate by the action of isochorismate synthase (ICS) via isochorismate (the isochorismate pathway takes place in chloroplasts). In all plants (except *Arabidopsis*), SA is produced from the phenylalanine by the action of phenylalanine ammonia-lyase (PAL) via *t*-cinnamic acid and benzoic acid (Modified and redrawn from Jayakannan et al. 2015. Abbreviations: *NPR1* non-expressor of PR-1, *SAG* 2-O-b-D-glucosylbenzoic acid)

chorismate by the action of isochorismate synthase (ICS) via isochorismate (Wildermuth et al. 2001). An unresolved issue is which pathway is used for SA synthesis when plants are subjected to O_3. Ogawa et al. (2005) reported that ^{14}C-labeled benzoic acid (a precursor of SA in the phenylalanine pathway) is metabolized to SA in tobacco plants exposed to O_3 (200 ppb, 6 h), whereas the activity and the mRNA level of ICS did not increase. By contrast, same experimental conditions induced an increase of *ICS1* expression and of ICS activity in *A. thaliana* plants showing that the pathway of O_3-induced SA biosynthesis differs between two species. Most SA synthesized in plants is either glycosylated and/or methylated in cells. The SA O-β-glucoside (SAG) is the dominant glucosylated conjugate of SA, and several studies suggest that it is inactive and must be converted to SA to induce defense responses. Methyl salicylate (MeSA) is the dominant methylated form of SA that, like SAG, is biologically inactive and acts as a mobile endogenous signal carrier that triggers induction of SAR upon converting back into SA. Among various forms of SA, only MeSA has been shown to travel in plant tissues (via phloem) locally as well as systemically after pathogen infections (Jayakannan et al. 2015). Experiments on O_3-exposed transgenic plants in which synthesis and metabolism of SA were altered showed that its presence is needed for (1) the stress symptom initiation/progression and (2) the induction of HR-like cell death. Örvar et al. (1997) reported that the expression of *NahG*, a bacterial salicylate hydroxylase gene, in the tobacco cultivar Xanthi resulted in an inability to accumulate SA and consequently in a reduction of lesion formation in response to O_3 exposure (250 ppb, 6 h). Similarly, Overmyer et al. (2005) observed that the O_3-sensitive *Arabidopsis* ecotype Cape Verde Islands (Cvi-0) and the *radical-induced cell death1* (*rcd1*) mutant (which hyperaccumulates SA in response to O_3) became more tolerant to O_3 (250–300 ppb, 6 h) in terms of the symptom development, when transformed with *NahG* or crossed with the *npr1* mutant (which lacks SA signaling). These results indicate that the O_3-induced production of SA acts as a signal that (1) maintains the cellular redox state, (2) induces and potentiates the activation of cellular defenses, and (3) has a vital role in SAR. At a functional level, some experiments provide evidence that the SA perception amplifies downstream signals that lead to the cell death in a process similar to what occurs during plant-pathogen interactions (Tamaoki 2008). Pellegrini et al. (2013) reported that O_3 triggers marked increases in SA (conjugated and free forms) in asymptomatic leaves of *M. officinalis* after 8 h from the beginning of exposure. In this case, SA may have a role in the upregulation of antioxidative systems during the lesion spread (as confirmed by the increase of ascorbate and glutathione content). The following necrotic cell death can be interpreted as a result of a gradual depletion of antioxidative capacity, which led to a drastic shift in the cellular redox balance and eventually to the cell death (reported after 48 h from the beginning of exposure). Similarly, Pasqualini et al. (2002) reported that, in an O_3-sensitive cultivar of tobacco exposed to a short-term O_3 treatment (200 ppb, 3 h), the maximum induction of SA was observed after 7 h of recovery. Di Baccio et al. (2012) documented an analogous pattern of SA accumulation during post-O_3 (200 ppb, 5 h) recovery in a cultivar of tomato confirming that O_3 exposure, such as the pathogen infection, induces SA synthesis within few hours from the beginning of

the exposure, as suggested by Kangasjärvi et al. (2005). SA exerts its role in a variety of plant developmental processes via cross talk with (1) other hormones (ET, ABA, and GAs), (2) molecules with hormonal function (JA, Pro, and BRs), and (3) ROS (especially H_2O_2). Mutual antagonistic or synergetic interactions among these molecules in (1) modulating plant responses during O_3 stress and (2) regulating leaf injury after O_3 exposure have been reported. However, detailed roles and their interactions in regulating O_3-induced stress or other forms of ROS-dependent cell death have not been completely elucidated yet. ET and SA are needed for the initiation/propagation of visible O_3 lesions, amplifying the oxidative signal (Kangasjärvi et al. 2005). In plant responses to O_3, SA is required for the execution of PCD (as discussed above), controlling and potentiating the oxidative burst together with ET. SA action is strictly dependent on ET synthesis/perception and thresholds of ROS/ET signaling pathway. Vahala et al. (2003) reported that the O_3-sensitive birch clone V5818 exposed to O_3 (200 ppb, 8 h) showed a high ET production without a late SA accumulation. Ogawa et al. (2005) observed that SA synthesis occurred later than ET production in O_3-exposed tobacco plants. Similarly, Pellegrini et al. (2013) noted a high early ET production just after 1 h from the beginning of exposure and a late SA accumulation (at 8 h from the beginning of exposure) during the post-fumigation phase when ET levels were not significantly different from those measured in controls. Subsequently, during the late post-fumigation phase (24 and 48 h), SA reached constitutive values, while ET increased. Taken together, these results might suggest that O_3-induced ET may antagonize SA accumulation. By contrast, Rao et al. (2002) reported that SA is required for O_3-induced ET production and that the synergistic action of SA and ET fine-tunes the kinetics and magnitude of lesion formation in *Arabidopsis* exposed to O_3 (300 ppb, 6 h). Thus, whether, and how, SA is involved in the regulation of ET biosynthesis can still be regarded as an open question. Currently, there are few studies dealing with ABA and O_3 (as discussed above), and the detailed roles/interactions of SA and ABA in regulating O_3-induced stress have been largely neglected. In general, SA is antagonistic to ABA during development of SAR (Jayakannan et al. 2015): exogenous application of ABA hampered the induction of SAR, whereas activation of SAR by SA suppressed ABA signaling (Yasuda et al. 2008). On the other hand, SA and ABA play a similar role in stomatal closure, although through a different pool of ROS, suggesting the interaction between SA and ABA may be either positive or negative depending on stress conditions. Pellegrini et al. (2013) noted a marked increase of ABA concentration at the end of O_3 treatment and a late SA accumulation (8 h from the beginning of exposure) during the post-fumigation phase when ABA levels were still higher than those measured in controls. Subsequently, during the late post-fumigation phase, SA reached constitutive values, while ABA values remained higher than those of controls. Detailed roles and interactions of SA and JA in regulating O_3-induced stress are discussed in more detail in the next section.

