Girdhar K. Pandey *Editor*

Elucidation of Abiotic Stress Signaling in Plants

Functional Genomics Perspectives, Vol. 2

Springer

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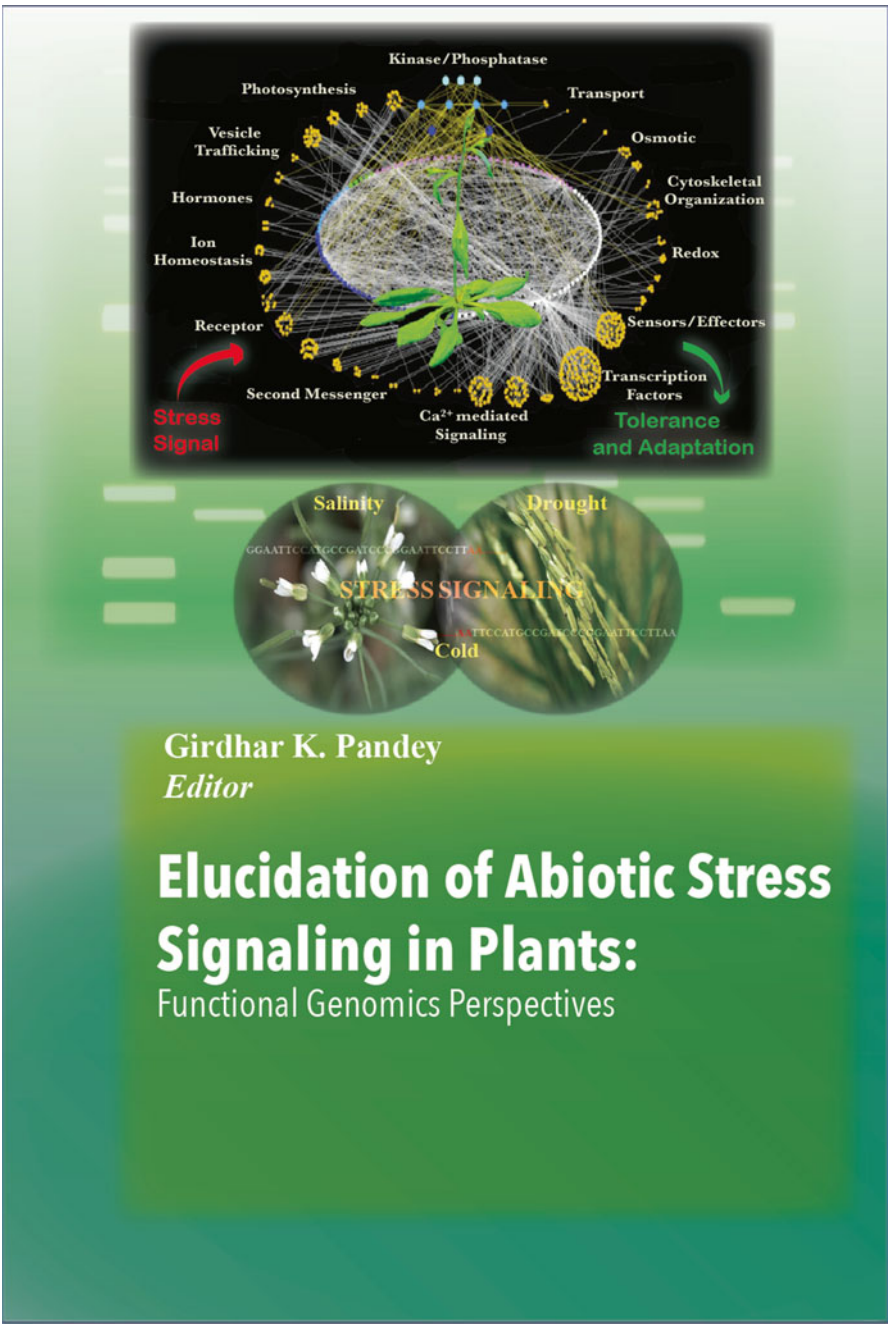

The above image represents a depiction of activation of different signaling pathways by diverse stimuli that converge to activate intricate signaling and interaction networks to counter stress (top panel). Since environmental stresses influence most significantly to the reduction in potential crop yield, progress is now largely anticipated through functional genomics studies in plants through the use of techniques such as large-scale analysis of gene expression pattern in response to stress and construction, analysis and use of plant protein interactome networks maps for effective engineering strategies to generate stress tolerant crops (top panel). The molecular aspects of these signaling pathways are extensively studied in model plant *Arabidopsis thaliana* and crop plant rice (*Oryza sativa*) (below).

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Preface

Plants are considered the backbone of life on earth. The colorful life on this planet has emerged as a consequence of over 3.5 billion years of unceasing evolution. Life on earth cannot sustain without plants, as they harness solar energy to produce sugars and oxygen, the primary constituents for supporting life. Humans are primarily dependent on plants and have developed a systematic discipline called "agriculture" to cultivate or domesticate plants over a period of time for food, biofuel, and fodder. At present time, crop productivity faces a major challenge from rapidly growing population and diminishing fertile land due to excessive anthropogenic activities. In addition, expanding human population and climate changes due to increased exploitation of natural resources imposes several major unfavorable conditions that reduce the crop productivity. These unfavorable conditions are primarily categorized as physical (or *abiotic*) and biological (or *biotic*) variables hindering normal growth and development in plants. Interestingly, stress perceived by one plant species may not be a stress factor for another plant species due to different growth habits and adaptation acquired during the course of evolution. Because of domestication and cultivation of crop plants by humans over a period of 10,000 years, many of these wild traits responsible for adaptive responses were lost, increasing the vulnerability of crop plants to biotic and abiotic stresses. Under abiotic stresses, limitation of water (drought), extremes of temperature (both high and low temperatures), nutrient deficiency, and soil contaminated with salt and heavy metals or pollutants are the major environmental factors contributing to crop losses worldwide.

In the past, agriculture has relied on breeding approaches to develop high yielding crop varieties which can grow optimally under stress conditions without affecting crop yield and productivity. In an effort to find an alternative tool faster than the traditional breeding approach, the last two decades has seen the advent and development of genetic engineering. This technique involves the identification, transfer, and stable integration of desired genes into genomes of crop plants to generate transgenic plants, exhibiting improved trait for tolerance against one or other stress factors in contained experimental conditions such as green houses.

However, plants are constantly exposed to a multitude of stresses at any given time in the natural environment, and not much has been achieved till now to generate crop varieties that can tolerate these multiple stresses without yield penalty. In order to develop stress-tolerant crop varieties with the ability to withstand multiple stresses in their environmental growth condition, an in-depth and systematic understanding of stress sensing, signal transduction, and generation of response is required.

Evolutionarily, the major distinction between plants and animals in sensing and responding to a plethora of stresses is due to their sessile versus mobile nature, respectively. In the case of animals, the primary response against a particular stress is avoidance of stress, whereas in plants, due to their immobilization, development of stress tolerance is the only escape response. Moreover, plants lack a well-defined brain and nervous system unlike their animal counterpart, leading to development of higher degree of plasticity in their communication skills by numerically expanding their signal transduction machinery. Despite the variances amid plants and animals, many of the signal transduction components can be found to be conserved. These include receptors, second messengers, signal-transducing molecules like kinases, phosphatases, small and large G-protein, and others, which finally affect the activity of either transcription factors to regulate the gene expression or transporters/channels, metabolic enzymes, and cytoskeletal proteins to directly change the physiology of the cell. Additionally, analogous to networking in the nervous systems, the signaling pathways in plants also exhibit scale-free web of networks instead of linear or definite pathways. These scale-free networks constitute extremely connected points called *nodes* and *hubs*, which are responsible for efficient processing, channeling, and integration of multiple signaling pathways at a given time to generate specificity as well as cross talk in the signaling networks.

Plants primarily rely on the complex, intertwined, and dynamic signal transduction pathways for developing a higher order of networks. This involves sophisticated control circuits like the nervous system of animals, where they learn, generate memory, alter behavior, and develop intelligence, which make them ready for future challenges. In nutshell, the complex interplay of signal transduction networks and machinery in plants leads them to sense, process, and integrate the signals they confront in their environment. Plants also develop behavioral changes accordingly or develop cognition and storage of processed information to adapt in rapidly changing or variable environment.

Identification of the role of a single or set of genes involved in signal transduction pathway has enabled researchers to understand and develop linear or complex signaling pathways, or maps in response to particular stimuli. However, because of the complete genome sequencing of many plant species including crop plants, a drift towards understanding the stress-signaling pathways involved in single or multiple stresses using high-throughput approaches has emerged. In the post-genomic era, the development of *-omic*-based approaches such as transcriptomic, proteomic, metabolomic, interactomic, and phenomic in several model organisms have laid the foundation of functional genomics. This area of plant science deals with the

understanding of large network of genes and proteins and integration of transcript data to proteins which then go to metabolite, and the complex and dynamic interaction develops a response or phenotype.

Elucidation of Abiotic stress signaling in Plants: *Functional Genomics Perspectives* comprises 30 chapters divided into two volumes (Volume I and II) in which some of the world's most well-known plant biologists have contributed in the field of stress signaling in plants with a special emphasis on functional genomics aspects. This book provides timely research in the field of stress-mediated signaling to develop a better and holistic understanding of stress perception and its transduction followed by the generation of response. In spite of the advent of different approaches to develop stress-tolerant crops towards multiple stress conditions in the field, the success in achieving this goal is still unsatisfactory. This is because stress tolerance is a very complex process involving plethora of components starting from stress sensing to generation of final adaptive response. As mentioned above, there are several factors, which act as nodes and hub in the signaling pathways, also serving as master-control switches in regulating a myriad of stress-signaling pathways by affecting diverse target genes or gene products to finally bring about a stress tolerance response. Therefore, in-depth understanding of these master-control switches and key components in signal transduction pathway will be highly beneficial for designing crop plants tolerant to multiple stresses in the field.

Towards achieving this goal, this book is divided into two volumes comprising five sections. Volume I consists of two sections with 14 chapters. The first section "Functional Genomics Approaches in Signal transduction" discusses three chapters on various approaches used to understand the signal transduction networks. These chapters will aware the readers on practical aspect of various "Omic"-based approaches such as transcriptomic, proteomic, phosphoproteomic, metabolomic, interactomic, and phenomic to understand the functions of genes and gene networks in signaling under stress.

The next section "Components of Signal Transduction" comprises 11 chapters discussing the different components of signal transduction pathways. The first three chapters focus on calcium signaling by describing the genes encoding for CAX (calcium-H^+-exchanger) involved in sequestration of calcium ions into vacuoles and maintenance of Ca^{2+} homeostasis. Chapters 5 and 6 discuss the role of Ca^{2+} signal decoding components like sensor and effector proteins. Here, CBLs, CIPKs, and CDPKs gene families have been extensively worked out in model plant Arabidopsis under abiotic stress condition and their role in other crop plant is being elucidated. Chapter 7 describes the role of ROS as redox signaling component in regulating multiple stress responses and in manipulation of ROS levels for imparting stress tolerance in crop plants. The role of MAP kinases as crucial signaling components in biotic as well as abiotic stresses has been discussed in Chapter 8. MAP kinases act as converging points for several signaling pathways, involving the phosphorylation-based relay of information to regulate a large number of targets such as transcription factors, other kinases, and cytoskeletal proteins in stress

signaling. The functional role of small and large G-protein acting as molecular switches to regulate both biotic and abiotic stresses has been discussed in Chapter 9. Chapter 10 deals with the molecular analysis of ABA receptor and ABA signaling in both biotic and abiotic stresses and genetic engineering of ABA receptor for developing stress-tolerant crop varieties. Auxin has been very well known as a plant growth regulator for several decades, and its emerging role in regulating stress signaling and responses is covered extensively in Chapter 11. SA (salicylic acid) is majorly involved in regulating biotic stress, but its role is also appreciated well in abiotic stresses as described in Chapter 12. In Chapter 13, the newly emerging role of methyl glyoxal (MG), which is a cytotoxin generated from both enzymatic and nonenzymatic pathways of metabolic reaction, has been discussed during several abiotic stresses. Chapter 14 discusses the role of immunophilins in diverse biological processes including development and stress management.

Volume II is divided into three sections encompassing 16 chapters. The first section of volume II emphasizes the gene expression regulation of stress signaling, with four chapters discussing the role of transcription factors (mediator complex in Chapter 1 and transcription factors of legumes in Chapter 2) and non-coding and small RNA (Chapters 3 and 4) in regulating abiotic stress responses.

Section two of volume II, comprises ten chapters, discusses the functional genomics aspect of heat/high temperature (Chapter 5), cold/freezing (Chapter 6), drought and dehydration (Chapter 7), flooding and submergence (Chapter 8), salinity (Chapter 9), UV-light (Chapter 10), heavy metal (Chapter 11), nitrogen (Chapter 12), and aging/senescence (Chapter 13) stress signaling responses. In this section, a detailed emphasis has been given in elaborating the respective stress-signaling pathway with a goal of potential candidate genes, which could be used for development of tolerant crop varieties by genetic manipulation and molecular breeding approaches. Moreover, cross talk or overlap in execution of several common signaling components open the scope for taming multiple stresses in future biotechnological intervention.

In the last section of volume II, Chapters 14–16 focus on the development of stress-tolerant crops and sustainable agriculture by utilizing the genes of signal transduction pathways. With the in-depth understanding of several signal transduction components and signaling pathways, the ultimate goal is to utilize the mechanistic knowledge and translate into useful tools to generate the crop varieties by either genetic manipulation of these signaling components or utilization of this knowledge for molecular marker-assisted breeding, ultimately augmenting stress tolerance in crop plants without compromising crop productivity.

Despite rigorous attempts, not every aspect of signaling pathways and components could be discussed here. Nevertheless, I strongly believe that two volumes covering signal transduction machinery and their components in stress condition, with a special emphasis to functional genomics, will be enormously useful to students, teachers, and research scientists.

I am indebted to all the contributors of this work, which could not be possibly compiled without their significant contributions. At last, I would like to express my sincere thanks to Dr. M. C. Tyagi and Dr. Amita Pandey for critical reading and help in copy-editing of this book. I also express my thanks to Ms. Manisha Sharma for designing the theme page.

New Delhi, India Girdhar K. Pandey, Ph.D.

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About the Editor

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Girdhar K. Pandey born in Almora, Uttrakhand, India. He received his B.Sc. (Hon.) in Biochemistry from Delhi University in 1992 and M.Sc. in Biotechnology in year 1994 from Banaras Hindu University (BHU). Subsequently, he joined Ph.D. in the School of Life Sciences, Jawaharlal Nehru University (JNU) and worked in the field of calcium signal transduction under abiotic stresses in plants. He was awarded the Ph.D. degree in year 1999 and then pursued postdoctoral career at Department of Plant and Microbial Biology, University of California Berkeley in year 2000. There, he extended his work in the field of calcium-mediated signaling in Arabidopsis by studying CBL-CIPKs, phosphatases, channels/transporters, and transcription factors involved in abiotic stresses. He has been working as Associate Professor in the Department of Plant Molecular Biology, Delhi University South Campus since October 2007.

Pandey's research interests involve detail mechanistic interplay of signal transduction networks in plant under mineral nutrient deficiency (mostly potassium, calcium, and nitrate) and abiotic stresses such as drought, salinity, and oxidative stresses induced by heavy metals. His laboratory is working on the coding and decoding of mineral nutrient deficiency and abiotic stress signals by studying several signaling

components such as phospholipases (PLA, PLC, and PLD), calcium sensors such as calcineurin B-like (CBL) and CBL-interacting protein kinases (CIPK), phosphatases (mainly PP2C and DSP), transcription factors (AP2-domain containing or ERF, WRKY), transporters and channels proteins (potassium and calcium channels/transporters), small GTPases, and Armadillo domain containing proteins in both Arabidopsis and rice. The long-term goal of his research group is to establish the mechanistic interplay and cross talk of mineral nutrient-deficient conditions and different abiotic stress signaling cascades in Arabidopsis and rice model system by using the advance tools of bioinformatics, genetics, cell biology, biochemistry, and physiology with greater emphasis on functional genomics approaches.

He has been awarded with Far Eastern Regional Research Organization (FERRO) fellowship to work at Beltsville Agricultural Research Center (BARC), United States Department of Agriculture, Beltsville, MD (1998). Later, he was awarded with Indian National Science Academy (INSA)-Deutsche Forschungsgemeinschaft (DFG) bilateral exchange visiting scientist fellowship in 2011. Also Department of Biotechnology (DBT), India, has awarded him with prestigious DBT-CREST Award (Cutting-edge Research Enhancement and Scientific Training) in 2011–2012. See Pandey's web page for further information about his lab and research work: https://sites.google.com/site/gkplab/home; http://www.dpmb.ac.in/index.php?page=girdhar-pandey.

Part I
Gene Expression Regulation of Stress Signaling

Chapter 1
Role of Plant Mediator Complex in Stress Response

Subhasis Samanta and Jitendra Kumar Thakur

Abstract Class II gene loci of eukaryotes are transcribed by RNA Polymerase II, which functions in coordination with several other proteins like transcription factors, general transcription factors, and cofactors. Recently, Mediator complex, a multi-subunit, megadalton size protein complex has gained lots of attention as an important component of RNA pol II transcriptional machinery because of its essentiality in the regulation of most of the class II genes. Like yeast and other metazoans, plants also possess the Mediator complex across the kingdom, and its isolation and subunit analyses have been reported from the model plant, *Arabidopsis*. Recent times have experienced a flurry of scientific papers containing the functional information of individual Mediator subunits in plants, although many were reported earlier without consideration of their association with the Mediator complex. Among its diverse functional aspects, several reports have established the Mediator complex as an important integrative hub of different biotic and abiotic stress signaling pathways, which have been discussed in this chapter from the functional genomics perspectives. Although reports are emerging in support of its inclusion as a component of the basic transcriptional machinery, the gene selective roles of the individual Mediator subunits are proven and indisputably accepted.

Keywords Transcription • RNA Polymerase II • Mediator complex • Mediator subunit • Biotic stress • Abiotic stress • Defense signaling • *Arabidopsis* • Rice

Abbreviations

BR	Brassinosteriod
ChIP	Chromatin immunoprecipitation
JA	Jasmonic acid
LC-MS/MS	Liquid chromatography-mass spectrometry
MED	Mediator
MudPIT	Multidimensional protein identification technology

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RNAP II	RNA Polymerase II
SA	Salicylic acid
TAP	Tandem affinity purification

1.1 Introduction

The process of transcription in eukaryotic organism is a highly orchestrated and immensely complex phenomenon and mediated by a plethora of proteins with the prime role played by RNA Polymerase II (RNAP II) (Lee and Young 2000). RNAP II with the basal transcription factors forms the heart of the transcription machinery. Over the time, several cofactors have been discovered, which offer the basic transcriptional machinery diverse regulatory avenues in terms of controlling gene expression (Woychik and Hampsey 2002). Among these cofactors, Mediator, a multi-subunit protein complex, has been proved to be quintessential in RNAP II-mediated gene expression (Myers and Kornberg 2000; Conaway et al. 2005; Kornberg 2005; Malik and Roeder 2005). Mediator complex, an ensemble of around 25–30 Mediator subunits, could be imagined as a bridge connecting the basic transcription machinery with the *cis*-element bound transcription factors (Fig. 1.1). However, Mediator does not act simply as a scaffold protein, rather as a subtle and complex modulator of gene expression during transcription. Although far from a clear and detailed understanding, the binding of transactivator or repressor with the Mediator complex might bring about certain conformational changes, which are transmitted to the RNAP II resulting into the desired changes in the level of gene expression. Apart from transcription factors (transactivator and repressor), Mediator complex also acts as docking site for several other proteins, which elicit their regulatory roles through Mediator-induced structural changes on RNAP II machinery (Meyer et al. 2010; Taatjes 2010). Since the discovery of plant Mediator in 2007, its subunits have been implicated in several biological processes. Recently, role of Mediator in growth and development was reviewed (Kidd et al. 2011). Here, in this chapter, we discuss the current status of Mediator research in plants from functional genomics perspectives with special emphasis on its role in biotic and abiotic stresses.

1.2 Discovery of Mediator Complex

Until now, Mediator complex has only been reported in eukaryotes. The complex was first isolated from the yeast as a factor required for enhanced transcription in a cell-free, in vitro transcriptional system, composed of RNAP II and general transcription factors in *Saccharomyces cerevisiae* (Kim et al. 1994; Myers et al. 1998) as well as in *Saccharomyces pombe* (Spahr et al. 2000). Later, the complex was isolated from almost all the eukaryotic organisms ranging from human (Fondell et al. 1996; Ito et al. 1999), *Drosophila* (Park et al. 2001), *Caenorhabditis* (Park et al. 2001) to even plant (Backstrom et al. 2007). The yeast Mediator complex was

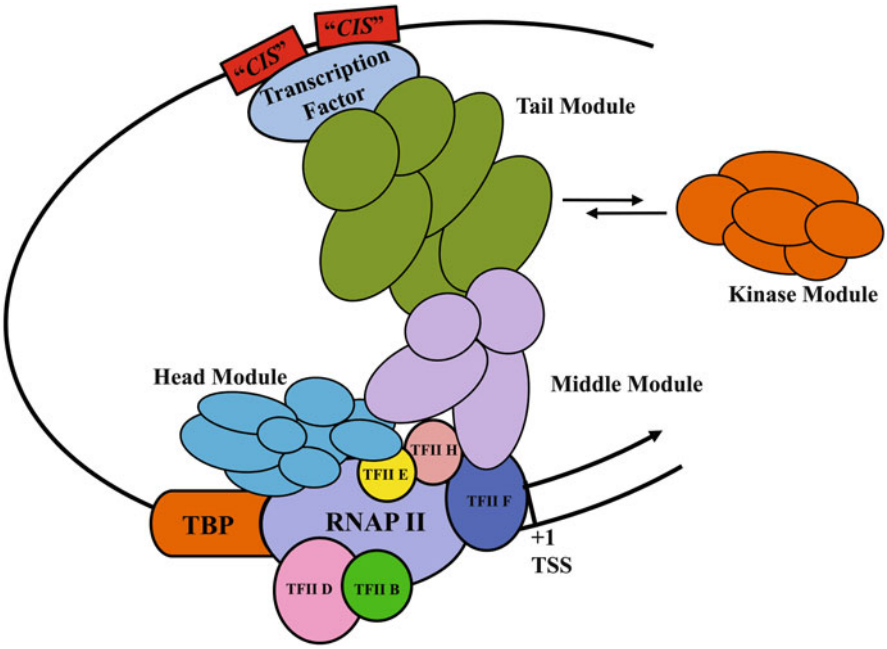

Fig. 1.1 Modular structure of Mediator complex and its interaction with transcriptional machinery. Head, Middle, and Tail modules form the Mediator complex along with a separable kinase module. Generally, the tail module interacts with the *cis*-element bound transcription factors, whereas the Head and Middle modules bind to the components of the basic transcriptional machinery of class II genes. In response to different signals, the Mediator complex helps the transcription factors transmit the messages encrypted in the regulatory DNA elements and engages the transcription apparatus to the promoter of the transcribing genes. **RNAP II**: RNA Polymerase II, **TBP**: TATA-box binding protein, **TFII**: transcription factor II, **TSS**: transcription start site

isolated using the principles of traditional biochemistry, i.e., fractionation of total protein through a series of chromatography based on different principles, and then immunoprecipitating the Mediator complex from the Mediator enriched chromatographic fractions. Using similar techniques, the first biochemical purification of Mediator complex among plants was reported from *Arabidopsis* (Backstrom et al. 2007). After protein fractionation by two different chromatographic techniques, the final step was performed by immunoprecipitation with antibody raised against a Mediator subunit, AtMED6. Apart from *Arabidopsis*, the bioinformatics analyses encompassing 16 plant species across the entire plant kingdom revealed the ubiquitous presence of this important regulatory complex in every plant groups included in the study (Bourbon 2008; Mathur et al. 2011). The presence of almost all the fungal/metazoan Mediator subunits in one or other plant species using HMM (Hidden Markov Model) profile of Mediator subunits was predicted (Bourbon 2008; Mathur et al. 2011). However, some plant-specific Mediator subunits are also reported. Thus, it seems that Mediator subunits have emerged at the very early stages of eukaryotic evolution and some extra subunit might have been added or lost in different lineages in course of evolution (Conaway and Conaway 2011).

1.3 Functions of Mediator Complex

RNAP II along with the components of preinitiation complex (PIC) is the minimum requirement to start any successful transcription event at the initiator region of a gene. In order to achieve increased or activated level of transcription, the requirement of Mediator complex has been proved quintessential almost for every gene of eukaryotes (Myers and Kornberg 2000). In fact, Mediator complex was first discovered as an entity required for enhanced transcription of an in vitro transcription system, which included RNAP II and other accessory factors (Kim et al. 1994). Very recently, critical role of Mediator was explained in the function of super-enhancers in increased level of gene expression to establish and maintain cell identity (Loven et al. 2013; Whyte et al. 2013). However, the inhibitory role of Mediator complex in the repression of gene functions has also been reported and is discussed in a later section. But, the controversial aspect of Mediator function as a cofactor or a basal transcription factor is still debatable (Taatjes 2010). There are evidences, which support the dual role of Mediator function, i.e., as a part of the basal transcriptional machinery as well as a selective regulator of gene function. The Mediator complex can support basal level of transcription as evidenced by its significant roles in the assembly of PIC and in the initiation of transcription (Mittler et al. 2001; Baek et al. 2002). On the other hand, Mediator complex enhances the RNAP II recruitment to the protein coding genes and provides stability to the transcription machinery assembled at the promoter region (Cantin et al. 2003; Baek et al. 2006). The repression of almost all the protein coding genes in yeast conditional mutant *MED17* corroborates the essentiality of Mediator complex in RNAP II-mediated transcription (Thompson and Young 1995; Ansari et al. 2009). In plants, the essentiality of Mediator complex in RNAP II-mediated gene expression became evident when 84 % of downregulated genes in *nrpb2–3* (second largest subunit of RNAP II) and *MED20A* mutant *Arabidopsis* plants were found to be common (Kim et al. 2011). Thus, literature evidences suggest that the Mediator complex is as important as the RNAP II and could be regarded as an integral component of the basal transcriptional machinery in eukaryotes. Nevertheless, reports of severe specific functional abnormalities, be it in growth and development or in the response to biotic and abiotic stresses, when a particular Mediator subunit gene is deleted, are proving that Mediator subunits do possess specific functions (Kidd et al. 2011). Although initial emphasis was laid in the crucial role of Mediator in the assembly of transcription initiation complex (Cantin et al. 2003; Johnson and Carey 2003; Wang et al. 2005), the more recent reports suggest its function in almost every steps of transcription such as promoter escape (Malik et al. 2007; Cheng et al. 2012; Jishage et al. 2012), elongation (Takahashi et al. 2011; Conaway and Conaway 2013; Galbraith et al. 2013), termination (Mukundan and Ansari 2011, 2013), and other related RNA-processing events (Kim et al. 2011; Huang et al. 2012; Oya et al. 2013). In last few years, Mediator has also been implicated in epigenetic modification of chromatin leading to changes in gene expression (Ding et al. 2008; Kagey et al. 2010; Zhu et al. 2011; Fukasawa et al. 2012; Liu and Myers 2012; Tsutsui et al. 2013; Zhang et al. 2013a; Lai et al. 2013).

1.4 Modular Organization and Composition of Mediator Complex in Plants

Mediator is a multi-protein complex, which is composed of several subunits. The number of subunits varies according to the species. The yeast Mediator complex is composed of 25 subunits whereas the metazoans possess 25–30 Mediator subunits (Boube et al. 2002; Bourbon 2008). On an average, the plants contain around 30–35 Mediator subunits (Backstrom et al. 2007; Mathur et al. 2011; Pasrija and Thakur 2012). However, expansion of some subunits has also been observed in plants. Apart from the orthologs of the yeast Mediator subunits, the plants also contain a unique set of Mediator subunits, which are not present either in yeast or in metazoans. Plants are sessile organisms and the Mediator complex assisted gene regulation seems to be more complicated in plants. This could be corroborated by the fact that plants possess increased number of transcription factors (Riechmann et al. 2000; Riechmann and Ratcliffe 2000). As the Mediator complex elicits its gene regulatory action by forging a bridge between the *cis*-element bound transcription factors and the RNAP II, the increased number of Mediator subunits might have evolved to interact with increased number of transcription factors in plants. Another important discovery is the presence of increased number of paralogs of some Mediator subunits in plants. Certain species of yeast like *Candida glabrata* and the metazoans do possess paralogs of MED15 and kinase module genes, respectively. But the possession of nine paralogs of MED15 in *Populus trichocarpa* and quite a few in other plant species is a distinguishing feature for the plant Mediator complex in general (Mathur et al. 2011; Pasrija and Thakur 2012). At present, the presence of all the paralogs of a particular Mediator subunit at the same time has not been validated. However, from the functional perspective, the spatial and temporal regulation of the expression level of different paralogs of a particular Mediator subunit has been reported (Mathur et al. 2011; Thakur et al. 2013). The rice *OsMED31_1* exhibits pronounced expression level in the leaves whereas *OsMED31_2* exhibits higher expression level only during early stages of panicle development. In *Arabidopsis*, there is only one *AtMED31* gene and it shows higher expression in reproductive organs including flower and seed. In rice, *OsMED15_1* showed seed preferential expression whereas *OsMED15_2* is expressed at similar level in vegetative and reproductive tissues (Thakur et al. 2013). Thus, the presence of multiple paralogs and the spatiotemporal regulation of Mediator subunits make the Mediator structure more dynamic depending upon the external milieu and the growth and developmental phases of the plants. Mediator subunits have been grouped into four modules according to the biochemical and structural evidences obtained from the 3D structure of the yeast Mediator complex (Asturias et al. 1999; Dotson et al. 2000; Chadick and Asturias 2005) assembled from the EM structure of the purified yeast Mediator complex. The following is a brief account of the Mediator complex subunits according to their arrangement in specific modules.

1.4.1 Head Module

The head module consists of MED6, MED8, MED11, MED17, MED18, MED19, MED20, MED22, MED28, and MED30. The head module subunits can establish direct contacts with RNAP II and with other components of the basic transcriptional machinery and, alone could stimulate transcription rate over the basal rate, but it does not support activator-dependent transcription (Takagi et al. 2006; Cai et al. 2010). Disruption of the head module leads to the dissociation of the Mediator complex from the promoter of the transcribing genes (Lariviere et al. 2006). Apart from the direct interaction of head module with the RNAP II (Soutourina et al. 2011), it also interacts with the components of the basic transcriptional machinery. The interaction between head module and TFIIH is probably mediated by an interface created by MED11/MED22 heterodimer of the head module, whereas the interaction with TBP is mainly through MED8 (Kim et al. 1994; Lariviere et al. 2006; Imasaki et al. 2011; Seizl et al. 2011). In yeast, MED17 performs the task of maintaining a link with the middle module through its interaction with the MED21 from the middle module. Similarly, MED17 also interacts with the reversible kinase module via its interaction with CDK8 (Guglielmi et al. 2004). MED17 is the most important Mediator subunit of the head module, as mutation in this gene in yeast affects the expression of most of the protein coding genes just like the deleterious effects caused by mutations in RPB1 subunit of RNAP II (Thompson and Young 1995). Given the fact that head module subunits establish direct contacts with the components of the RNAP II machinery and form the core of the Mediator complex, the head module subunits are thought to be the most conserved Mediator subunits of the complex. In general, structural analysis of the Mediator complex has been impeded by the low expressibility of the Mediator subunit proteins and by the inherent difficulties in the in vitro assembly of the Mediator complex. However, assembly of the head module has become feasible with the recent advances in heterologous protein expression technology, and a low resolution, EM structure of the head module has already been reported (Cai et al. 2010). More recently, a seven subunit partial backbone structure of the head module has been resolved with the help of X-ray crystallography (Imasaki et al. 2011). Only a limited number of head module Mediator subunits have been addressed functionally in plants. Among the significant ones, AtMED8 has been implicated in flowering and root hair biogenesis (Kidd et al. 2009; Sundaravelpandian et al. 2013) whereas AtMED17, 18, and 20 were found to be involved in siRNA and non-coding RNA production (Kim et al. 2011).