A "self-amplifying feedback loop" concept has been proposed to explain the interaction between SA and H_2O_2 during stress (Xia et al. 2015). In particular, H_2O_2 induces accumulation of SA (catalyzing the conversion of benzoic acid into SA), and SA enhances H_2O_2 levels (inhibiting catalase activity through specific binding

to the enzyme or by inducing the formation of this molecule by phenol-dependent peroxidase; Diara et al. 2005). Pellegrini et al. (2013) reported that the marked increase in SA content of *M. officinalis* plants exposed to O_3 was coincident with the second H_2O_2 peak, suggesting a H_2O_2-SA interaction pattern. This agrees with the studies that proved the essential role of SA perception in (1) SAR, (2) the activation of an HR cell death pathway, and (3) the lesion initiation and progression in response to O_3 (as discussed above).

9.3.2 Jasmonic Acid (JA)

JA has been recognized as an endogenous regulator that plays a crucial role in (1) different developmental processes and (2) responses to several stress factors (including pathogens and wounding, Okada et al. 2015). JA is a fatty acid-derived cyclopentanone that is synthesized by the sequential action of several plastid, peroxisome, and cytoplasmic enzymes, via the octadecanoid pathway (Dar et al. 2015, Fig. 9.6). JA and its methyl ester (methyl jasmonate, MeJA) are the most studied of the linoleic acid-derived signaling molecules in higher plants that are collectively referred to as oxylipins or jasmonates (Dar et al. 2015). Exposure to O_3 stimulates jasmonate biosynthesis in plants (Rao et al. 2000): it has been reported that the reaction of O_3 with unsaturated lipids of the plasma membrane leads to the production of peroxidative processes and, consequently, of substrates for octadecanoid pathways, such as α-linoleic acid (Mudd 1997). In the oxidative cell death cycle, jasmonates (1) participate in the containment of the ROS-dependent lesion propagation (Devoto and Turner 2005; Kangasjärvi et al. 2005), (2) protect tissues from ROS-induced cell death (Overmyer et al. 2000), and (3) counteract ET and SA effects (Rao et al. 2002). Pellegrini et al. (2013) observed that in O_3-treated *M. officinalis* plants, JA peaked at 8 h from the beginning of exposure and remained high throughout the entire recovery period. Di Baccio et al. (2012) documented an analogous pattern of JA accumulation during post-O_3 recovery in a cultivar of tomato. Tuominen et al. (2004) reported that the level of JA increases only quite late in *A. thaliana* plants exposed to O_3 (300 ppb, 6 h), during the lesion formation. These results suggest that JA is primarily formed in the wounds (i.e., the borders of foliar lesions), as JA accumulation was observed only in plants that were impaired during the O_3 exposure. Also at a genic level, Castagna et al. (2007) reported an O_3-induced increase in transcript abundances of two JA biosynthetic genes [allene oxide synthase 2 (*AOS2*) and allene oxide cyclase (*AOC*), which have been reported to be strictly correlated to JA concentration] in two tomato genotypes exposed to O_3 (200 ppb, 4 h), only during the recovery period. Similarly, Di Baccio et al. (2012) observed that the mRNA of a gene encoding an acidic tomato pathogenesis-related protein (PR) was strongly upregulated in two tomato lines following O_3 exposure (3 h and 6 h after the end of treatment). These results confirm that JA acts as a component of anti-cell death pathway limiting and modulating the lesion spreading in later stages. Generally, jasmonates are vital in providing defense against biotic stresses,

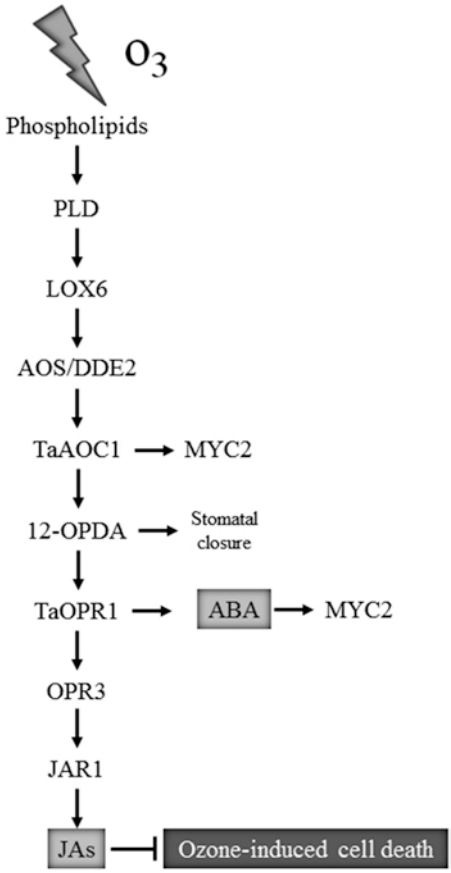

Fig. 9.6 Proposed pathways for jasmonic acid biosynthesis in ozone-exposed plants. Ozone stress induces membrane damage. A phospholipase D (PLD) releases α-linoleic acid from membrane lipids that is oxygenated by lipoxygenase (LOX6). It is converted to 12-oxophytodienoic acid (12-OPDA) by allene oxide synthase/delayed dehiscence 2 (AOS/DDE2) and by *Triticum aestivum* allene oxide cyclase 2 (TaAOC1). Jasmonic acid (JA) is synthesized from OPDA through reduction [12-oxophytodienoate reductase 1 (OPR3) and *Triticum aestivum* 12-oxophytodienoate reductase 1 (TaOPR1)] and three steps of β-oxidation [jasmonate resistant 1 (JAR1)]

especially necrotrophic pathogens. Resistance against pests (such as herbivores, predatory, or parasitic arthropods) and pathogens can be enhanced by the jasmonate treatment that is an integral part of the plant defense signaling (Rohwer and Erwin 2008). In the case of an abiotic stress such as O_3, Örvar et al. (1997) reported that pretreating tobacco plants with jasmonates inhibited O_3-induced cell death. Similarly, in *Arabidopsis*, jasmonate treatment halted O_3-lesion spread and reduced the amount of SA produced in response to the pollutant (Rao et al. 2000). Kock et al. (2000) proposed that the O_3 sensitivity of the poplar clone NE388 (300 ppb, 6 h d^{-1} for 4 consecutive days) resulted from JA insensitivity. Not all JA-insensitive mutants are, however, sensitive to O_3: Nickstadt et al. (2004) noted that the jasmonate-insensitive (*jin1*) mutant of *Arabidopsis* is tolerant to episodic O_3 treatment (250 ppb, 6 h). This finding suggests that JA has at least two distinct roles in O_3 responses: JA can (1) overcome the promoting effect of ET for ROS generation, resulting in the containment of lesion propagation and spread (Santino et al. 2013), and (2) act as a negative regulator of the oxidative cell death, promoting lesion formation and HR