1.4.2 Middle Module

The Mediator subunits, MED4, MED7, MED9, MED10, MED21, and MED31 form the middle module. Although MED1 is an important middle module constituent in yeast and metazoans as it regulates many important genes by binding to their respective transcription factors, so far bioinformatics analyses from different

studies in different organisms have never been able to find its orthologs in plants except in a distantly related red algae (Ito and Roeder 2001; Bourbon 2008; Mathur et al. 2011). The apparent absence of plant MED1 suggests that either MED1 has been lost in course of evolution or its function might have been acquired by some other Mediator subunit. Middle module subunits, MED1 and MED10, interact with the tail module subunit, MED14, which happens to be at the interface of middle and the tail modules (Li et al. 1995; Lee et al. 1999; Guglielmi et al. 2004). The interaction between MED21 and MED3 also strengthens the connection between middle and tail module (Guglielmi et al. 2004). A combination of biomolecular techniques including small angle X-ray scattering revealed that a high degree of intrinsic flexibility and the elongated shape are the characteristic features of the middle module (Koschubs et al. 2010). MED4 and MED7 are probably the most important middle module subunits as they form three heterodimeric subcomplexes, Med7N/21, Med7C/31, and Med4/9 (Koschubs et al. 2010). Large-scale structural changes in the Mediator subunits are effected by a flexible hinge formed by MED7 and MED21 in the middle module (Baumli et al. 2005). The Med7C/31 is characterized by a novel conserved fold and is essential for activator-dependent transcription (Koschubs et al. 2009). Most of the middle module subunits are conserved in plants too, except a "poly Pro" region in AtMED31C, followed by a nuclear localization signal, which is absent in yeast and human (Mathur et al. 2011). Except AtMED21 whose involvement in pathogenesis is discussed in latter section, function of no other plant Mediator subunits from the middle module has been characterized.

1.4.3 Tail Module

The tail module is arguably the least conserved and functionally most significant Mediator module. The subunits from the tail module maintain direct contacts with the *cis*-element bound transcription factors and accordingly recruit the DNA bound Mediator complex to the RNAP II machinery. The module includes MED2/29/32, MED3/27, MED5/24/33, MED14, MED15, MED16, and MED23. Structural analysis revealed that MED14 occurs at the interface of middle and tail module. In yeast, heterodimer of MED2 and MED3 interacts with MED15 to form a triad (Zhang et al. 2004). Similarity analyses among the tail module subunits revealed that MED2 and MED3 of plants, are more similar to human as compared to yeasts (Mathur et al. 2011). Size of MED15 in plants is bigger than that of fungi and animals, though its amino terminal KIX domain is conserved in them (Thakur et al. 2013). Functionally, the KIX domain seems to be very important domain of MED15 proteins and so, structurally the most well-investigated one (Thakur et al. 2008, 2009, 2013, 2014, Lariviere et al. 2012). A myriad of transcription factors have been reported to interact with MED15 via KIX domain regulating diverse pathways in different organisms (Malik and Roeder 2005; Thakur et al. 2014). Despite poor structural similarity except in the N-terminal transcription factor interacting KIX domain among the MED15 proteins, the crucial amino acid residues of the KIX domain are surprisingly conserved among human, yeast, and *Arabidopsis*, three

important model organisms from three different kingdoms (Mathur et al. 2011). The importance of the tail module subunits in the transcriptional regulation could be well imagined by the fact that the maximum numbers of subunits, whose functions are elucidated, belong to this module. Although no interaction has been reported among them, an intriguing hypothesis regarding the formation of a triad consisting of MED15, MED16, and MED14 in plant defense signaling has been recently put forward (Zhang et al. 2013b). Apart from its gene-specific role, the tail module has recently been implicated in many different aspects of transcriptional regulations as a separate entity. The TATA-box containing and SAGA-regulated genes are much more dependent on the tail module for their transcription as compared to the TFIID-dependent gene expression (Ansari et al. 2012; Ansari and Morse 2012). Interestingly, a role of Mediator tail module in the maintenance of heterochromatin region of chromosome telomere has also been reported (Peng and Zhou 2012).

1.4.4 Kinase or CDK8 Module

The stimulatory role of Mediator complex in gene regulation has become complicated with the discovery of kinase module, which can reversibly associate with the core part of the Mediator complex. The kinase module is composed of MED12, MED13, Cyclin-Dependent Protein Kinase 8 (CDK8), and Cyclin C (CycC). All the kinase module subunits were discovered in yeasts in a screen for suppressor of RNAP II CTD mutation (Liao et al. 1995). Basically, the association of kinase module with the core complex inhibits its interaction with the RNAP II machinery (Akoulitchev et al. 2000; Knuesel et al. 2009a). Also, initial genetic studies revealed negative effect of this module on a subset of genes (Holstege et al. 1998; Samuelsen et al. 2003). However, recent reports contradicted these observations and showed the positive regulation of some genes by the Mediator complex, which had the kinase module associated with it (Donner et al. 2007, 2010; Belakavadi and Fondell 2010). Thus, the CDK8 kinase module can modulate the transcription factor activity in both positive and negative way (Taatjes 2010). Among the regulators of CDK8 kinase activity, MED12 has been established as the most significant one as CDK8 requires MED12 for its kinase activity (Knuesel et al. 2009a). Moreover, MED12 might directly interact with transcription factors for recruiting CDK8 to the chromosomal loci. MED13 helps in association and recruitment of kinase module to the Mediator complex via its interaction with the tail module (Knuesel et al. 2009a). Mediator also regulates kinase activity of CDK8 on chromatin by restricting its association with it (Knuesel et al. 2009b). The bioinformatics analyses have revealed the presence of kinase module in almost all the plant groups analyzed. Like in mammals and other metazoans, paralogs of the kinase module subunits have also been discovered in plants, which raise the possibility of combinatorial control of Mediator function in plants too. Since the kinase module bound Mediator complex accounts for only a small fraction of the total Mediator, the absence of kinase module

subunits in the first-ever Mediator complex purified from *Arabidopsis* is not so surprising (Backstrom et al. 2007). Among the four members of the kinase module, CDK8, Cyclin C, and MED12 have not been found to interact with any other subunit of the Mediator complex (Guglielmi et al. 2004). However, a comprehensive analysis in different organisms needs to be done before making any conclusion.

1.4.5 Plant-Specific and Module-Unassigned Mediator Subunits

Positions of MED25 and MED26 have not been understood yet. Similarly, the plant-specific Mediator subunits, MED34, MED35, MED36, and MED37, have not been assigned any module (Backstrom et al. 2007). Other two plant-specific Mediator subunits, MED32 and MED33, identified during biochemical purification of Mediator complex from *Arabidopsis*, have reported to be apparent homologs of MED2 and MED5, respectively (Mathur et al. 2011). MED26, which remained unreported for a long time from any plant species, has been reported from all the plant species using a rigorous HMM search except algal group (Mathur et al. 2011). Most of these MED26 proteins have been described as transcriptional elongation factors especially in rice and *Arabidopsis* databases, probably because of the presence of TFIIS helical bundle in them (Mathur et al. 2011). This helical bundle is a characteristic feature of RNAP II elongation factors, TFIIS and Elongin A. Thus, MED26 of the Mediator complex contributes to the elongation step of RNAP II-mediated transcriptional event, which is unusual as compared to the canonical role of the Mediator complex in the assembly of initiation complex (Takahashi et al. 2011; Conaway and Conaway 2013). As of now, in plants, MED25 is the most well-characterized Mediator subunit, which has been described to function in different biotic and abiotic stresses and in diverse developmental processes like root development, flowering, and fruit development. As the plants are sessile organisms, perhaps the gene regulatory mechanisms in plants are more diverse and complicated. Several transcription factors function as the master regulator of the cellular and physiological processes, and so their complex network can contribute immensely to the complexity of gene regulation. The genome analysis of plants revealed that plants contain more number of transcription factors as compared to animals (Riechmann et al. 2000; Riechmann and Ratcliffe 2000). As Mediator functions by interacting with the transcription factors, the increased number Mediator subunits in plants might have evolved to cover more number of transcription factors. Also, plant-specific Mediator subunits might be targeted by plant-specific transcription factors conferring the plants better transcript alteration ability in response to diverse internal and external cues. On the other hand; as the basic, overall structure of the Mediator complex is same in all the organisms, most of the plant-specific subunits, if not all, will predictably occupy the tail module of the Mediator complex, bestowing the plants with seemingly unlimited gene regulatory potential.

1.5 Transcriptomics of Mediator Genes

Most of the total protein-coding genes in eukaryotes require the contribution of Mediator complex even to sustain basal level of transcription proves unequivocally that Mediator constitutes the part of the basal transcriptional machinery (Ansari et al. 2009; Kim et al. 2011; Lacombe et al. 2013). At the same time, the increasing numbers of reports describing the effects of mutation in specific subunit on the transcription of specific set of genes strongly suggest that Mediator could also act as selective gene regulator (Taatjes 2010; Kidd et al. 2011). This raises the possibility of regulation of specific *MED* genes in response to specific signals. In order to address this, expression analyses of *MED* genes were performed by different research groups in animals and plants. In human endothelial progenitor cells, expression of *MED12* and *MED30* increased and decreased, respectively, after L-arginine treatment (Rienzo et al. 2010). Additionally, Mediator subunit genes were also found to undergo alternative splicing in tissue-specific manner (Rienzo et al. 2012). In plants too, alternatively spliced isoforms of *MED* transcripts are predicted. In rice, *MED* genes are more pronouncedly expressed in seeds of different stages as compared to shoot and root (Mathur et al. 2011). This is in accordance with the enrichment of seed storage-specific promoter elements on certain *MED* genes indicating the important regulatory role of MED subunits during seed development and maturation. A genome level transcriptome analysis of *MED* transcripts in response to different stresses like drought, cold, and salinity did not reveal much perturbations except that in *OsMed37_6*, which exhibited around twofold changes in transcript abundance in response to different stresses (Mathur et al. 2011). In *Arabidopsis*, some hormones such as brassinosteroid (BR) and ABA affect the stoichiometric concentrations of a set of MED subunits by regulating their transcript abundance (Pasrija and Thakur 2012). However, other hormones like auxin, jasmonic acid (JA) affect very few *MED* genes. *AtMED37*, which has been discovered as a plant-specific Mediator subunit, is highly up-regulated in response to BR treatment. A significant transcriptomic reprogramming of the Mediator subunit genes in *Arabidopsis* happens in response to stresses like salinity, cold, high light and continuous dark and is summarized in Table 1.1 (Pasrija and Thakur 2012). Additionally, in *Arabidopsis*, tissue- and organ-specific analyses revealed changes in transcriptome profile of several *MED* genes during development and maturation of tissues and organs (Pasrija and Thakur 2013). On the basis of their studies, apart from spatiotemporal regulations of individual Mediator subunits, enrichment of specific structural arrangement composed of specific Mediator subunits during certain developmental stages can be predicted. In the following section, we describe the change in the transcript abundance of individual Mediator subunits according to their module occupancy, at different developmental stages and in response to different environmental cues.

Table 1.1 Transcript perturbation of Mediator subunit genes of *Arabidopsis* in response to different abiotic cues

Mediator genes	Salt	Cold	High light	Dark
Head module				
MED6	+	nc	nc	−
MED8	+	nc	+	−
MED11	nc	nc	nc	−
MED17	+	nc	+	−
MED18	+	−	nc	−
MED19	+	nc	nc	nc
MED20	+	−	nc	−
MED22	+	−	nc	−
MED28	nc	nc	nc	−
Middle module				
MED4	+	nc	+	−
MED7	nc	nc	nc	nc
MED9	+	nc	nc	−
MED10	+	nc	nc	−
MED21	nc	nc	nc	−
MED31	+	nc	+	−
Tail module				
MED2	nc	nc	nc	−
MED3	−	nc	nc	−
MED5	+	nc	+	nc
MED14	+	nc	+	nc
MED15	nc	nc	+	+
MED16	+	nc	nc	nc
MED23	+	−	+	−
Kinase module				
MED12	+	nc	+	nc
MED13	nc	nc	+	nc
CDK8	+	nc	+	−
Cyclin C	nc	−	nc	−
Module-unassigned subunits				
MED25	nc	nc	+	−
MED34	−	nc	nc	nc
MED35	nc	nc	+	−
MED36	nc	nc	−	−
MED37	+	+	+	+

"+" : up-regulation, "−" : downregulation; "*nc*" : no change

1.5.1 MED Genes Coding for Head Module Subunits

Among the core Mediator subunits, in rice, *OsMED8_1* showed increased transcript abundance (around threefold) in the early panicle stages. Other important core Mediator subunits, *OsMED6*, *17*, *20*, *22*, *28*, and *30* maintain a steady-state level

irrespective of any developmental stages indicative of its basal role in transcription (Mathur et al. 2011). However, *OsMED11_1* showed more than twofold up-regulation in early stages of seed development. In *Arabidopsis*, more than 2.5-fold up-regulation of *AtMED6* and *AtMED17* is the notable changes among the head module Mediator subunits in response to BR treatment (Pasrija and Thakur 2012). Among the other significant expression changes of the head module subunits in response to phytohormone treatments, more than twofold buildup in the transcript level of *AtMED18* in response to JA deserves special mention. AtMED18 has been reported to be involved not only in flowering but also in regulation of organ number and shape (Zheng et al. 2013). It would be interesting to find out whether AtMED18 regulates these processes through JA-regulated pathways or not. More than twofold up-regulation of *AtMED17* in response to high light is an indication of its important gene regulatory role in light-dependent plant processes (Kim et al. 2011; Pasrija and Thakur 2012). The downregulation of *AtMED18* and the up-regulation of *AtMED20* in response to cold and salinity stresses (Table 1.1), respectively, may connect the Mediator functions to the growth and development of the plant under harsh environmental conditions.

1.5.2 MED Genes Coding for Middle Module Subunits

The study of expression pattern of *MED9* revealed its up-regulation during seed development in *Arabidopsis* while it declined in rice in the equivalent stages (Mathur et al. 2011). Similar to *AtMED9*, *AtMED10* also showed increased transcripts during the advanced stages of seed development. More than 2.5-fold increase in the *AtMED9* transcript level in response to BR treatment may hint at its important role during seed development. Another, middle module Mediator subunit, *AtMED21_1* showed approximately twofold upsurge in the advanced stages of seed development, which conforms to the already reported role of AtMED21 in embryo development and cotyledon expansion whereas OsMED21 might be involved more in the early stages of panicle development (Mathur et al. 2011). Among the other notable changes, *OsMED31_1* showed around threefold induction in leaf as compared to root. A slight increase in the transcript level of *AtMED4* and *AtMED31* in response to auxin treatment was noted. Among the physical stresses, NaCl increases the transcript level of *MED4* quite significantly, more than twofold.

1.5.3 MED Genes Coding for Tail Module Subunits

Among the tail module Mediator subunits, *MED3*, *5*, *14*, and *15* showed differential expression during different stages of reproductive developments, both in rice and *Arabidopsis* (Mathur et al. 2011). Mediator subunit *MED14*, also known as *STRUWWELPETER* (*SWP*), is characterized by its stronger expression level in the

leaf and has been implicated in the control of cell cycle duration and root elongation (Autran et al. 2002; Krichevsky et al. 2009). Between the two rice *OsMED14* paralogs, *OsMED14_1* showed more than 11-fold upsurge just after pollination and then gradually dropped down. However, the expression enhancement of *AtMED14_1* was specifically confined to advanced stages of seed development. *OsMED5_2* shows downregulation during reproductive developments. Significant up-regulation of *OsMED15_1* during different stages of seed development supports its probable role in seed development (Thakur et al. 2013). In *Arabidopsis*, *AtMED15_1* shows very high expression in mature leaves as compared to the young ones (Pasrija and Thakur 2013). Surprisingly, its transcript level goes down with the maturation of the flower. It will be interesting to dissect the function of AtMED15_1 in leaf and flower. In earlier reports, AtMED16 has been implicated to be involved in freezing tolerance (Warren et al. 1996; Knight et al. 1999). But the expression analyses of both *AtMED16* and *OsMED16* do not show any alteration in their transcript abundance across different stages, and also under the cold stress condition. Function of MED16 has been predicted to be at the post-translational stage of C-Box binding transcription factors (CBFs) involved in cold stress signaling pathways (Knight et al. 2009). Like its role in cold signaling, more than twofold increase of *MED16* transcript in response to salinity stress may imply its role as a converging point of both salt and cold signaling pathways. On the other hand, we noted significant downregulation (>40 %) of tail module subunit genes like *AtMED15*, *AtMED14*, and *AtMED5* in response to auxin treatment (Pasrija and Thakur 2012). Although auxin and BR are known for their synergistic effects on plant growth and development, the same study reported the up-regulations of a set of Mediator genes, including the tail module subunit, *AtMED15* in response to BR treatment. However, overall, there is an overlap in the *MED* gene transcriptomic changes barring its antagonistic nature in response to these hormones. The increase in the transcript level of *AtMED15* under both dark and light conditions needs to be studied further (Table 1.1).

1.5.4 MED Genes Coding for Kinase Module Subunits

Kinase module Mediator subunits, MED12 and MED13, also known as GRAND CENTRAL (GCT) and CENTER CITY (CCT), respectively, have been reported to take part in the pattern formation of *Arabidopsis* embryos, and determine the central and peripheral identity of the same (Gillmor et al. 2010; Ito et al. 2011). Moreover, AtMED12 has been shown to be a positive regulator of flowering process (Imura et al. 2012). In compliance of the reported functions, the expression level of *MED12* increased significantly at globular embryo stage in both *Arabidopsis* and rice. On the other hand, the reported 2.5-fold increase of *AtMED12* in response to BR treatment might also shed some light on its role in embryo development (Pasrija and Thakur 2012). The important regulatory role of AtMED12 in light and salt signaling pathways could also not be ruled out because of its twofold up-regulation in response to high light and salt conditions. However, the Mediator subunit *OsMED13* did not

show any change in the expression level in both, *Arabidopsis* and Rice. *OsMED13_1* expresses usually more in the leaf as compared to the root (Mathur et al. 2011). Around 40 % reduction in the expression level of *AtMED13* was observed in response to auxin treatment, which, perhaps, plays a significant role in its tissue-specific function (Pasrija and Thakur 2012). Though AtCDK8 or HEN3 has been reported to be involved in stamen and carpel development in *Arabidopsis*, the expression analyses of *Cyclin C* and *CDK8* genes revealed no significant alteration in their expression pattern during different stages of reproductive development (Wang and Chen 2004). In rice, *OsCDK8* expresses more in the leaf when compared with its expression in root. Also, more than twofold downregulation of *AtCycC* of the kinase module during cold stress may be worth mentioning here.

1.5.5 MED Genes Coding for Plant-Specific and Module-Unassigned Subunits

Several plant-specific Mediator subunits like *MED34*, *35*, *36*, and *37*, which have not been assigned any module yet, are expressed more in reproductive stages as compared to vegetative parts implying its tissue-specific functions. Another module-unassigned Mediator subunit is MED25, which has extensively been characterized to be involved in both biotic and abiotic stress responses (Kidd et al. 2009; Elfving et al. 2011). In *Arabidopsis*, the positive regulatory role of AtMED25 in flower development has been documented by several research groups (Cerdan and Chory 2003; Kidd et al. 2009; Inigo et al. 2012a, b). In rice, its expression remains constant during panicle initiation, increases nearly twofolds during seed maturation stages (Mathur et al. 2011). The induction of *AtMED37* in response to BR and low light suggests a probable link between shade and brassinosteroid signaling, and the process may be mediated by Endoplasmic Reticulum-Associated Degradation (ERAD) (Hong et al. 2008). The up-regulation of *AtMED37* in response to cold and salinity stresses (Table 1.1) provokes an intriguing hypothesis that AtMED37 may act as an integrative hub of many different signaling pathways, which is supported by the near ubiquitous, high expression level of *AtMED37* in all the organs tested so far (Pasrija and Thakur 2013). Another Mediator subunit, *MED36* expresses highly in the root of *Arabidopsis* and is anticipated to be involved in root-specific gene regulatory functions (Pasrija and Thakur 2013). In rice, *OsMED26* is expressed more in root as compared to leaves.

1.6 Role of Mediator in Biotic Stress

Plants in its natural environments are being constantly challenged by myriad of insects, pests, and other pathogens, which together constitute the biotic stresses. A survivor plant must activate its defense arsenal quickly and efficiently in order to

triumph over the invading and inflicting biotic agents. Any orchestrated and rapid response is achieved by the timely expression of defense genes, which is usually implemented and coordinated by the combined action of RNAP II machinery and Mediator complex. Emerging reports have started establishing Mediator complex as an essential component of defense gene regulatory pathway (An and Mou 2013). In comparison to other responses where few subunits are involved, maximum number of subunits are reported to be involved in defense signaling (Table 1.2). The first one reported to be involved in defense response was AtMED25 (Kidd et al. 2009). AtMED25 bears similarity with human MED25, where it also plays the role in defense response (Leal et al. 2009). In *Arabidopsis*, it directly affects the jasmonate-dependent expression of *PDF1.2*, *HEL*, *CHIB*, and *ESP* genes and provides resistance against the leaf infecting necrotrophic fungi, *Alternaria brassicicola* and *Botrytis cinerea* (Kidd et al. 2009). The complementation of *Atmed25* by its homologs from wheat strengthened the view that functions of some of the Mediator subunits may be conserved in higher plants (Kidd et al. 2009). AtMED25 takes part in ERF1- and ORA59-dependent activation of the *PDF1.2* gene as well as MYC2-dependent activation of the *VSP1* gene, which are some important target genes of JA signaling pathway (Çevik et al. 2012). In fact, MED25 physically associates with the basic helix-loop-helix transcription factor, MYC2 in promoter regions of MYC2 target genes to elicit its positive effect on their transcription (Chen et al. 2012). A group of 12 transcription factors (TFs) including AP2/ERF, bHLH, MYB, WRKY, and bZIP have been shown to interact with AtMED25. Among these transcription factors, many have previously been demonstrated to be involved in JA signaling pathway (Çevik et al. 2012). Thus, it is tempting to speculate that defense signaling evoked by different pathogens might be integrated at MED25 level for their proper channeling into the transcription apparatus via Mediator complex.

AtMED8 is one of the Mediator subunits, which has been reported to be involved not only in plant development, but also in its defense response. The plants carrying mutation in *AtMED8* behave quite similar to *Atmed25* but show more pronounced susceptibility towards *A. brassicicola*. Intriguingly, there is no genetic interaction between these genes, leading to the speculation that these Mediator subunits, AtMED25 and AtMED8, might be acting in two independent pathways controlling the same phenotype (Kidd et al. 2009).

Embryonic lethality of homozygous *Arabidopsis* lines carrying T-DNA insertion in *AtMED21* suggests the essentiality of MED21 in plant's life (Dhawan et al. 2009). The RNA interference lines of *MED21* are highly susceptible to *A. Brassicicola* and *B. Cinerea*. The detailed study revealed that MED21 interacts with RING E3 ligase, Histone Monoubiquitination 1 (HUB1), which mediates the H2B ubiquitination, and thus establishes a link between Mediator and the chromatin remodeling (Dhawan et al. 2009). The induced expression of both *MED21* and *HUB1* in response to chitin treatment, an important constituent of fungal cell wall that function as pathogen-associated molecular pattern (PAMP), suggests their probable role in defense signaling. Just like *Atmed21* mutants, loss of function mutation in *HUB1* makes the plant susceptible to *B. cinerea* and *A. brassicicola*. It

Table 1.2 Role of Mediator subunits in biotic and abiotic stresses in plants

Stress type	Gene name	Functions	References
Biotic	*AtMED25/PFT1*	Regulates the gene expression of jasmonate pathway genes and provides resistance against *Alternaria brassicicola* and *Botrytis cinerea*. It interacts with several transcription factors including MYC2, AP2/ERF, bHLH, MYB, WRKY, and bZIP.	Kidd et al. (2009), Cevik et al. (2012), Chen et al. (2012)
	AtMED8	Same like AtMED25. The mutant shows more disease susceptibility as compared to AtMED25 towards *A. Brassicicola*.	Kidd et al. (2009)
	AtMED16/SFR16	Involved in SA and JA pathways of disease signaling. Regulates expression of *PR1*, *PR2*, *PR5*, *GST11*, *EDR11*, and *SAG21* genes. Also regulates expression of the jasmonate pathway genes. The mutants are susceptible to *Pseudomonas syringae* and necrotrophic fungi like *A. brassicicola* and *B. cinerea*.	Wathugala et al. (2012), Zhang et al. (2012)
	AtMED21	*MED21* gets induced in response to fungal elicitors and provides resistance against *A. brassicicola* and *B. cinerea*. It interacts with Ring E3 ligase, HUB1, and regulates H2B ubiquitination.	Dhawan et al. (2009)
	AtMED15/NRB4	Involved in SA response pathway of disease signaling.	Canet et al. (2012)
	AtMED14/SWP	Regulates expression of *NPR1*, *EDS1*, *PAD4*, *ICS1*, *EDS5*, *NIMIN2*, *WRKY38*, and *WRKY62* genes of SA pathway and confers resistance against *Pseudomonas syringae pv. tomato* (*Pst* DC3000). Also regulates the expression of SAR genes, *PR1*, *PR2*, *PR5*, *GST11*, *EDR11*, and *SAG21*.	Zhang et al. (2013b)
Abiotic	*AtMED25*	AtMED25 regulates responses to salinity stress and drought stress, antagonistically. It interacts with different transcription factors like DREB2A, ZFHD1, and MYB, which are also known to be involved in salt and drought tolerance.	Elfving et al. (2011)
	AtMED16/SFR16	Regulates partly the expression of *LTI78*, *COR15A*, and *KIN1/2* genes in response to cold stress.	Knight et al. (1999, 2008, 2009)

seems that MED21 integrates signals from transcription regulators and HUB1-mediated chromatin modification to regulate transcriptional machinery.

Prior to its identification as a part of the Mediator complex, AtMED16 was initially discovered as SFR6 (sensitive to freezing). Mutation in *SFR6* compromised the ability of the plant to withstand lower temperature (Warren et al. 1996). Subsequently, an array of papers has documented its important roles not only in cold and drought responses (Knight et al. 1999, 2009; Wathugala et al. 2011) but also in the regulation of flowering and circadian clock in plants (Knight et al. 2008). Detailed analyses of mutants carrying defective *MED16* revealed compromised regulation of defense genes controlled by salicylic acid and jasmonic acid pathways (Wathugala et al. 2012). The *sfr6* mutant plants are more susceptible to *Pseudomonomas syringae* attack and exhibit lower expression level of defense-related genes coding for proteins like PR proteins and defensins. In another study, the expression levels of the important SAR (systemic acquired resistance) markers like *PR1*, *PR2*, *PR5*, *GST11*, *EDR11*, and *SAG21* were severely reduced in *Atmed16* mutant (Zhang et al. 2012). Hence, MED16 acts as a positive regulator of SA-induced gene expression. From the detailed studies of MED16-GFP recombinant protein, it was reasoned that the non-accumulation of NPR1, which resides at the nodal point of SA-induced gene expression pathway, might be responsible for the improper regulation of SAR genes in *Atmed16* mutant during pathogen attack (Zhang et al. 2012). Similarly, the *MED16* mutation also blocks the induction of the JA/ethylene (ET)-induced gene expression making the plants vulnerable to necrotrophic fungi like *Alternaria brassicicola* and *Botrytis cinerea*. Hence, MED16 might act as a converging point for both salicylic acid and jasmonate signaling pathways.

Another tail module Mediator subunit, AtMED15, also dubbed as NRB4 (Nonrecognition of BTH4, a salicylic acid analog), has recently been shown to be involved in SA-dependent defense signaling (Canet et al. 2012). The plants carrying mutation in *MED15* do not show any noticeable phenotype change except its attenuated response to salicylic acid (SA), reminiscent of the effects of *npr1* mutation in plant's defense signaling. NPR1 (non-expresser of *PR* genes) plays a pivotal role and takes the center stage in the SA signaling pathway (Dong 2004). However, neither a genetic interaction nor a biochemical interaction has been reported between MED15 and NPR1. That *nrb4-1*/*npr1-70* plants show additive phenotypes indicates that they might work independently in SA acid signaling pathway. Moreover, *nrb4* affects neither localization of NPR1 nor its stability. Thus, mechanistically, MED15/NRB4 functions downstream of NPR1 in the regulation of SA response pathway. But, how MED15/NRB4 mediates the SA-responsive gene expression in plants warrants still more detailed investigations.

The latest member to join the growing number of Mediator subunits playing important roles in defense response in plants is AtMED14/SWP (Zhang et al. 2013b), which has earlier been shown to be involved in meristem pattern formation and control of cell cycle duration (Autran et al. 2002). A mutation in *AtMED14* subunit gene suppresses the salicylic acid-induced expression of defense genes. AtMED14 does not interfere with the binding of NPR1, the master regulator of defense gene regulation, to the promoter of defense gene, *PR1*. However, it prevents

the expression of *PR1* leading to the speculation that AtMED14 might be responsible for the recruitment of RNAP II to the promoter of *PR1* gene. Further investigation is needed to delineate the exact mechanism involved in this process.

Thus, the tail module as a whole plays significant role in the regulation of defense genes during pathogen attack. But the mechanisms employed by these three different Mediator subunits (MED14, MED15, and MED16) differ considerably in controlling the pathogenesis-related genes. The *MED16* mutation differentially affects the expression of different positive and negative regulators of SAR whereas *MED14* mutation inhibits both the positive and negative regulators of the SAR. Moreover, defense-related transcriptomic change in case of *Atmed14* is much smaller as compared to that of *Atmed16*.

1.7 Role of Mediator in Abiotic Stress

Being sessile organisms, plants cannot run away to safer places during inclement weather. On the other hand, growth and development of a plant is profoundly influenced by the environment. In order to survive, a plant must translate the vagaries of the surrounding environments into proper molecular cascades relaying the signals to the transcriptional machinery ensuring the adaptability of the plants to the changed milieu. Of late, Mediator has emerged as an integrative hub for the different signaling pathways leading to the transcriptional regulation by RNAP II. So, it is quite obvious that the Mediator plays crucial roles in the integration of signals evolved in response to stresses like drought, cold, salinity etc. So far two Mediator subunits, which too play important roles in biotic stresses, have been reported to be involved in abiotic stresses (Table 1.2). The importance of MED25 in salt signaling is conserved across the plant species (Elfving et al. 2011). In a yeast two-hybrid screen, utilizing the activator interacting domain (ACID) domain (551–680 a.a.) of AtMED25, Bjorklund's group identified three transcription factors, DREB2A, ZFHD1, and MYB like proteins as the probable interacting partners of AtMED25 (Elfving et al. 2011). Mutations in the genes of these transcription factors make the plants more sensitive to salt. Mechanistically, these transcription factors, in response to salt stress, might be recruiting Mediator and the RNAP II machinery to their respective target promoter affecting the salt-responsive transcriptomic changes in plants. On the other hand, surprisingly, AtMED25 negatively regulates drought tolerance in plants (Elfving et al. 2011). Plants with mutation in *AtMED25* display huge increase in the expression level of drought-responsive marker genes like *RD29A*, *RD29B*, and *DREB2A*. In wild type plants, AtMED25 was projected as a co-repressor interacting with the repressor domain of DREB2A making the plants vulnerable to drought stress. Thus, it is a unique example, where the same Mediator subunit, AtMED25 controls similar kind of stresses in antagonistic manner.