cell death and, consequently, senescence. Generally, the major role postulated for JA is its antagonistic action toward ET- and SA-dependent signaling pathways (Kangasjärvi et al. 2005). It is known that ET stimulates and JA inhibits the spreading of cell death (Kazan 2015). Tuominen et al. (2004) documented an early and rapid accumulation of ET during the spread of O_3-induced cell death in *A. thaliana* exposed to O_3 that suppresses the protecting function provided by the JA pathway allowing cell death to proceed during the propagation phase of lesion formation. At the tissue level, the function/balance of ET and JA signaling has both temporal and spatial components in the regulation of oxidative cell death. In first cells, the SA- and ET-driven processes prevail, but further away from the site of initiation, JA pathways become progressively more induced, overcoming first processes, and containment of cell death follows. For this reason, it is possible that antagonistic and/or synergistic functions are also separated in place and/or time. Pellegrini et al. (2013) noted that in O_3-treated *M. officinalis* plants, ET evolution was induced within 1 h, and an accumulation of JA was observed within 3 h after the end of exposure. JA accumulating later during this response antagonizes the spread of cell death promoted by ET, resulting in the containment of O_3-induced lesions. Thus, both ET and JA syntheses seem to be locally restricted, and it is possible that the antagonistic action of ET and JA might occur only in borders of spreading lesions. Similarly, Di Baccio et al. (2012) observed that JA synthesis occurred later than ET production in O_3-exposed tomato plants. These results suggest that a mutually antagonistic interaction between JA and ET occurred in the regulation of spreading O_3 cell death. Overmyer et al. (2000) reported that ET levels in a JA-insensitive (*jar1*) mutant of *Arabidopsis* exposed to O_3 (250 ppb, 6 h) were similar to those of the wild type, suggesting that the antagonistic effect of ET seems to act at the level of JA signaling rather than of JA biosynthesis/accumulation. In particular, Vahala et al. (2003) documented an ET and JA accumulation in the O_3-sensitive clone of *Betula pendula* in response to O_3 treatment, whereas, in the O_3-tolerant clone, the JA concentration did not increase. The suggested involvement of JA in lesion containment may at first seem contradictory to JA accumulation in the sensitive clone; however, because the JA biosynthesis appears to be limited by substrate availability, it is also possible that the JA accumulation is a consequence of cell death. As discussed above, the substrate for JA synthesis could originate from membranes in dying cells, thus resulting in increased JA synthesis, which then halts the ET-dependent lesion propagation by decreasing ET sensitivity of cells (Kangasjärvi et al. 2005). Antagonistic interactions among JA and SA have been also observed in plants subjected to O_3. In particular, the regulation of ROS production may play a critical role in the interaction between JA and SA signaling pathways (Xia et al. 2015). In fact, JA appears to protect tissues from ROS-induced cell death counteracting effects of SA and ET (as discussed before, Kangasjärvi et al. 2005). At a genic level, Rao et al. (2000) reported an antagonistic relationship between JA and SA signaling pathways in controlling the magnitude of O_3-induced HR-like cell death in a JA-insensitive mutant (*jar1*) and in a mutant of *Arabidopsis* blocked in JA biosynthesis (*fad3/7/8*). At a functional level, Di Baccio et al. (2012) documented an opposite accumulation patterns for JA and SA in two tomato lines exposed to O_3 (the largest absolute content

was measured at 6 h and 9 h of recovery time after the end of treatment, respectively). The relationship between JA and SA signaling pathways is dependent on relative concentrations of each hormone. Low SA concentrations can also act synergistically with JA in the regulated induction of defense mechanisms. Pellegrini et al. (2013) observed that in O_3-treated *M. officinalis* plants, JA and SA peaked at 8 h from the beginning of exposure. Similarly, Kock et al. (2000) reported that an O_3-sensitive hybrid poplar clone accumulates SA and JA in the response to a single pulse of this contaminant. These findings demonstrate (1) an involvement of these compounds in modulating and limiting the lesion spreading and (2) overwhelming similarities between O_3- and pathogen-induced responses.

9.3.3 Proline (Pro)

Pro is a proteinogenic amino acid that has multifunctional roles in higher plants (Szabados and Savouré 2010). It is synthesized via the sequential action of enzymes mainly from glutamate. In higher plants, two Pro synthesis pathways have been identified: (1) glutamate and (2) ornithine. Pro is synthesized mainly from glutamate by the action Pro dehydrogenase or Pro oxidase enzymes. Alternatively, Pro can also be produced from ornithine via aminotransferase. The catabolism of Pro occurs in the mitochondria (Iqbal et al. 2014). For a long time, Pro was considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress. However, recent results highlight its multiple functions in stress adaptation, recovery, and signaling. Pro can act as an enzyme protectant enhancing activities of several enzymes and protecting the protein integrity (Filippou et al. 2014). As a scavenger of hydroxyl radicals and as a single oxygen quencher, Pro may be important in preventing an oxidative damage caused by ROS (Rejeb et al. 2014). Pro can act as a cell redox balancer, stabilizing cellular homeostasis, and as a stabilizer for subcellular structures, providing carbon and nitrogen for growth (Szabados and Savouré 2010). Pro metabolism can also influence the programmed cell death acting as a signaling molecule to (1) modulate mitochondrial functions, (2) influence the cell proliferation, and (3) trigger specific gene expression (Iqbal et al. 2014). These roles of Pro have been extensively demonstrated in plant responses to drought and salt and metal stress at functional and genic levels (Gill and Tuteja 2010; Dobra et al. 2010; Iqbal et al. 2014; Filippou et al. 2014). Generally, a dramatic accumulation of Pro due to increased synthesis or decreased degradation was observed. However, little is known about the responses of Pro content to O_3 stress. Moreover, this few information is generally based on experiments where plants have been exposed to chronic O_3 treatment. Zheng et al. (2014) documented a marked increase in Pro levels in two cultivars of *Zea mays* exposed to chronic O_3 treatment (85 ± 5 ppb, 8 h d^{-1} for 35 consecutive days). Similarly, El-Khatib (2003) reported that increasing O_3 levels (50–100 ppb, 5 h d^{-1} for 5 consecutive days) induced an accumulation of Pro in some common Egyptian plant species. Consequently, further investigations are required in order to understand (1)

the role of Pro as potential inhibitor of programmed cell death, (2) the role of Pro as signaling molecule in response to O_3, and (3) the interaction between different phytohormones in regulating Pro metabolism for O_3 tolerance.

9.3.4 Brassinosteroids (BRs)

BRs are a group of naturally occurring plant steroids (in free form or conjugated to sugars and fatty acids) and are important for a broad spectrum of cellular and physiological processes. Like IAAs, BRs (1) promote cell elongation, (2) control plant height, and (3) produce ROS (as second messengers by activation of NADPH oxidases, Bartoli et al. 2013). Progress has been made in elucidating the BR signal transduction pathway, and in the recent past, BRs have emerged as a new paradigm in the category of phytohormones. BRs can induce plant tolerance to a variety of abiotic stresses, such as high and low temperature, drought, and salt excess. BRs may also play a role in responses to pathogens. Recent studies demonstrated that the BR-induced resistance to bacterial and fungal pathogens in tobacco and rice plants was not correlated with enhanced SA accumulation or increased expression of genes associated with SA-regulated SAR (Nakashita et al. 2003). However, mechanisms underlying for BR-mediated stress responses are not fully understood. It is difficult to analyze genetically the role and the action mechanism of BRs in plant stress tolerance because of the strong and pleiotropic phenotypes of BR biosynthesis and signaling mutants (Xia et al. 2009). It is proposed that changes induced by BRs are mediated through the repression and/or de-repression of specific genes (Hasan et al. 2011). Puckette et al. (2009) reported that the regulation of BR biosynthesis and the interaction between BRs and IAAs caused a global decrease in the transcription of auxin-responsive genes via the *BREVIS RADIX* (*BRX*) protein in O_3-resistant accession (JE154) of *Medicago truncatula* exposed to episodic O_3 treatment (300 ppb, 6 h). Little is known about the role of BRs in response of plant to O_3 and generally to oxidative stress (Bajguz and Hayat 2009). Further investigations are required in order to understand (1) the role of BRs as a signaling molecule in response to O_3 and (2) the interaction between phytohormones in regulating BR metabolism for O_3 tolerance. Molecular studies present the notion that the cross talk between BRs and other phytohormones exists. Puckette et al. (2009) documented that BR/IAA interactions may play a critical role in determining the resistance to O_3 in two accessions of *M. truncatula*.

9.4 Conclusions and Perspectives

Overall, plant hormones ET, ABA, SA, and JA and other important signaling molecules have crucial roles in the modulation of apoplastic ROS signaling in response to O_3. The cross talk among all these players and their complex and interconnected

signaling pathways depicts the extraordinarily regulatory potential that plants have to promptly respond to environmental stimuli. The O_3 exposure is a valuable tool to improve the biochemical and molecular studies of phytohormones. As previously observed by Beauchamp et al. (2005), O_3 is a good plant stress "model" agent for several reasons: (1) exposure can be conducted under well-defined conditions; (2) experiments may be easily repeated mimicking the same conditions; (3) doses of O_3 can be varied over a wide range.

Understanding the integration of O_3-induced responses with ROS signaling and hormonal interactions can provide powerful tools for improving the (1) plant growth, (2) productivity, and (3) product quality for the future plant production in a changing climate. The future challenge for plant O_3 research is to identify the component involved in O_3/ROS perception and to elucidate cross talk pathways to understand interaction mechanisms in the whole signal network.

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Chapter 10
Engineering Phytohormones for Abiotic Stress Tolerance in Crop Plants

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Abstract Abiotic stresses including salinity, drought, extreme temperatures, and heavy metals are posing serious threats to agricultural yields as well as the quality of produce. This necessitates the production of cultivars capable to withstand the harsh environmental conditions without substantial yield losses. Owing to the complexity underlying stress tolerance traits, conventional breeding techniques have met with limited success and demand effective supplements to feed the growing food demands worldwide. This necessitates the development and deployment of novel and potent approaches, and engineering of phytohormone metabolism could be a method of choice to produce climate resilient crops with higher yields. Phytohormones are considered critical for regulating and coordinating plant growth and development; however, in recent years, they have received great attention for their multifunctional roles in plant responses to environmental stimuli. Creditable research has shown that phytohormones including the classical ones – auxins, cytokinins, ethylene, gibberellins, and newer members including brassinosteroids, jasmonates, and strigolactones – may prove to be potent targets for their metabolic engineering for producing abiotic stress-tolerant crop plants. This chapter presents short description of the roles of phytohormones in abiotic stress responses and tolerance followed by reviewing attempts made by the plant biotechnologists for

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engineering of phytohormone metabolism, signal, transport, and perception to develop abiotic stress-tolerant crop plants.

Keywords Abiotic stress • Genetic engineering • Plant hormone • Plant tolerance

10.1 Introduction

Recent climate changes aggravated by the modern anthropogenic activities have put unprecedented pressure on global crop production and yield. Various environmental stress factors including salinity, drought, water logging, extreme temperatures (heat, chilling, and freezing), heavy metals, light (intense and weak), and radiation (UV-A/B) have become severe threat to agricultural yields and quality of produce (Hasanuzzaman et al. 2013; Vardhini and Anjum 2015). This has increased the need for stress-tolerant cultivars adapted to adverse environmental conditions, and modern plant biotechnologies offer an effective solution in this context, as they enable to overcome the limits of classical breeding techniques (Zalabak et al. 2013). Genetic engineering, particularly in post-genomic era, has shown its tremendous potential in producing plants with higher yield and improved nutritional quality besides conferring tolerance against stresses.

Applied plant biotechnology, which relies largely on the expression of genes involved in signaling and regulatory pathways (Shinozaki et al. 2003), genes encoding proteins (Wang et al. 2004), or their pathway enzymes (Kumar et al. 2010) which confer stress tolerance, is a sound platform for producing abiotic stress-tolerant crops. Abiotic stresses such as drought, high salinity, and extreme temperatures induce the expression of large number of genes, and this stress-mediated induction is regulated through complex transcriptional networks. Molecular advancements in recent past such as microarray technologies have revealed the key genes functioning in these transcriptional networks on large scale, and these genes are potent candidates for the development of transgenic abiotic stress-tolerant plants (Todaka et al. 2015); besides, transcriptomics has contributed significantly to the elucidation of stress responses (Urano et al. 2009).

Several classes of small molecules, known as plant hormones, phytohormones, or plant growth regulators, are critical for regulating and coordinating the plant growth and development (Davies 2010). They act either close to or remote from their site of synthesis to mediate genetically programmed developmental changes or responses to environmental stimuli (Sreenivasulu et al. 2006; Colebrook et al. 2014). Phytohormones are very important for providing the adaptive responses during stress conditions, a critical requirement for their survival as sessile organisms (Argueso et al. 2009; Wang et al. 2009; Colebrook et al. 2014). In recent past, phytohormones have received great attention owing to their multifunctional roles in plants' responses to adapt to and counteract the deleterious effects of abiotic stress in crop plants. Creditable research has shown that phytohormones including the classical well-known auxins, cytokinins,

ethylene, gibberellins, and newer members including brassinosteroids, jasmonates, and strigolactones may prove to be potent targets for their metabolic engineering for producing abiotic stress-tolerant crop plants.

This chapter reviews the roles of phytohormones in abiotic stress response and tolerance in brief, while engineering of phytohormone metabolism, signal, transport, and perception to produce abiotic stress crop plants has been discussed with considerable depth.