MED16, originally discovered as SFR6 before being identified as a part of Mediator complex, has been reported as an important regulatory component of cold response in *Arabidopsis* (Knight et al. 1999, 2008; Wathugala et al. 2011). The

mutant plants fail to embrace freezing temperature following its exposure to subzero temperature. At the molecular level, the plants are incapable of switching on the COR (cold on regulation) regulation including the expression of *LTI78*, *COR15A*, and *KIN1/2*. Microarray analysis of *sfr6* mutant plant revealed that a group of cold regulatory genes, which are regulated by CRT/DRE motifs are misregulated (Knight et al. 1999). The CRT/DRE motif containing genes involved in freezing tolerance are under the control of CBF transcription factors (Boyce et al. 2003). However, neither expression of *CBF* genes nor the localization of their proteins is affected in *sfr6* mutant plants. Thus, it provokes the intriguing speculation that MED16/SFR6 might modulate the activity of CBFs post-translationally (Knight et al. 2009).

Conclusion and Future Perspective

Mediator research in plants is relatively new as compared to its study in fungi and metazoans. In spite of that, significant developments have been made in plant Mediator research in last couple of years. Isolation of Mediator complex from *Arabidopsis* has been reported and proven the usefulness of bioinformatics predictions. Many Mediator subunits were characterized in mutant screening even before their identification as a bona fide component of Mediator complex. In recent times, many Mediator subunits have been reported to be involved in biotic and abiotic stresses. As a matter of fact, the Mediator complex has emerged as an integrative hub for different biotic and abiotic signaling pathways. In most of the cases, phenotypes of a particular Mediator subunit mutation have been described but its association with transcription factors and the set of genes under its control are yet to be achieved. What is lacking more is the understanding of how the Mediator components interact with components of the basic transcriptional machinery resulting in the activated transcription. Recently, many of the hitherto unknown but interesting aspects of Mediator functions have been unveiled in other organisms broadening the horizon of understanding of Mediator functions. Mediator not only takes part in the recruitment of RNAP II on the promoters of the active genes but also in transcript elongation, termination of transcription, chromatin remodeling, regulation of chromatin architecture, alternative splicing, miRNA biogenesis, and non-coding RNA production. Although there is an overall structural conservation among the Mediator complexes isolated from different organisms, there will be lots of minute and subtle differences, which may bring differential functioning of the Mediator complex in different organisms. That necessitates the isolation of Mediator complex from economically important crop species and to study the mechanistic and functional details of the Mediator complex as a whole as well as its individual subunits. As far as the structure is concerned, no plant Mediator structure has been resolved till date. There are multiple paralogs of many Mediator subunits, and the number is more in case of plants. Another level of complicacy may arise due to selection of a particular paralog for complex formation, which most probably is controlled in a temporal and

spatial manner. The presence of more than one paralog at a time in the Mediator complex has not been reported by any group. The more interesting question, which is just being started to be answered is how stable is the Mediator structure in terms of the amount of subunits present at a given point. Researchers are now very much certain that Mediator structure changes depending on the stoichiometric concentration of Mediator subunits, which again is controlled by different biotic and abiotic stimuli. So, complex isolation and its structural comparison from different stages of growth and development hold the key to the question of how the structural changes in the Mediator are translated into transcriptomic changes of a species in response to different developmental and environmental cues. Armed with the tools of modern molecular biology like tandem affinity purification (TAP), multidimensional protein identification technology (MudPIT), liquid chromatography-mass spectrometry (LC-MS/MS), high-throughput ChIP sequencing (HT-ChIP), the aforementioned questions are anticipated to be answered in accelerated speed in the near future.

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Chapter 2
Towards Understanding the Transcriptional Control of Abiotic Stress Tolerance Mechanisms in Food Legumes

Rebecca Ford, Saleem Khan, and Nitin Mantri

Abstract A multitude of environmental and subsoil conditions cause abiotic constraints to the growth and productivity of legume food species. These stresses often occur simultaneously, leading to compounded effects of low and unreliable yields. Since legumes are a major food source, particularly in regions of major population growth, it is imperative that better tolerant and adapted varieties are developed. For this, transgenic approaches integrated within traditional breeding programs are proposed to offer substantial productivity gains through fast-tracking the development and deployment of well adapted and tolerant varieties to regions of greatest need. For this to occur, knowledge of the major tolerance genes and more importantly their regulators is required. Accordingly, recent functional genomics approaches have begun to shed light on the transcriptional, and hence regulatory and mechanistic controls governing tolerances to several of the major abiotic stresses, such as drought, temperature and salinity within temperate legume food species. Functional validation of these regulatory signals, their action on downstream genes and associated pathways is underway within several large international programs. This chapter will review these advances in knowledge to date within the model and crop grain legume species, to identify and characterize the molecular targets for the future selection and breeding of sustainably tolerant crops. Specifically, this chapter aims to summarize progress towards identifying and understanding the functions of the WRKY transcription factors involved in instigating and regulating abiotic stress tolerance mechanisms and their potential for improving abiotic stress tolerance within temperate legume food species.

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Abbreviation

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
AP2/ERF	APETALA2/Ethylene-responsive factor
DREB	Dehydration-responsive element binding
IAA	Indole Acetic Acid
LAP	Legume anthocyanin production
MYB	Myeloblastosis
NCED	9-cis-epoxycarotenoid dioxygenase
PEG	Polyethylene glycol
TF	Transcription factor
TMV	Tobacco mosaic virus
WRKY	Tryptophan Arginine Lysine

2.1 Regulation of Abiotic Stress and Tolerance

The primary causes of crop loss globally are the extremes of water availability (mainly drought), temperature (heat and cold) and soil toxicities and deficiencies (mainly salinity). These limit the growth and development and agro-geographical distribution of crops worldwide, which causes significant reduction in productivity. Plants respond to adverse environmental conditions through many physiological, biochemical and molecular changes, which enable plants to survive and reproduce (Mantri et al. 2010a). These are governed by an array of genes encoding proteins with diverse functions. The ability of a plant to grow and survive under such conditions is dependent on its ability to adapt growth and metabolic processes, governed through complex networks of molecular switches and regulators. This includes the ability to perceive the stress, respond to the stress through instigation of nearby and/or systemic signals (signal transduction) and the expression of relevant tolerance genes and metabolites. Hence, abiotic stress tolerance traits are multigenic and quantitative in nature and difficult to manipulate or select for within the constraints of traditional breeding approaches. It would be unlikely that selection of a single or few functional genes would result in sustained tolerance since the impacts of abiotic stresses are varied and complex. In the case of salinity tolerance, effects caused by altered ionic strengths and osmotic pressures around membranes lead to potential ion compartmentation and/or exclusion and even changing hormone levels that stimulate cell division for avoidance through altered root physiology (Lv et al. 2012; Mantri et al. 2012). Therefore, selection or manipulation of the stress perceivers and gene pathway regulators is proposed to be a far more efficient approach. In particular, the group of genes that encode regulatory proteins may be useful for the management of

crops under stress conditions (Cabello et al. 2014). This group includes protein kinases, enzymes involved in phospholipid metabolism, transcription factors and other signalling molecules. Understanding their function is critical for determining the possibilities to manipulate or select for improved stress tolerance.

There has been a recent flurry of publications demonstrating the isolation and characterization of abiotic stress-responsive genes and their potential transcriptional controllers from temperate legumes using several methods. This has included partial elucidation of the transcriptional responses and controls to drought, salinity and cold in chickpea using microarrays (Mantri et al. 2007, 2010b) and most recently the more in-depth identification of cold stress tolerance genes and transcription factors in chickpea using cDNA-AFLP (Dinari et al. 2013). There exists tremendous opportunity to employ the high throughput and next-generation sequencing technologies to start to characterize the key functional roles of many genes and gene regulators that have been identified.

2.1.1 Transcription Factors

Among the regulatory proteins, transcription factors (TFs) play a major role in defensive gene expression involved in tolerance mechanisms (Puranik et al. 2012). TFs contain key proteins that interact with *cis*-acting elements within the stress-responsive gene promoters and enhancer sequences to regulate through activation or repression of downstream gene networks. Hence, TFs are the subject of many studies aimed at determining how they may best be manipulated to change the regulation of the whole suit of genes under their control through up- or downregulation (Liu et al. 2013).

TFs are grouped into large gene families related to their characteristic DNA-binding domains (DBDs), such as AP2/ERF, B3, NAC, SBP and WRKY. They are also characterized based on their role in responding to a particular stimulus/stimuli and controlling physiological and metabolic responses involved in adaptive plant growth and development. Members of a family may respond uniquely to different stress stimuli (Yamasaki et al. 2013). TFs that control the complex signals for flowering in pea that are associated with the FT locus have recently been well characterized (Hecht et al. 2011).

TF expression has been characterized in several legume and non-legume species using large-scale quantitative reverse transcription-PCR (Czechowski et al. 2004; Caldana et al. 2007; Kakar et al. 2008; Libault et al. 2009). Using this information, several attempts have been made to quantify TF gene expression in different plant parts to assess for tissue specificity related to function (Gruber et al. 2009; Libault et al. 2009). Additionally, the availability of whole genome sequences has paved the way for elucidating the complex network of gene expressions and regulations in response to abiotic stresses. Sequences are available for *Medicago truncatula* (http://www.plantgdb.org/MtGDB/), *Lotus japonicus* (http://www.plantgdb.org/LjGDB/), *Glycine max* (http://www.plantgdb.org/GmGDB/) and *Cicer arietinum* (Varshney et al. 2013; Jain et al. 2013).

The model legume *M. trucatula* was originally used to undercover abiotic stress-specific TFs and their functions. For example, *WXP1*, an *AP2/EREBP* TF was found to enhance drought tolerance in transgenic plants by increasing cuticular wax and thereby improving the water retaining capacity (Zhang et al. 2005). The same gene and a closely

related paralog (*WXP2*) also enhanced drought tolerance in transgenic *Arabidopsis* plants, as well as enhanced freezing tolerance in the *WXP1* overexpressing lines (Zhang et al. 2007). Further, *MtHAP2-1* was shown to be a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 (Combier et al. 2006).

A *Legume Anthocyanin Production 1* (*LAP1*) gene that serves as an *MYB* TF was identified and constitutively expressed in transgenic alfalfa. *LAP1* induced massive accumulation of anthocyanin pigments comprising multiple glycosidic conjugates of cyanidin. Constitutive expression of *LAP1* induced many genes involved in anthocyanin biosynthesis including glucosyltransferase *UGT78G1*, that when expressed transgenically resulted in increased anthocyanin accumulation when plants were exposed to abiotic stress (Peel et al. 2009).

In another study, a *M. truncatula* 16,000+ gene microarray was used to identify 84 TF sequences differentially expressed in the root apex in response to salt stress. Analysis of salt-stress regulation in root apexes versus whole roots showed that several TF genes have more than 30-fold expression differences including specific members of *AP2*/*EREBP*, *HD-ZIP* and *MYB* TF families. Several salt-induced TF genes also respond to other abiotic stresses such as osmotic stress, cold and heat, suggesting that they participate in general stress response pathways (Gruber et al. 2009).

Despite the historical lag in availability of genomic information for the food legumes, the recent development of online transcriptomic databases for model legumes and reference species holds great promise to aid with TF identification and functional assignment in these orphan species. In particular, "LegumeIP" is a freely available tool (plantgrn.noble. org/LegumeIP) that contains large-scale gene expression data from *L. japonicas* and *M. truncatula* microarrays, as well as RNA-Seq-based gene expression data from *G. max* and time-course expression data from nodule, flower, root and leaf tissues (Li et al. 2012). The ability to perform systematic synteny analyses and construct gene family phylogenies across these species and *A. thaliana* will better enable the accurate classification of TF family members from the legumes once the sequences become available.

The following summarizes the current status of knowledge regarding TF characterization in some important food legumes.

2.1.2 Soybean

The availability of the soybean genome sequence has allowed large-scale identification and annotation of regulatory TFs for functional studies. This has enabled capturing of stress-responsive TFs and their regulatory networks and identification of the full complement of TF encoding genes (Mochida et al. 2009). A total of 5,035 TF models have been found within the soybean genome and grouped into 61 families. The relevant annotations of soybean TF genes can be accessed via the soybean TF database (soybeantfdb.psc.riken.jp).

In functional studies, overexpression of the *GmHSFA1* TF gene conferred tolerance to heat stress (Zhu et al. 2006). Further, 131 *bZIP* genes were identified from soybean and their response to abscisic acid (ABA), drought, salt and cold was analysed. From these, Soybean *GmbZIP44*, *GmbZIP62* and *GmbZIP78* functioned as negative regulators

of ABA signalling and conferred salt and freezing tolerance in transgenic *Arabidopsis* (Liao et al. 2008). Recently, a novel bZIP transcription factor gene, *GmbZIP1* was shown to enhance multiple abiotic stress tolerances (Gao et al. 2011).

Members of the ethylene response factor (*ERF*) TF family regulate gene expression in response to biotic and abiotic stresses. In soybean, 98 unigenes that contained a complete *AP2*/*ERF* domain were identified and their phylogeny, gene structures, and putatively conserved motifs in soybean *ERF* proteins were analysed and compared with those of *Arabidopsis* and rice. Expression analysis showed that nine unigenes belonging to six *ERF* family subgroups were induced by both biotic/abiotic stresses and hormone treatment, suggesting that they were involved in crosstalk between biotic and abiotic stress-responsive signalling pathways. Overexpression of two full-length genes from two different subgroups enhanced the tolerances to drought, salt stresses, and/or pathogen infection (Zhang et al. 2008).

Several TF genes from the *DREB* (dehydration-responsive element binding) gene subfamily of the *AP2*/*EREBP* family have also been identified and characterized in soybean. For example, the overexpression of *GmDREB2* activated expression of downstream genes in transgenic *Arabidopsis*. This resulted in enhanced tolerance to drought and high-salt stresses but did not cause growth retardation (Chen et al. 2007). In addition, constitutive expression of *GmDREB3* in transgenic *Arabidopsis* caused growth retardation, whereas its expression under control of the stress-inducible *Rd29A* promoter minimized negative effects on plant growth under normal growth conditions. This indicated that a combination of the *Rd29A* promoter and *GmDREB3* might be useful for improving tolerance to environmental stresses (Chen et al. 2009).

A new member of the soybean *AP2*/*ERF* TF family, *GmERF3*, was also analysed for biotic and abiotic stress tolerance. Ectopic expression of the *GmERF3* gene in transgenic tobacco plants induced the expression of some *PR* genes and enhanced resistance against infection by *Ralstonia solanacearum*, *Alternaria alternata* and tobacco mosaic virus (TMV), and gave tolerance to high salinity and dehydration stresses. This suggested that *GmERF3* might play dual roles in the responses to biotic and abiotic stresses (Zhang et al. 2009a).

NAC TFs play important roles in plant growth, development and stress responses. In soybean, *GmNAC11* acts as a transcriptional activator, whereas *GmNAC20* functions as a mild repressor. Overexpression of *GmNAC20* enhanced salt and freezing tolerance in transgenic *Arabidopsis* plants whilst *GmNAC11* overexpression only improved salt tolerance (Hao et al. 2011). In another recent study, *GmNAC5* was induced by mechanical wounding, high salinity, and cold treatments but was not induced by ABA (Jin et al. 2013).