10.2 Abiotic Stress: Serious Threat to Global Crop Production

Abiotic stresses pose major threats for crop productivity to food security (Huang et al. 2013). In 2005, FAO reported that there was 6–20 % loss due to abiotic stress, i.e., US$120 billion due to different stresses like drought, flood, and nutrient deficiencies. As pointed out by Goel and Madan (2014), abiotic stresses account for more than 50 % reduction in crop yields. Nowadays, due to changing environment, plants compete with different types of abiotic stress, which limit the growth and productivity of crop plants. During the stress, plants experience with multiple stresses at the same time during growth and development which leads to the effect on yield of crop plants (Tuteja 2007). This reveals that abiotic stress is a serious threat to global food productions. Plants developed a variety of defense mechanism to adapt from stress environment, although it is not enough to defend them. Due to stress, plants experience cellular dehydration and accumulate reactive oxygen species which ultimately affects membrane potentials and macromolecules like carbohydrates, proteins, and DNA (Doltchinkova et al. 2013). Therefore, to fulfill the demand, crop production must be increased exponentially. For the better crop productions, plant biotechnologists are trying different techniques to develop abiotic stress-tolerant plants. In modern era, genetic engineering has proved a prominent tool for the development of abiotic stress-tolerant plants to achieve the goal. Using these techniques, some varieties are already in the pipeline to be released as abiotic stress tolerant. Singh and his co-workers (2012) developed a transgenic tobacco, which is resistant to salinity and heavy metal stress. In this tobacco, they transferred a gene from rice. Thus, genetic engineering of phytohormones could be a new hope to develop abiotic stress-tolerant plants which will be beneficial for boosting crop yield and thereby achieving target.

10.3 Roles of Phytohormones in Abiotic Stress Tolerance

It is well documented and discussed with great depth in previous chapters that they provide the necessary adaptation to plants to abiotic stress tolerance. Phytohormones help to alter the gene expression by preventing the degradation of transcriptional

regulators (Santner and Estelle 2010). Currently, many of the researchers are focused on developing abiotic stress-tolerant plants through phytohormone engineering. Genetic engineering of cytokinin revealed the development, morphogenesis, and many physiological aspects. It has an important role toward productivity and increased tolerance to various stresses (Zalabák et al. 2013). Reguera et al. (2013) reported that phytohormone engineering, i.e., cytokinin, leads to increase drought tolerance in rice. Similarly, Klay et al. (2014) reported that ethylene response factor leads to enhance salt and cold tolerance in tomato. Colebrook et al. (2014, for details, see review) reported that gibberellins (GA) have a significant role in response to abiotic stress. Increasing the GA biosynthesis and signaling induces tolerance toward submergence, whereas the reduction of GA levels restricts plant growth and development to many stresses like cold, salt, and osmotic stress. Like GA, ABA has also the bigger role in the development of abiotic stress tolerance in plants. It plays many regulatory roles toward physiological process in plants and provides tolerance to abiotic stresses like water, drought, cold, light, temperature, etc. In a recent research, Singh and his co-workers (2015) reported that phytohormone engineering of ABA revealed increased salt and mannitol stress tolerance in *Arabidopsis*. Transgenic *Arabidopsis* plants, accumulating high level of ABA, showed better physiological appearance than wild ones in respect to water loss, chlorophyll content, and photosynthetic potential which ultimately provides high tolerance to salt, mannitol, and drought. Thus, genetic engineering of phytohormones could be a very potent tool for plant biotechnology in the future and is discussed in the following section.

10.4 Engineering Phytohormones for Producing Abiotic Stress-Tolerant Crops

Perturbed phytohormone fluxes and subsequent signal transduction cascade have been revealed as one of the key stress responses evolved by plants. Signal transduction cascades that interact with the baseline pathways transduced by phytohormones are triggered by perception of abiotic stresses (Harrison 2012). The fluctuations of stress-responsive hormones help alter the cellular dynamics and thus play a central role in coordinately regulating the growth responses under stress conditions (Kohli et al. 2013). Since, phytohormones are tagged as key regulators of not only plant growth and development but also act as mediators of the response to environmental stress (Sreenivasulu et al. 2012), the hormone metabolism and signaling process thus are potential targets for manipulations to obtain enhanced abiotic stress tolerance. The mutants and transgenic lines/tissues with altered activities of phytohormone metabolic enzymes or perception machinery have reaffirmed their crucial involvement in different traits including increased tolerance to various abiotic stresses. Over the past decade, many genes have been identified

that are either involved or affect phytohormone synthesis, transport, metabolism, and perception.

10.4.1 Genetic Engineering of ABA Metabolism

Though ABA regulates various physiological processes of plant development at its basal levels, ranging from stomatal opening to seed development, dormancy, and synthesis of storage proteins and lipids (Sreenivasulu et al. 2010), however, its role in stress tolerance is taking a center stage. It is now credited as an essential messenger involved in adaptive response of plants against stressful conditions (Umezawa et al. 2006). ABA is perhaps the most studied plant hormone for plant stress responses as summarized in some excellent recent reviews (Sreenivasulu et al. 2007, 2012; Raghavendra et al. 2010; Todaka et al. 2015). Extensive research has established the role of ABA in adaptive responses of plants to drought and salinity stress, and its levels gets substantially elevated under water-deficit conditions around the roots (Wilkinson et al. 2012). In response to environmental stresses, endogenous ABA levels increase rapidly, activating specific signaling pathways and modifying gene expression levels as well as inducing ABA-inducible genes (Lu et al. 2013; O'Brien and Benkova 2013). It lead the signals to shoots indicating stressful conditions around roots triggering water-saving antitranspirant activities such as stomatal closure and reduced leaf expansion (Wilkinson and Davies 2002). It regulates the expression of different stress-responsive genes involved in the accumulation of compatible osmolytes, in the synthesis of LEA proteins, dehydrins, and other protective proteins besides antioxidant enzymes (Chaves et al. 2003; Verslues et al. 2006). Through the characterization of ABA auxotrophic mutants, majority of the ABA pathway enzymes have been discovered (Koornneef et al. 1998; Seo and Koshiba 2002) as pointed out by Xiong and Zhu (2003) that most of these genes are transcriptionally upregulated under abiotic stress conditions.

Various genes involved in ABA biosynthesis, catabolism, and signaling network have been advocated as interesting candidate genes for breeding stress-tolerant crops. The ABA synthesis starts with zeaxanthin in plastids, a product of MEP pathway. The de novo biosynthesis of ABA occurs in leaves, stems, and roots of most plant species primarily in plastids; however, the last two steps occur in cytoplasm where xanthoxin is converted to ABA (Seo and Koshiba 2002). Nonetheless, the ABA biosynthesis is feedback controlled, and the rate-limiting step of ABA biosynthesis is the formation of xanthoxin via oxidative cleavage by the enzyme 9-cis-epoxycarotenoid dioxygenase (*NCED*) (Krannich et al. 2015). The last step in ABA synthesis is catalyzed by abscisic aldehyde oxidase (*AAO*), which requires a sulfurylated form of a molybdenum cofactor (*MoCo*) for its activity (Bittner et al. 2001; Seo et al. 2004), and the molybdenum cofactor sulfurase (*LOS5*) gene encodes the *MoCo* sulfurase involved in the regulation of ABA biosynthesis (Xiong et al. 2001).

LOS5 gene is particularly important in regulating the final step of ABA biosynthesis, and its enhanced expression has been reported to be induced by drought, salt, and ABA treatment (Xiong et al. 2001).

These aforementioned molecular mechanisms have been targeted for metabolic engineering, and the engineering of different ABA pathway genes has improved plant abiotic stress tolerance. Li et al. (2013) overexpressed the *MoCo* sulfurase gene in soybean which resulted into noticeably higher biomass production and yield with overall enhanced drought tolerance attributed to higher ABA accumulation, reduced water loss through decreased stomatal aperture, and induced antioxidant enzymatic machinery. *AtZEP*-overexpressing transgenic *Arabidopsis* plants showed smaller stomatal aperture, enhanced de novo ABA biosynthesis, and higher tolerance to osmotic stress than their wild-type counterparts (Park et al. 2008). Xiao et al. (2009) observed significantly higher crop yield and better spikelet fertility of transgenic rice plants overexpressing *LOS5* gene, growing under drought conditions. Overexpression of *Arabidopsis LOS5* markedly enhanced the expression of aldehyde oxidase (AO) activity, leading to ABA accumulation and increased drought tolerance of transgenic maize plants (Lu et al. 2013). The transgenic maize lines with higher ABA contents exhibited decreased water loss and maintenance of higher relative water content through reduced stomatal aperture, with lower electrolyte leakage, lipid peroxidation levels, and H_2O_2 contents and concomitant higher activities of antioxidant enzymes and proline content (Lu et al. 2013). In similar vein, overexpression of another gene *NCED* increased the levels of endogenous ABA, induced stomatal closure, and ultimately led to enhanced drought tolerance in transgenic plants of *Arabidopsis* (Iuchi et al. 2001), tobacco (Qin and Zeevaart 2002), creeping bent grass (Aswath et al. 2005), and tomato (Thompson et al. 2000, 2007; Tung et al. 2008).

Additionally, Kim et al. (2014) observed that the constitutive expression of *OsPYL/RCAR5* (PYRABACTIN RESISTANCE 1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTOR) in rice driven by *Zea mays ubiquitin* promoter induced the expression of many stress-responsive genes and resulted in improved drought and salt stress tolerance in rice. However, this was accompanied with negative impacts on seed yield (Kim et al. 2014). Similar to this overexpression of *PYL1*/*RCAR*, *PYL5*/*RCAR8*, or *PYL8*/*RCAR3* gave more tolerance to drought in *Arabidopsis* (Mehrotra et al. 2014). In an interesting study, Mao et al. (2010) overexpressed ABA signaling network gene serine/threonine protein kinase (*SnRK2.4*), and transgenic *Arabidopsis* overexpressing this gene exhibited enhanced tolerance to drought, salt, and freezing stresses, accompanied by decreased rate of water loss, enhanced higher relative water content, strengthened cell membrane stability, improved photosynthesis potential, and significantly increased osmotic potential. However, unlike many other studies, the overexpression-mediated multi-stress tolerance was not accompanied with any growth retardation of transgenic plants under well-watered conditions (Mao et al. 2010), indicating this gene to be a potent target for utilization in transgenic breeding to improve abiotic stress tolerance in crops.

10.4.2 Genetic Engineering of Auxin Metabolism

Auxin represents one of the most important groups of plant hormones, known for their pleiotropic effects on plant growth and development. It regulates numerous complex plant processes such as apical dominance, lateral/adventitious root formation, tropisms, fruit set and development, vascular differentiation, as well as embryogenesis (Friml 2003). Probably the most versatile phytohormone, auxin not only performs many essential physiological processes critical for plant growth and development but also govern and/or coordinate plant growth in stressed conditions (Kazan 2013). As envisaged by De Smet et al. (2010), the existence of an auxin biosynthesis, signaling, and transport apparatus in single-celled green algae indicates the evolutionary role played by auxin during adaptation of plants to diverse land environments. Through recent research, it is evident that auxin also plays a role as an integral part of plant responses to unfavorable and stressful conditions (Sharma et al. 2015).

Environmental stress conditions are known to induce differential expression of genes involved in various auxin-related pathways, as pointed out by various authors (Jain and Khurana 2009; Song et al. 2009; Ha et al. 2014). Aux/IAA and auxin response factor (ARF) are two important protein families that are well recognized for their roles in auxin-mediated responses (Song et al. 2009). The authors reported that the results based on DNA chip and real-time PCR analysis confirmed that many genes of these families were responsive to various abiotic stresses, indicating an interaction between plant growth and abiotic stress.

Overexpression of an auxin efflux carrier *OsPIN3t* under the control of 35S promoter resulted in increased drought tolerance of rice plants, and the knockdown of *OsPIN3t* caused crown root abnormalities in seedling stage, indicating the involvement of this gene in control of polar auxin transport (Zhang et al. 2012). Despite the fact that auxin is being studied for over 100 years, its biosynthesis, transport, and signaling pathways are still not very clear (Ke et al. 2015). However, some interconnecting pathways have been proposed so far to synthesize auxin in plants, including four tryptophan (Trp)-dependent and a Trp-independent pathway (Mano and Nemoto 2012). Trp-dependent auxin biosynthesis comprises of the conversion of Trp to indole-3-acetaldoxime, indole-3-acetaide, indole-3-phyruvic acid (IPA), and tryptamine (TAM) (Zhao et al. 2002; Mashiguchi et al. 2011), and the YUCCA family of flavin monooxygenase (FMO)-like proteins catalyzes the rate-limiting step in the TAM pathway (Won et al. 2011). Recent studies have demonstrated the crucial involvement of *YUCCA* genes in auxin biosynthesis in plants ranging from mosses to monocots as well as dicots (Kim et al. 2013; Landberg et al. 2013; Ke et al. 2015). Additionally, YUCCAs are also reported to function in the IPA pathway in *Arabidopsis* (Mashiguchi et al. 2011). The transgenic poplar plants overexpressing *Arabidopsis YUCCA6* gene under the control of SWPA2, an oxidative stress-

inducible promoter, exhibited auxin overproduction and elevated transcript levels of early auxin-responsive genes and increased tolerance to drought and oxidative stresses (Ke et al. 2015). Previously, Kim et al. (2013) observed increased plant height, longevity, and enhanced drought tolerance in *Arabidopsis* and potato resulted from overexpression of *YUCAA6*.