Trihelix TFs condition light-regulated responses and other developmental processes involved in abiotic stress signalling. Two trihelix TF genes, *GmGT-2A* and *GmGT-2B*, from soybean conferred stress tolerance through regulation of common and specific sets of genes (Xie et al. 2009). Lastly, the role of soybean *MYB* TFs in response to abiotic stresses has also been evaluated. Three *GmMYB* genes (*GmMYB76*, *GmMYB92* and *GmMYB177*) whose expression changed in response to ABA, salt, drought and/or cold stress were chosen for functional analysis from about 156 soybean *GmMYB* genes. The transgenic *Arabidopsis* plants overexpressing *GmMYB76* or *GmMYB177* showed better performance than the *GmMYB92-transgenic* plants in response to salt and freezing tolerance (Liao et al. 2008).

2.1.3 Chickpea

A chickpea NAC gene *CarNAC5* was found to be expressed in many chickpea tissues including seedling leaves, stems, roots, flowers, seeds and pods, but mostly accumulated in flowers. The *CarNAC5* was strongly expressed during seed maturation, in germinating seeds, and strongly induced by drought, heat, wounding, salicylic acid (SA) and indole-3-acetic acid (IAA) treatments (Peng et al. 2009a). Subsequently, among other NAC TFs, *CarNAC3*, was also shown to be significantly induced by drought stress, ABA, ethephon and IAA (Peng et al. 2009b) and the expression of *CarNAC1* was strongly induced by drought, salt, cold, wounding, H_2O_2, ethephon, salicylic acid, indole-3-acetic acid and gibberellin (Peng et al. 2010). From the members of *ERF/AP2* proteins that play a crucial role in growth and stress response, expression of *CAP2* from chickpea enhanced growth and tolerance to dehydration and salt stress in transgenic tobacco (Shukla et al. 2006).

2.1.4 Peanut

In peanut, six *ERF* TF genes designated as *AhERF1–6* were cloned and their expression patterns were analysed under cold, salt and drought stress. Of these, the expression of *AhERF4* and *AhERF6* was rapidly and substantially enhanced under abiotic stress. Whereas *AhERF3* was downregulated in leaves under salt stress and *AhERF2* was downregulated in leaves under drought stress. Interestingly, the expression of *AhERF3* and *AhERF5* exhibited contrary expression patterns in peanut leaves and roots upon PEG treatment. These results suggested that different *ERF* TFs might have different functions in abiotic stress acclimation in peanut (Chen et al. 2012a).

Previously, a drought-induced NAC gene, *AhNAC2*, was isolated from peanut and transgenic *Arabidopsis* overexpressing *AhNAC2* was hypersensitive to ABA in root growth, seed germination and stomatal closure compared to the wild-type plants. The transgenic lines exhibited enhanced tolerance to drought and salinity stress, and the expression levels of 12 stress-related genes in the *AhNAC2* transformed plants were higher than the wild type (Liu et al. 2011). Finally, the oxidative cleavage of *cis*-epoxycarotenoids catalysed by *9-cis-epoxycarotenoid dioxygenase* (*NCED*) is considered to be the rate-limiting step in ABA biosynthesis. The constitutive expression of peanut *AhNCED1* gene in wild-type *Arabidopsis* resulted in increased ABA accumulation in transgenic plants in response to drought stress (Wan and Li 2006).

2.1.5 Cowpea

In cowpea, a stress-inducible gene for NCED, *VuNCED1*, involved in ABA biosynthesis under water stress was identified from drought-tolerant cowpea (Luchi et al. 2000). In a bid to generate genomic resources in cowpea, sequencing and analysis of the gene-rich, hypomethylated portion of the cowpea genome identified over

250,000 gene-specific sequence reads (GSRs) with an average length of 610 bp (Timko et al. 2008). A total of 62 out of the 64 well-characterized plant TF gene families were represented in the cowpea GSRs, which may provide a resource for functional markers linked to abiotic stress tolerance traits in cowpea. Recently, a comparative genomics approach was used to identify 18 conserved *V. unguiculata* miRNAs belonging to 16 distinct miRNA families. Using these potential miRNA sequences 15 potential target genes were predicted and all of them were identified as transcription factors (Paul et al. 2011).

2.2 Phaseolus

A root-specific *bZIP* TF was shown to be responsive to water deficit stress in tepary bean (*Phaseolus acutifolius*) and common bean (*P. vulgaris*) (Rodriguez-Uribe and O'Connell 2006). Meanwhile, a *NAC* family TF member *AtNAP* important for leaf senescence was characterized in *Arabidopsis* (Guo and Gan 2006). In this study, an orthologous *NAC* TF from *P. vulgaris*, PvNAP, was identified that shared the same leaf senescence-specific expression pattern as *AtNAP. P. vulgaris* leaves at five distinct developmental stages, were analysed and the *PvNAP* transcript was detected in senescing leaves only. In addition, AtNAP homologues in *P. vulgaris* were able to restore the *Arabidopsis AtNAP* null mutant to wild type (Guo and Gan 2006). Recently, analysis of genes in response to dehydration stress in *P. vulgaris* identified *CA1* with sequence similarity to the *ERF* family *AP2/EREBP* and was tenfold induced by drought stress (Kavar et al. 2008).

2.3 WRKY TFs and Their Roles in Abiotic Stress Tolerance

The WRKY (W=Tryptophan, R=Arginine, K=Lysine, Y=Tyrosine) family are one of the largest TF families and regulate many plant processes (Ulker and Somssich 2004; Zhang and Wang 2005). Members can either activate or repress multiple downstream gene networks along with MAP kinases, MAP kinase kinases, 14-3-3 proteins, calmodulin, histone deacetylases and other protein partners. WRKY TFs possess a DNA-binding domain of 60 amino acids with an evolutionary conserved WRKYGQK signature at the N terminal and a C terminal that contains a zinc finger motif (Eulgem et al. 2000). WRKY proteins have DNA-binding activity at a specific W BOX motif (C/T)TGAC(T/C) in their promoter region (Eulgem et al. 2000). Other sequences adjacent to the WRKY domain also assist in DNA-binding activity (Luise et al. 2013; Ciolkowski et al. 2008).

Structural classification of the WRKY family is based on configuration of the zinc finger motif, the number of WRKY domains and the size of the intron (Zhang and Wang 2005). The family is classified into three main groups 1, 2 and 3, which are further divided into subgroups 2a+2b, 2c, 2d and 2e (Eulgem et al. 2000; Zhang and Wang 2005) (Table 2.1). The family originated two billion years ago

Table 2.1 WRKY transcriptional factor family groups and functions

WKRY gene	Group/subgroup	Organism	Source of induction	Role in abiotic stress	References
At WRKY33	1	*Arabidopsis thaliana*	Drought, salt	Activate drought tolerance gene CesA8	Wang et al. (2013)
AtWRKY25	1	*Arabidopsis thaliana*	NaCl, heat	Increased salinity and heat tolerance	Jiang and Deyholos (2009)
AtWRKY34	1	*Arabidopsis thaliana*	Cold	Increased pollens viability during cold stress	Zou et al. (2010)
PtrWRKY2	1	*Poncirus trifoliata*	Low temperature	Positive regulation of cold stress	
AtWRKY26	1	*Arabidopsis thaliana*	High temperature	Upregulation of heat stress	Li et al. (2011)
GmWRKY1, 5, 15, 21, 30	1 and 2	*Glycine max*	Cold	Overexpression during cold stress	Zhou et al. (2008)
PtrWRKY1	2	*Poncirus trifoliata*	Drought, cold	Increased drought and cold tolerance	Şahin-Çevik and Moore (2013)
CgWRKY1	2	*Citrus grandis*	Drought, cold	Increased drought and cold tolerance	Şahin-Çevik and Moore (2013)
GmWRKY6, 41	2	*Glycine max*	NaCl, dehydration	Overexpression under salinity stress	Zhou et al. (2008)
Hv-WRKY38	2	*Hordeum vulgare*	Cold and dehydration	Positive regulation of cold and drought stress	
BhWRKY1	2	*Boea hygrometrica*	Dehydration, ABA	Increased expression of *BhGolS1*/drought tolerance	
OsWRKY11	2	*Oryza sativa*	Drought, heat	Binding with HSP101 and increased drought tolerance	
GmWRKY13, 17, 27	2	*Glycine max*	Drought, salt	Early positive regulation of drought stress	Zhou et al. (2008)
OsWRKY08	2	*Oryza sativa*	NaCl, PEG, ABA	Upregulation of osmotic stress in transgenic *Arabidopsis*	

CaWRKY40	2-a	*Capsicum annum*	Heat	Increased resistance to hot environment	
AtWRKY8	2-c	*Arabidopsis thaliana*	NaCl	Positive regulation of salt stress	Hu et al. (2013)
DgWRKY1	2-c	*Dendranthema grandiflorum*	Salt, drought	Upregulation of salinity and drought stress	Liu et al. (2013)
AtWRKY39	2-e	*Arabidopsis thaliana*	Heat	Enhanced PR1 expression/heat tolerance	Li et al. (2010)
TcWRKY53	3	*Thlaspi caerulescens*	PEG, NaCl	Negative regulation of osmotic stress	
OsWRKY45	3	*Oryza sativa*	ABA, PEG, Dehydration	Overexpressed during dehydration	Qiu and Yu (2009)
AtWRKY63	3	*Arabidopsis thaliana*	ABA, drought	Increased drought tolerance	
GsWRKY20	3	*Glycine soja*	Drought, salt, ABA	Positive regulation of drought and salt stress	Luo et al. (2013)
BcWRKY46	3	*Brassica campestris*	Salt, cold, ABA	Increased salt and cold tolerance in transgenic tobacco	

and has subsequently increased in frequency and distribution throughout the plant kingdom (Ulker and Somssich 2004), playing roles in stress signalling and defence (Eulgem and Somssich 2007; Lai et al. 2008; Chavan and Kamble 2013; Chen et al. 2012a; Madrid et al. 2010). Involvement in plant biotic signalling has been extensively studied, however, much less is known about their involvement in abiotic stress tolerances. A summary of this knowledge, with a specific focus on each stress and application of knowledge to improving legume food species follows.

2.3.1 Drought

In *A. thaliana*, the AtWRKY33 TF interacts with the W BOX of the drought resistance gene *CesA8* to regulate its transcription (Wang et al. 2013). This was similar to expression of the rice OsWRKY45 TF in response to dehydration (Qiu and Yu 2009) and induction of PtrWRKY1 from *Poncirus trifoliata* and CgWRKY1 from *Citrus grandis* in response to drought (Şahin-Çevik and Moore 2013).

Of the soybean WRKY TFs, expression of GmWRKYs 13, 17 and 27 occurred in the early response to drought and those numbered 21, 41, 54 and 62 were induced under prolonged drought conditions (Zhou et al. 2008). Subsequently, in a wild soybean (*Glycine soja*), GsWRKY20 was highly induced under drought, salt and cold treatments in leaf and root tissues of transgenic *Arabidopsis* and showed increased drought tolerance by subsequent reduction in number of stomata and water loss (Luo et al. 2013). In *Medicago truncatula*, the genome-wide analyses of MtWRKY showed that 19 MtWRKYs belong to group 1 and 49 MtWRKYs were recognized in a bigger WRKY group 2. Only 12 MtWRKYs were identified in-group 3 (Song and Zhibiao 2014). The functional validation and possible roles in defence activation of MtWRKYs has not yet been achieved.

2.4 Salinity

In *A. thaliana*, AtWRKY25 and AtWRKY33 were upregulated quickly in leaf and root tissues (Jiang and Deyholos 2009) and AtWRKY8 was highly expressed in root tissues under salinity (Hu et al. 2013). Similarly, the transient high expression of soybean GmWRKY6, 13, 17, 27 and 41 was reported under salinity stress (Zhou et al. 2008). It was recorded that soybean WRKYs increased salt tolerance through regulating the salinity stress-related transcriptional factors DREB2A and STZ/Zat10 genes (Zhou et al. 2008).

2.4.1 Temperature

In *A. thaliana*, the viability of mature pollen increased with the induction of AtWRKY34 after cold treatment and pollen sensitivity towards cold stress was reduced after overexpression of AtWRKY34 in wild-type lines (Zou et al. 2010).

Meanwhile, AtWRKY25 was highly expressed under continuous high temperature stress (Li et al. 2009), and using mutant studies Li et al. (2010) showed that AtWRKY39 expression under thermal stress enhanced expression of heat responsive and the salicylic acid-inducible pathogenic-related protein PR1 gene for improved heat tolerance. Additionally, highlighting the very unique functional nature of individual WRKY family members, expression of AtWRKY26 increased and AtWRKY33 decreased under thermal stress (Li et al. 2011). Meanwhile, in the food legumes, the soybean GmWRKY1, 5, 15, 21, 30, GmWRKY43, GmWRKY48 and GmWRKY62 were upregulated under low temperatures (Zhou et al. 2008).

The functional biology and temperature activation of *Arabidopsis* WRKY TF (AtWRKY25, AtWRKY26 and AtWRKY33) indicated that in temperature stress, heat stress-related genes like Hsps, MBF1c and Zat10 are activated which further increased heat tolerance of the plant (Li et al. 2011). The AtWRKY25, 26 and 33 all belong to group 1 of the WRKY family (Eulgem et al. 2000; Zhang and Wang 2005).

2.5 Progress Towards Establishing Tolerance-Related Function of Candidate Transcription Factors

A diversity of functional genomics approaches have been employed to better characterize and validate the actions of TFs involved in particular abiotic stress tolerance responses. In *Arabidopsis*, the WRKY25 and 33 genes were found via microarray analysis to aid in NaCl tolerance, the function of these genes and an upstream-inducible region were validated through transgenic overexpression and found to be stimulated with ABA (Jiang and Deyholos 2009). In the legume model species *Medicago truncatula*, the functional response levels of over 1,000 TF transcripts were established using a 384-well plate qRT-PCR pipeline to produce a useful resource that has subsequently been used to study the function of TFs under several abiotic stress stimuli (Kakar et al. 2008). More recently, DeepSuperSAGE followed by RT-qPCR was used to identify and validate the functional responses of several transcription signals involved in the early perception of water deprivation in dehydrated soybean roots, including members of the WRKY and NAC families (Ribamar et al. 2013). In chickpea, TFs that lie within microsatellite loci have been used to genome map with, to determine those that may be functionally important (Kujur et al. 2013). This is a smart approach to hone in on a subset of TFs directly related to the functional control of a particular trait of interest, including abiotic stress tolerance in crop plants.

2.6 Concluding Remarks

Apart from a limited number of studies and mostly in the model legume species, to date there is very little knowledge of the functional role of TFs in abiotic stress responses and regulation in food legumes. Much investigation, using forward and reverse genetics techniques is required to address specific research questions to

uncover the key transcriptional drivers and their regulators. Comprehensive program aimed at the elucidation of the genetic controls governing abiotic stress tolerances and the subsequent incorporation of these genes and mechanisms through transgenics into elite breeding lines and subsequently cultivars are beginning to emerge, for example, the chickpea program at ICRISAT. This includes research towards developing cultivars tolerant to drought, salinity and low temperatures. Genes already under investigation for transfer include those encoding for enzymes required for the biosynthesis of osmoprotectants, modifying membrane lipids, LEA proteins and detoxification enzymes, microRNAs (Mantri et al. 2013). However, more programs are predicted for the near future to address other mandate legume crops and to uncover stress-inducible transcription factors that may be used for tolerance induction and selection following functional validation.