10.4.3 Genetic Engineering of Cytokinin Metabolism

Cytokinins (CKs) are ubiquitous phytohormones that are considered as key regulators of plant development, physiological processes, and plant morphology. The mutants and transgenic cells and tissues with altered activity of cytokinin metabolic enzymes or perception machinery have highlighted their crucial involvement in different agriculturally important traits, such as productivity, and increased tolerance of crop plants to various stresses (Zalabák et al. 2013). Genetic engineering approaches were used to confirm the role of CKs in plant morphogenesis, and results revealed that CK organ imbalance results into morphological abnormalities and a crucial effect on shoot/root ratio (Werner et al. 2003).

The CK function has been linked to different abiotic stresses (Hare et al. 1997), for instance, there are reports of CK sensing in long-term responses to cold stress as well as enhanced tolerance to freezing stress of CK receptor mutants (Jeon et al. 2010). Drought conditions exerted a reduction in isoprenoid CK levels in the xylem sap of plants (Shashidhar et al. 1996); on the other hand, elevated levels of BAP, with apparent inhibitory effects on leaf senescence coupled with higher accumulation of proline, were detected in xylem sap of maize exposed to drought (Alvarez et al. 2008). Salinity or osmotic stress shows a strong impact on expression levels of *Arabidopsis* CK receptors (Zalabák et al. 2013). Downregulation of *AHK2* and *AHK4* and upregulation of *AHK3* as well as its orthologue were observed in roots and shoots of *Medicago sativa* (Coba de la Peña et al. 2008; Argueso et al. 2009). Owing to the overexpression of stress-responsive genes in *ahk2*/*ahk3* double mutants, Tran et al. (2007) pointed out the nature of AHKs as apparent stress signaling negative regulators. Tight regulation of genes involved in CK metabolism has been mentioned during plants' adaptation to salinity and osmotic stresses in maize (Zalabák et al. 2013).

It is evident that drought conditions decrease the endogenous levels of CK in plant tissues, and elevated levels of CK might promote plants' adaptation and minimize the yield penalties. Therefore, attempts have been made to increase CK levels via genetic engineering to confer stress tolerance in transgenic plants. The inducible promoters have been used successfully for producing highly drought-tolerant plants without negative impacts on the growth of cassava (Zhang et al. 2010a) and tomato (Ghanem et al. 2011). On similar lines, Rivero et al. (2007) and Peleg et al. (2011) reported enhanced drought tolerance and higher grain yield than non-transformed counterparts of tobacco and rice, respectively. In an interesting experiment,

improved growth of transgenic plants was achieved under osmotic stress conditions after isopentenyl transferase gene fusion to the 3′ transcription ends of other genes under constitutive promoters (Guo et al. 2010).

Werner et al. (2010) generated transgenic *Arabidopsis* and tobacco plants over-expressing cytokinin oxidase/dehydrogenase (CKX) gene with enhanced root-specific degradation of CK, and the resultant transgenic plants formed a larger root system and exhibited higher survival rate under severe drought conditions. Under conditions of sulfur or magnesium deficiency, leaf chlorophyll content was less affected in transgenic plants. This approach holds significance and can be explored to improve drought tolerance, nutrient efficiency, and nutrient content of crop plants.

10.4.4 Genetic Engineering of Ethylene Metabolism

Ethylene (ET) is one of the most important regulatory molecules that are related to environmental responses in plants. ET is a gaseous phytohormone and has been documented for its involvement in plant stress responses, in addition to its roles in germination, fruit ripening, organ abscission, pathogen, and senescence (Chen et al. 2005; Klay et al. 2014). There are various reports confirming that the accumulation of ET or its precursor ACC (aminocyclopropane-1-carboxylic acid) is sharply induced by abiotic stress conditions such as soil salinity (Zapata et al. 2007) and water stress (Narayana et al. 1991). The transcript profiling of expanding *Arabidopsis* leaves under mild osmotic stress conditions revealed that an ET and gibberellin-dependent regulatory circuit modulated the growth under these conditions (Skirycz et al. 2010). This was accompanied by the rapid accumulation of 1-ACC, and this accumulation has been proposed to activate a cascade of the growth regulatory circuit in *Arabidopsis* (Claeys and Inzé 2013). Recent findings have drawn a general conclusion that ET and 1-ACC act as positive growth regulators under various conditions in rice (Fukao and Xiong 2013; Wang et al. 2013).

ET has been proposed to function via modulation of gene expression, which operates in part at transcriptional level by *ERF* (ethylene response factor) considered as the effectors of ethylene signal (Klay et al. 2014). A linear ET transduction pathway has been proposed in *Arabidopsis* which corresponds to a succession of components from ET receptor integrated in endoplasmic reticulum to TFs localized in nucleus (Chen et al. 2005; Guo and Ecker 2004). The ERF-TFs, comprising a huge multigene TF-family, regulate the expression of ET-dependent genes and are considered as the most prominent components directing the plant responses to ET signal (Klay et al. 2014). Various members of this family are critical in regulating the expression in response to abiotic stresses (Zhang et al. 2008). Notably, these ERFs have the ability to work as both activator and repressor elements depending upon the situation (Fujimoto et al. 2000; Zhang et al. 2004). Several reports have

confirmed the involvement of different ERF genes in abiotic stress responses of plants including *Arabidopsis* (Yang et al. 2011).

Klay et al. (2014) studied the expression patterns of a *Solanum lycopersicum* ERF-gene S*l*-ERF.B.3, which encodes for tomato TF of ERF family, to determine its regulation in response to abiotic stresses including salinity, drought, flooding, heat, and cold. RT-PCR-based results showed that *Sl-ERF.B.3* is an abiotic stress-responsive gene and is induced by cold, heat, and flooding but downregulated by salinity and drought. Further, as compared with wild type, S*l*-ERF.B.3 antisense transgenic plants exhibited a salt stress-dependent growth inhibition (Klay et al. 2014).

Overexpression of different ERFs has been attempted in different plant species for conferring abiotic stress tolerance. Rice mutants overexpressing the response factor jasmonate and ERF-1 (*JERF1*) exhibited enhanced drought tolerance, and this effect was correlated with the expression of stress-responsive genes, including key enzymes of proline biosynthesis (Zhang et al. 2010b). Similarly, better drought and salinity tolerance was observed in *Arabidopsis* (Cheng et al. 2013) and tomato (Pan et al. 2012) plants overexpressing *ERF*-1 and ERF-5, respectively. Further, ET overproducer 1-like (*ETOL1*)-overexpressing rice plants was found more tolerant to drought and submergence (Du et al. 2014). It can be concluded that ET metabolism and signaling pathways influence various stress responses in plants (Krannich et al. 2015). The ET genes, in particular those related with growth maintenance under water-deficit conditions, have significant agronomic relevance for providing efficient resources for crop improvement and can negate the yield penalties.

10.4.5 Genetic Engineering of Brassinosteroid Metabolism

Brassinosteroids (BRs) are a group of steroidal phytohormones, structurally similar to animal and insect steroids. These are ubiquitous throughout the plant kingdom and regulate a range of physiological responses such as cell elongation, photomorphogenesis, xylem differentiation, seed germination (Sasse 2002), and stress responses (Krishna 2003). Owing to the multifunctional roles of BRs, the genetic manipulation of BR metabolism offers a unique possibility of significantly increasing crop yields and protecting plants from environmental stresses (Divi and Krishna 2009). Various BR-induced molecular changes that are related with stress tolerance have been identified such as overexpression of stress-responsive genes (Dhaubhadel et al. 1999; Kagale et al. 2007), maintenance of protein synthesis (Dhaubhadel et al. 2002), induction of antioxidant enzymes and osmoprotectant accumulation (Ozdemir et al. 2004; Divi and Krishna 2009), and higher photosynthetic efficiency (Krishna 2003; Ogweno et al. 2007).

Consequently, T-DNA knockout mutants of *OsGSK1*, a rice ortholog of the BR-negative regulator *BIN2*, exhibited enhanced tolerance to cold, heat, salt, and drought stresses as compared to their wild-type counterparts (Koh et al. 2007). A

gene HSD1 encoding a protein with homology to animal 11-b-hydroxysteroid dehydrogenase (HSD) was identified in *Arabidopsis* by Li et al. (2007). The HSDs are attributed for regulating growth and development in animals, though their enzymatic functions are still not clearly known in plants. Though a relationship of *AtHSD* with BR biosynthesis or signaling has yet to be established, the overexpression of *AtHSD1* in *Arabidopsis* resulted in the constitutive expression of BR response genes and produced phenotypes similar to plants overproducing BRs or BRI1 (BR-insensitive 1), with increased growth and seed yield as well as increased tolerance to salinity stress and reduced seed dormancy (Li et al. 2007). These findings suggest that *AtHSD1* is linked to the BR pathway and it has potential for improving crop yield and stress tolerance in plants. As it is clear that BRs have the potential to enhance crop yield and confer tolerance to abiotic stresses, their genetic manipulations present a sound platform for producing high-yielding stress-tolerant crops. However, there is a long way to make these strategies a commercial success. In this direction, the identification of species-specific BR-related genes and trial and error of gene manipulation with respect to the candidate gene and targeted organ or tissue may be of great help. A list of transgenic plants with altered expression of phytohormone-related genes with enhanced abiotic stress-tolerant phenotypes has been presented in Table 10.1.

10.5 Conclusion and Perspectives

It can be concluded that the phytohormone engineering has the potential for producing high-yielding and abiotic stress-tolerant crops which provides new opportunities to maintain sustainable crop production to feed the whole world under changing environmental conditions. Although, with rapid development of genomic technology, significant attempts have been done in recent years toward deciphering the plant abiotic stress responses, many challenges still lie ahead to uncover the complexity of stress signal transduction pathways. More extensive work is required to be done at the genetic level of biosynthetic pathway of hormones like IAA, mechanism of upregulation of ABA biosynthesis genes by the abiotic stress and hormone homeostasis of GA, and exact pathway of many hormones like SA.

Plant hormones are involved directly or indirectly in wide spectrum of abiotic stresses, and creditable research has validated that they play a critical role in plant defense and plant-environment interactions. Undoubtedly, the phytohormone engineering is highly promising for plant biologist, but there is still a long way to reach its complete prospective. Among the greatest challenges that remain to be addressed is the development of stable phytohormone-engineered abiotic stress-tolerant crops but without growth or yield penalties. The multi-gene transfer approaches may also prove beneficial for producing crop plants with tolerance to multiple or combination of abiotic stresses.

Table 10.1 Altered expression of phytohormone-related genes and enhanced abiotic stress-tolerant phenotypes of resultant transgenics

Plant hormone	Gene	Function of gene	Expression/knockout	Phenotype of transgenics	References
ABA	MoCo sulfurase	Regulation of the last step of ABA biosynthesis	↑	Transgenic soybean showed higher biomass production, yield, enhanced drought tolerance, higher ABA accumulation, reduced water loss that are attributed to decreased stomatal aperture and induced antioxidant capacity	Li et al. (2013)
	AtZEP	Important during the first step of ABA biosynthesis	↑	Enhanced de novo synthesis of ABA, higher tolerance to osmotic stress	Park et al. (2008)
	LOS5	Regulation of the last step of ABA biosynthesis	↑	Transgenic rice showed higher crop yield, better spikelet fertility under drought stress	Xiao et al. (2009)
			↑	Transgenic maize with enhanced AO expression, ABA accumulation, and increased drought tolerance	Lu et al. (2013)
	NCED	Important role in rate-limiting step of ABA biosynthesis for feedback control	↑	Increased levels of endogenous ABA, induced stomatal closure, and increased drought tolerance	Iuchi et al. (2001); Qin and Zeevaart (2002), Aswath et al. (2005), Thompson et al. (2000, 2007), and Tung et al. (2008)

	SnRK2.4	Important serine/threonine protein kinase in ABA signaling network	↑	Transgenic *Arabidopsis* with enhanced tolerance to drought, salt, freezing stress, decreased water loss, improved photosynthesis potential, and osmotic potential	Mao et al. (2010)
Auxin	*OsPIN3t*	Auxin efflux carrier, important in polar auxin transport	↑	Increased drought tolerance in rice	Zhang et al. (2012)
	YUCCA6	Important gene in auxin/IPA biosynthesis	↑	Overproduction of auxin, increased tolerance to drought and oxidative stress	Ke et al. (2015)
			↑	Increased plant height, longevity, and enhanced drought tolerance	Kim et al. (2013)
CK	*ARRs*	Cytokinin transcription factors	Knockout mutants *arr*	Increased salt tolerance in *arr* double mutants with increased expression of K transporter	Mason et al. (2010)
	CKX	Cytokinin degradation	↑	Transgenic *Arabidopsis* plants overexpressing cytokinin oxidase/dehydrogenase gene showed enhanced drought tolerance	Werner et al. (2010)
	IPT	Catalyze the rate-limiting step of CK synthesis	↑	Transgenic tobacco exhibited enhanced drought tolerance	Rivero et al. (2007)
ET	*ERF-1 (JERF1)*	Response factors for ethylene as well as jasmonates	↑	Rice plants showed increased drought tolerance	Zhang et al. (2010b)
	ETOL1		↑	Increased tolerance to drought and submergence	Du et al. (2014)

(continued)

Table 10.1 (continued)

Plant hormone	Gene	Function of gene	Expression/knockout	Phenotype of transgenics	References
BRs	*OsGSK1*	BR-negative regulator	Knockout of *OsGSK1*	Increased tolerance of knockout mutants to cold, heat, salt, and drought stresses	Koh et al. (2007)
	AtHSD1	Role in BR biosynthesis	↑	Overproduction of BR, increased growth rate and seed yield, increased salinity tolerance	Li et al. (2007)

↑: Overexpression

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