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Chapter 3
Insights into the Small RNA-Mediated Networks in Response to Abiotic Stress in Plants

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Abstract Under natural conditions, plants are constantly exposed to various environmental stresses such as drought, extreme temperature, salt, UV, mechanical, or nutrient starvation. To cope with these adverse conditions, plants have evolved cascade of molecular networks to perceive and transduce the stress signals, resulting into the reprogramming of gene expression. The stress-regulated reprogramming of gene expression at post-transcriptional regulation has been emphasized with the discovery of small regulatory RNAs. Plant small RNAs represent non-coding RNAs in the size range of 20–24 nucleotides and categorized into hairpin RNAs (hpRNAs) and siRNAs. The first category includes miRNAs, lmiRNAs, and nat-miRNAs while the siRNA group includes hc-siRNA, secondary siRNAs and nat-siRNAs. Studies have shown that small RNAs, especially miRNAs, are dynamically regulated by a variety of abiotic stress conditions. Such sRNAs target a variety of downstream targets including regulatory proteins as well as metabolic enzymes and thus play pivotal role in the regulation of plant abiotic stress response. Stress appears to regulate miRNA biogenesis as well as its activity. Several miRNA gene:target pairs respond to multiple stress conditions and are conserved in various plant species indicating that miRNAs may define pivotal regulatory nodes involved in the regulation of the plant stress response. On the other hand, miRNAs also show variety-/cultivar-specific stress response indicating that they themselves are under a very dynamic regulation. The world of small RNAs is gradually unfolding and much remains to be explored, nevertheless, it has been conclusively demonstrated that small RNAs define a new dimension in the molecular regulatory network regulating the plant stress response.

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3.1 Introduction

Plants are constantly challenged by a plethora of stress conditions due to their sessile nature. In order to survive unfavorable conditions, plants have evolved a complicated stress tolerance mechanism. Unfavorable fluctuations in global environment result in significant loss in crop productivity. Thus, understanding of the molecular basis of stress tolerance is critical for the management of crop productivity. Plants have evolved a complicated and robust stress mechanism, which involves interplay of multiple genetic elements. Small RNAs have emerged as one of the most important regulators of global gene regulatory networks. The past few years have witnessed large number of research studies implicating small RNAs in the transcriptional, post-transcriptional, and translational regulation of gene expression in response to abiotic stresses such as salinity, drought, temperature fluctuations, and nutrient starvation. The importance of small RNA-mediated regulatory modules in response to abiotic stress has been implicated by the evidence that mutants such as *hyl1*, *dcl1*, *hen1*, *sta1*, *sic1*, and *cpb80*/*abh1*, which are compromised in the small RNA biogenesis are extremely sensitive to abiotic stresses (Lu and Fedoroff 2000; Hugouvieux et al. 2001; Lee et al. 2006; Zhang et al. 2008a; Zhan et al. 2012). Subsequently, several sRNA especially miRNAs were found to be dynamically regulated under various abiotic stress conditions. Several stress-responsive small RNA regulatory subnetworks are conserved in different plant species suggesting their pivotal role in the evolutionarily conserved regulatory schemas. With the advent of high-throughput technologies, several studies have identified many stress-responsive small RNAs in diverse crop species under variety of stress regimens. Here, we review, with special emphasis on miRNA-mediated regulatory modules, the current understanding of the small RNAs pertaining to their classification, biogenesis, mode of action, and molecular networks involved in regulation of abiotic stress response.

3.2 Small RNA Classification, Biogenesis, and Mode of Action

The sources of regulatory non-coding RNAs in plants could be either endogenous (transcriptome) or exogenous (as induced by viruses or introduced dsRNAs). All small RNAs can be primarily classified into two main groups, as hpRNAs (hairpin RNAs)

for those originating from single hairpin structures and siRNAs (small interfering RNAs) for those derived from dsRNA precursors. Classification is based on the differences in their biogenesis and/or functions (Axtell 2013). hpRNAs are broadly classified into miRNAs and non-miRNAs depending on whether they originate from a precisely processed hairpin resulting in a single/few functional small RNAs or from an imprecisely processed hairpin that do not follow the criteria for miRNA annotation. miRNAs can be lineage-specific miRNAs (found only in one species or closely related species), natural *cis*-antisense transcripts (nat-miRNA), and long miRNAs (lmiRNA). Nat-miRNAs and lmiRNAs are so far reported only from grasses.

Majority of MIR genes are transcribed as an independent transcriptional unit by RNA Pol-II, which is recruited to the promoters of MIR genes by the mediator complex (Kim et al. 2011). Several transcription factors have also been identified, which regulate the expression of miRNA genes (Yamasaki et al. 2009; Bari et al. 2006; Zhang et al. 2011a; Yant et al. 2010). Moreover, Cell Division Cycle 5 (CDC5) and At-Negative on TATA less2 (NOT2) were also recently identified as the positive regulators of MIR transcription (Zhang et al. 2013; Wang et al. 2013a). The resulting pri-miRNA transcripts are stabilized by the addition of 5′cap and 3′ polyA tail similar to the mRNA transcripts (Xie et al. 2005; Jones-Rhoades and Bartel 2004). The processing of pri-miRNA to mature miRNA requires several steps catalyzed by multiple proteins. First, the FHA domain containing protein, DAWDLE (DDL) ensures stabilization and proper access of pri-miRNA by DCL1 (Yu et al. 2008). Similar to the pre-mRNA, pri-miRNAs are also bound by CAP-BINDING PROTEINS (CBP20 and CBP80) to ensure correct splicing, a process involving SERRATE (SE) and spliceosome (Raczynska et al. 2010; Wang et al. 2013a; Laubinger et al. 2008). More recently, Raczynska et al. (2014) showed that both CBP20 and CPB80 interact with SE and are involved in the preferential selection of 5′ splice site of first intron similar to cap-binding complex. One more protein identified to function in pri-miRNA splicing and modulation of DCL1 transcript levels is STA1 (STABILIZED1) (Chaabane Ben et al. 2012). Splicing and processing are interdependent processes and it's not clear whether they occur simultaneously or sequentially (Bielewicz et al. 2013; Rogers and Chen 2012). The pri-miRNA stem-loops are then acted upon by the miRNA-processing complex consisting of Dicer Like RNase III endonucleases (DCLs) (Margis et al. 2006) along with other proteins such as TOUGH (TGH) (Ren et al. 2012), SE (Grigg et al. 2005; Yang et al. 2006), HYPONASTIC LEAVES 1 (HYL1/DRB1) (Han et al. 2004; Vazquez et al. 2004), At-Negative on TATA less2 (NOT2) (Wang et al. 2013a), Cell Division Cycle 5 (CDC5) (Zhang et al. 2013), C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 (CPL1) (Manavella et al. 2012), and Cap-Binding Proteins (Laubinger et al. 2008) in the D-bodies (Fang and Spector 2007; Fujioka et al. 2007). Recently, Bologna et al. (2013) classified the miRNA processing into four mechanisms based on the direction and number of cuts required to release miRNA viz short base-to-loop, long base-to-loop, short loop-to-base, and long loop-to-base. As reported previously, a short base-to-loop pathway involves the detection of bulge followed by ~15 nt lower stem (Mateos et al. 2010; Song et al. 2010; Werner et al. 2010; Bologna et al. 2013). In second scenario, long base-to-loop, in addition to the first cut as in short base-to-loop, three cuts are required to release the miRNA, which leads to the

generation of low accumulating additional small RNAs. The precursor following the third pathway, short loop-to-base, has a 42 nt terminal region and a small loop. Here, the upper stem segment controls the processing and two cuts are required to release the miRNA. Finally in the last pathway, long loop-to-base, four sequential DCL1 cuts are required for processing (Addo-Quaye et al. 2009b; Bologna et al. 2009, 2013). Majority of miRNAs are processed by the catalytic action of DCL1 (Park et al. 2002; Reinhart et al. 2002). dsRNA-binding domain (DRB) protein, HYL1 and zinc-finger protein, SE are involved in the regulation of accurate processing of pri-miRNA by DCL1 (Kurihara et al. 2006; Dong et al. 2008; Manavella et al. 2012). While the other RNA-binding protein TGH binds to the pri- and pre-miRNA and contributes to the pri-miRNA–HYL1 interaction to enhance the DCL1 activity and not contribute to the precision of processing (Ren et al. 2012). HYL1 is further under the phospho-regulation by a C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 (CPL1) protein, which functions in the maintenance of hypo-phosphorylated state of HYL1 through interaction with SE (Manavella et al. 2012). In addition to the MIR transcription regulation, CDC5 is also required for efficient pri-miRNA processing through interaction with SE and DCL1 (Zhang et al. 2013). Similarly, NOT2 also interacts with SE, DCL1, and CBPs in addition to its interaction with PolII, suggest that it acts as a scaffold to connect MIR transcription and processing through efficient recruitment of DCL1 (Wang et al. 2013a). miRNA/miRNA* duplexes are methylated at 3′ nucleotides by methyltransferase, HUA ENHANCER1 (HEN1) (Yu et al. 2005; Abe et al. 2010). HEN1 is localized both in nucleus and cytoplasm and the exact site of methylation is not clear (Fang and Spector 2007). HASTY is required for the export of miRNA to the cytoplasm, although the nucleo-cytoplasmic ratio of miRNAs is not affected in the *hst* mutants (Park et al. 2005).

The mature miRNA strand is then incorporated into the AGO protein to form RISC. Most of miRNAs are bound by AGO1, and its activity is dependent on several effector proteins viz Heat Shock Protein 90 (HSP90) (Iki et al. 2010), SQUINT (SQN) (Iki et al. 2012), F-Box protein FBW2 (Earley et al. 2010), SUPERSENSITIVE TO ABA AND DROUGHT 2 (SAD2), and GW repeat containing protein SUO (Yang et al. 2012). miRNAs regulate their target protein by mRNA cleavage, translational repression, and DNA methylation. AGO1 protein has the slicer activity with which it cleaves the complementary mRNAs bound by miRNAs. Besides AGO1, other AGOs (AGO1, AGO2, AGO4, AGO7, and AGO10) have also been shown to possess the slicer activity (Mi et al. 2008, Montgomery et al. 2008, Takeda et al. 2008, Ji et al. 2011; Maunoury and Vaucheret 2011; Zhu et al. 2011). Degradome-sequencing analysis from diverse plant species indicated that mRNA cleavage is the chief mode of action used predominantly by large population of plant miRNAs (Zhou et al. 2010a; Wu et al. 2009; Addo-quaye et al. 2008). Besides cleavage, several reports demonstrated translational inhibition by miRNAs in plants as the alternative mode of action (Aukerman and Sakai 2003; Chen 2004; Brodersen et al. 2008; Beauclair et al. 2010). All these reports observed the decrease in the protein levels of miRNA target without affecting the transcript levels suggesting the interference at mRNA translation level. Further, characterization and identification of mutants defective in the miRNA-mediated repression at protein level further suggested the occurrence of this mechanism in

plants (Brodersen et al. 2008; Yang et al. 2012). Recent study indicated the involvement of an integral membrane protein AMP1 for the miRNA-mediated translational inhibition and AMP1 together with AGO1 are localized to endoplasmic reticulum (Li et al. 2013a). Further, the 24 nt lmiRNAs are associated with AGO4 and are involved in the cytosine methylation of MIR genes as well as their target loci (Wu et al. 2010).

Nat-miRNAs are conserved among monocots and derived from natural antisense strand of target genes. The precursors encoding nat-miRNAs usually contain large introns. Similar to the canonical miRNAs, they are processed by DCL1 and cleave their target in the middle of their complementary site (Lu et al. 2008b). Another class of 24 nt miRNAs named as long-miRNA (lmiRNA) are processed as a variant of 21 nt miRNA following base-to-loop pathway (Wu et al. 2010). In addition to DCL1, their processing also requires the activity of DCL3. They are loaded onto the AGO4 to methylate loci from which they were generated as well as their target genes in *trans* (Wu et al. 2010).

siRNAs, on the other hand, are classified into three main subgroups as heterochromatic siRNAs, secondary siRNAs, and NAT-siRNAs. Heterochromatic siRNAs (23–24 nt) mainly originates from intergenic and/or repetitive regions and mainly involved in de novo deposition of repressive chromatin marks. Biogenesis and function of these siRNAs require RDR2, DCL3, AGO4, and the plant-specific nuclear RNA polymerase IV. Secondary siRNAs are the ones whose ds precursor synthesis depends on an upstream small RNA trigger followed by subsequent RDR activity. A secondary siRNA is referred to as phased siRNA when the siRNA loci have a uniformly defined terminus, resulting in the production of a set of siRNAs. It would be called a *trans*-acting siRNA (tasiRNAs) when the secondary siRNA has more than one or more targets distinct from their locus. The biogenesis of tasiRNAs exploits both miRNA and 21-nucleotide siRNA biogenesis pathways. It requires all the factors necessary for miRNA and 21-nucleotide siRNA production. In *Arabidopsis*, three specialized miRNAs (miR173, miR390, and miR828) cleave the transcript from a *trans*-acting siRNA (*TAS*) gene, followed by converting either the 3′-cleaved (in the case of miR828 and miR173) or the 5′-cleaved transcript fragments (in the case of miR390) into double-stranded RNAs by RDR6. This dsRNAs are subsequently diced into phased 21-nucleotide siRNAs by DCL4 to generate multiple but distinct tasiRNA species. The tasiRNAs thus produced can further guide sequence-specific cleavage of their target gene transcripts through the RISC. To date, only four *TAS* gene families have been identified in *Arabidopsis*, and their biogenesis has been extensively characterized (Allen and Howell 2010). Recent study in apple identified two *TAS* gene families conserved with similar but unique tasiRNA generation profiles and target specificities (Xia et al. 2012a). miR159, miR828, and miR858 could collectively target up to 81 *MYB* genes potentially involved in diverse aspects of plant growth and development. Ten MYBs targeted by miR828 undergo siRNA biogenesis at the 3′ cleaved fragment generating over 100 sequence-distinct siRNAs that potentially target over 70 diverse genes (Xia et al. 2012a).

NAT-siRNAs (Natural antisense siRNAs) ds precursor is formed by hybridization of complementary RNAs, which could be generated from either *cis* or *trans* locus. The biogenesis of the primary NAT-siRNAs appears to be either DCL1- or DCL2 dependent. NAT-siRNAs whose precursors were transcribed from overlapping genes in opposite directions are the *cis*-NAT-siRNAs and those whose precursors are transcribed

from non-overlapping but complementary genes constitute the *trans-NAT-siRNAs*. Another less studied class called rasiRNAs (repeat-associated siRNAs) is a longer group of siRNAs with a length of 24–27 nucleotides. Endogenous plant siRNA species corresponding to three different retroelements, namely Tnt1 and TS SINE elements (*Nicotiana*) and AtSN1 (*Arabidopsis*) have been reported by Hamilton et al. (2002).

3.2.1 Identification and Expression Analysis

Expression of small RNAs can be assessed through various methods like northern blotting, microarrays, NGS (Next-Generation Sequencing), and real-time PCR. Because of the high-throughput nature of NGS, millions of small RNAs are sequenced per run at a time and thus generate a genome-wide quantitative expression profile of small RNAs in a given biological sample. It also serves as a useful tool for novel small RNA identification, since prior knowledge of sequence is not required. Deep sequencing-based studies of small RNA population have been widely adopted as an approach for identification of novel microRNAs in different plant species (Sunkar et al. 2008; Zhao et al. 2010; Joshi et al. 2010, Yang et al. 2011; Barrera-Figueroa et al. 2012; Gébelin et al. 2012, Peng et al. 2013). NGS method of small RNA library sequencing gives the advantage of comparing small RNAs (known or novel) abundance across different libraries with normalized tag value. Plant small RNA population is complex in comparison to that of animals as it has predominant class of siRNAs that poses a challenge in identification of low abundance miRNAs. However, it is still difficult to assess the expression of specific miRNA because of its short length, the presence of very similar sequences among family members and the presence of its precursor intermediates. Very recently, a genome-wide approach called SPARE (Specific Parallel Amplification of 5′ RNA Ends) has been developed to detect miRNA-processing intermediates. In this method, reverse transcription with a mixture of miRNA precursors-specific primers is performed followed by amplification to generate a library, which is subjected to deep sequencing. This has been employed to identify processing intermediates for most of the *Arabidopsis* miRNAs and can be adapted for other plant systems as well (Schapire et al. 2013).

3.2.2 sRNA Target Identification

Different approaches have been proposed in the recent years for identification of miRNA target genes. Since the complementarity of miRNA to its target genes is not 100 % exact, a single miRNA may target multiple genes and a single gene can be targeted by more than one miRNA. Target prediction searches mRNAs with perfect/near perfect complementarity to mature miRNA sequence and thus guide experimentation for plausible target candidates. However, experimental validation is required in order to eliminate false positives. Therefore, accurate identification of target molecules with experimental confirmation is of utmost importance in

understanding miRNA functions. This validation of miRNA targets can be employed at single gene level or at genome-wide level. 5′ RLM-RACE has been extensively used to detect the in vivo miRNA-mediated cleavage of target RNAs, which results in a cleaved target with a 5′ end that aligns to the tenth nucleotide (generally) of the miRNA (German et al. 2008). However, due to its single gene specific, costly and time-consuming nature, this technique is replaced by high-throughput sequencing technologies. PARE (Parallel Analysis of RNA Ends) or GMUCT (Genome-wide Mapping of uncapped transcripts) or Degradome sequencing, as popularly known, is a modified 5′ RLM-RACE (RNA Ligase-Mediated Rapid Amplification of cDNA ends) (German et al. 2009). Due to slicer activity, miRNA-directed cleavage products possess a 5′ monophosphate rather than a 5′ cap thereby making the cleaved products ligation competent. PARE is applicable to those sequences having a 5′ monophosphate and 3′ poly-A tails. Extraction of RNA followed by isolation of 3′ polyadenylated RNA, a 5′ RNA adaptor with *Mme*I recognition site is ligated to the monophosphorylated products. The ligated products are reverse transcribed, amplified, and cleaved with *Mme*I, followed by gel selection, 3′ dsDNA adaptor ligation, and amplification. The amplified products are gel purified and subjected to sequencing (German et al. 2009). PARE data is analyzed by different command line packages, the most popular one being "CleaveLand" (Addo-Quaye et al. 2009a). Cleaveland pipeline is an implementation of "perl" scripts which is designed to process small RNA target predictions and degradome data to give a concise output depicting the sliced RNAs and the cleavage sites (Addo-Quaye et al. 2009a).

3.3 Role of Small RNA in Abiotic Stress Response

3.3.1 Nutrient Starvation

Normal growth and development of plants depends on the adequate uptake of macro- and micronutrients from soils. Decrease in the crop productivity due to the limiting nutrients in the soil is a ubiquitous problem all around the world. Out of 17 essential elements, nitrogen (N), phosphorus (P), and potassium (K) are considered as the most important inputs for proper crop production. Plants are well equipped with the nutrient uptake, assimilation, and distribution mechanisms that play crucial roles under deficient, sufficient, and excessive conditions of nutrient availability. Recent work on this aspect highlighted the critical roles of small RNAs in the regulation of nitrogen, phosphorus, copper, and sulfur homeostasis from diverse plant species (Fig. 3.1).

3.3.1.1 Nitrogen Homeostasis

Nitrogen holds a very important status among the minerals, as it is required for energy transfer and as building blocks of nucleic acids, proteins, vitamins, etc. Plants absorb nitrogen mainly as nitrate ions from the soil. The first link of miRNA and *Arabidopsis* root nitrogen response was shown by Gifford et al. (2008), while

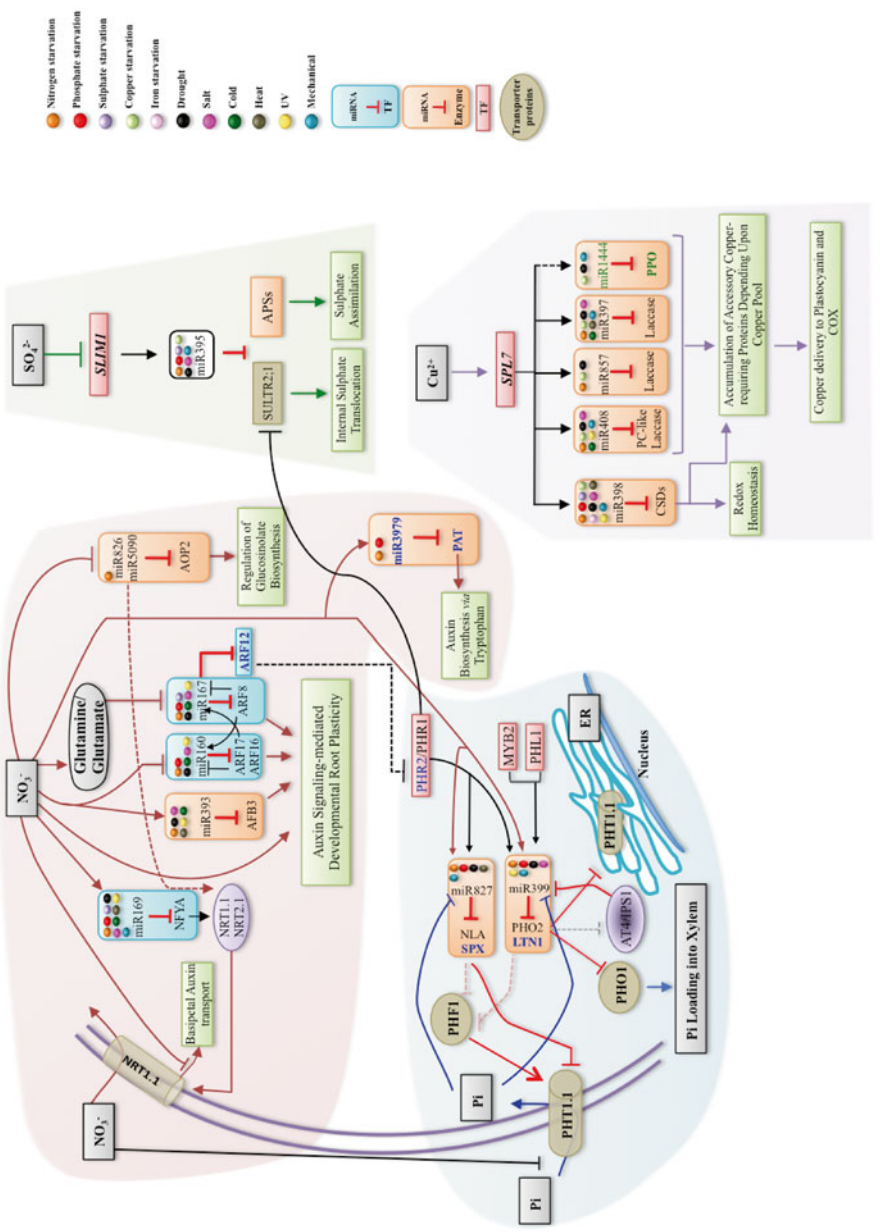
Nitrogen starvation
Phosphate starvation
Sulphate starvation
Copper starvation
Iron starvation
Drought
Salt
Cold
Heat
UV
Mechanical
miRNA
TF
miRNA
Enzyme
TF
Transporter proteins
SO_4^{2-}
SLIM1
miR395
APSs
SULTR2;1
Sulphate Assimilation
Internal Sulphate Translocation
Cu^{2+}
SPL7
miR1444
PPO
miR397
Laccase
miR857
Laccase
miR408
PC-like Laccase
miR398
CSDs
Redox Homeostasis
Accumulation of Accessory Copper-requiring Proteins Depending Upon Copper Pool
Copper delivery to Plastocyanin and COX
NO_3^-
Glutamine/ Glutamate
miR826
miR5090
AOP2
Regulation of Glucosinolate Biosynthesis
miR3979
PAT
Auxin Biosynthesis via Tryptophan
miR167
ARF8
ARF12
miR160
ARF17
ARF16
miR393
AFB3
Auxin Signaling-mediated Developmental Root Plasticity
PHR2/PHR1
MYB2
PHL1
ER
Nucleus
miR169
NFYA
NRT1.1
NRT2.1
Basipetal Auxin transport
NRT1.1
NO_3^-
Pi
PHF1
miR827
NLA
SPX
miR399
PHO2
LTN1
PHT1.1
AT4/IPS1
PHO1
Pi Loading into Xylem
PHT1.1
Pi

Fig. 3.1 miRNA-mediated regulatory circuit for nutrient homeostasis and their cross talk: Nitrate homeostasis is regulated by miRNA node at three points. First, miRNA169/NFYA module, depending upon nitrate pool, is involved in the transcriptional regulation of root nitrate transporters (NRT1.1 and NRT2.1), which further maintain the nitrate levels and basipetal auxin transport, then the other module with miR826/miR5090 and their common target AOP2 is involved in the regulation of consumption of nitrogen into glucosinolates and also regulate the NRTs. The third strategy deals with the nitrate-responsive auxin-mediated root plasticity wherein miR393/AFB3, miR167/ARF8, and miR160/ARF17-ARF16 are involved in the regulation of lateral root growth and root density in response to N availability. Further, nitrate antagonistically regulates the phosphate homeostasis since nitrate starvation downregulates miRNA399 and miR827, which otherwise are induced by phosphate starvation. Under phosphate starvation, the upregulation of miR399 and miR827 decreases the levels of AtPHO2 and NLA, thereby increasing their downstream targets PHT1.1, PHO1, and PHF1 to regulate the phosphate uptake and loading. Also the above two modules are regulated at the transcriptional level by ARF12 regulated PHR2 further suggesting the cross talk of nitrate and phosphate homeostasis. Sulfate homeostasis is regulated by miR395/SULTR2;1/APSs module, which is regulated by SLIM1. Further, PHR1 connects the phosphate and sulfate homeostasis by inhibiting SULTR2;1. Copper homeostasis involves SPL7 and its downstream regulated miR398/CSD, miR408/PC-like/laccase, miR857/miR397/laccase, and miR1444/PPO nodes involved in the regulation of copper containing proteins depending upon the available copper. These nutrient-responsive miRNA nodes are responsive to other abiotic stress conditions suggesting the cross talk of multiple stress response mechanisms. *Black*, *red*, *brown*, *blue*, *green*, and *purple lines* represent the transcriptional, post-transcriptional, nitrate, phosphate, sulfate, and copper-regulated pathway. Blue and green font represents findings specific to rice and populus. The model is based on well-studied miRNA genes and does not include data of all nutrient-responsive miRNAs reported in NGS-/microarray-based analysis

studying different root cell-specific response to nitrogen. Nitrogen represses miR167a expression to allow its target, AUXIN RESPONSE FACTOR 8 (*ARF8*) transcripts to increase in the cell-specific manner in pericycle. The quantitative adjustment of *ARF8*/miR167a transcriptional circuit controls the lateral root architecture in response to nitrogen supply. Further, the nitrogen-mediated control of root plasticity via the above circuit is abolished by the treatment of methionine sulfoximine (MSX), inhibitor of nitrate assimilation, suggesting that the response was mediated by the nitrogen metabolites rather than nitrate itself. Another module miR393/*AFB3* also contributes to plant developmental plasticity in response to nitrogen limitation by controlling root system architecture (Vidal et al. 2010). AUXIN SIGNALING F-BOX (*AFB3*) is crucial for primary root growth and lateral root density in response to nitrate as *afb3* mutant and miR393 overexpressor lines lack the nitrate-induced root inhibition and lateral root formation. Interestingly, both *AFB3* and *ARF8* were induced in pericycle, which is the site for emergence of lateral roots and regulate auxin signaling. miR393 was induced by nitrate specifically in *Arabidopsis* roots after 2 h while AFB3 showed peak induction at 1 h and subsequently it starts decreasing at 2 h as miR393 levels rises and cleaves *AFB3* mRNA. Thus, there is a lag in the nitrate-induced expression of miR393 vis-à-vis AFB3 to the extent that nitrate induces an early increase in AFB3, which is, then suppressed by miR393, which comes in late. *AFB3* is induced by external nitrate while miR393 is induced in response to the nitrogen signals generated after the reduction of nitrate and assimilation, thereby forming an incoherent feed-forward circuit to regulate *AFB3* levels as per the external and internal nitrogen levels. Recently, in rice a root preferential miRNA miR3979, which targets anthranilate phosphoribosyltransferase, involved in tryptophan biosynthesis, was reported to be downregulated under nitrogen starvation (Jeong et al. 2011). This pathway is also involved in the auxin biosynthesis (Cohen et al. 2003), so the authors speculated that miR3979 repression may induce auxin production, which controls the roots developmental plasticity under starvation. As auxin signaling controls the primary root growth in a concentration-dependent manner (Evans et al. 1994), the above three circuits highlight the involvement of miRNAs in the regulation of the auxin-mediated regulation of root architecture in response to nitrogen (Fig. 3.1). Furthermore, phenotypic root plasticity is regulated by the complex regulation by miR160/ARF17 and miR167/ARF8 nodes in *Arabidopsis* (Gutierrez et al. 2009). ARF8 overexpression leads to reduced miR167 and increased miR160 levels while ARF17 overexpression leads to the increased miR167 and reduced miR160 levels (Gutierrez et al. 2009). Several genome-wide small RNA analysis studies under nitrogen starvation revealed many nitrogen-responsive miRNAs in rice (Nischal et al. 2012), *Arabidopsis* (Liang et al. 2012), maize (Zhao et al. 2012, 2013), and soybean (Wang et al. 2013b). Several miRNA that behave more or less in a similar manner under nitrogen starvation were identified. For example, miR169 was found to be significantly downregulated under N deficiency (Liang et al. 2012; Pant et al. 2009; Zhao et al. 2012). Moreover, MIR169a overexpression transgenic plants accumulate less nitrogen and are hypersensitive to nitrogen deficiency (Zhao et al. 2011). Upon nitrogen starvation, miR169 downregulation results in the upregulation of NFYA transcription factor,

which then regulate the expression of nitrate transporter genes, low affinity nitrate transporter (AtNRT1.1), and high affinity nitrate transporter (AtNRT2.1). AtNRT1.1 is crucial for repression of lateral root growth under nitrogen deficient conditions by basipetal transport of auxin out of roots (Krouk et al. 2010). Recently, a very interesting miRNA regulatory module was demonstrated in nitrogen starvation in *Arabidopsis* involving miR826 and miR5090 and their common target ALKENYL HYDROXALKYL PRODUCING 2 (*AOP2*) (He et al. 2013). Both miR826 and miR5090 are induced upon nitrogen starvation and decrease *AOP2* transcript. AOP2 is involved in the modification of the side chains of methionine-derived nitrogen-rich metabolites glucosinolates (Kliebenstein et al. 2001; Neal et al. 2010). miR826 and miR5090 overexpression phenocopies the *aop2* mutants, as in all three cases the reduction in the level of glucosinolates was observed (He et al. 2013). Also, the miRNA overexpression transgenic plants are tolerant to low nitrogen due to longer primary and more lateral roots due to higher expression of NRT2.1, *ARABIDOPSIS* NITRATE REGULATED 1 (ANR1), and decreasing AOP2. Decrease in AOP2 leads to the reduction in the glucosinolates, which decreases the consumption of nitrogen into glucosinolates and saving nitrogen for other nitrogen containing metabolites necessary for plant growth under starvation (He et al. 2013). In summary, nitrate homeostasis is regulated at several steps: nitrate transport via the regulation of NRTs, nitrogen consumption, and nitrate-mediated auxin signaling, which is involved in the root adaptation under varying nitrate pool (Fig. 3.1).

3.3.1.2 Phosphate Homeostasis

Phosphorus is one of the essential nutrients required for optimal plant growth and reproduction. It is involved in several plant processes including energy transfer, photosynthesis, signal transduction, regulation of protein activities, and function as fundamental component of cellular structures including nucleic acids, membranes, etc. Plant absorbs phosphorus in the form of orthophosphate (Pi), which is scarce in soil due to precipitation with cations or its conversion to organic compounds by the soil microflora (Marschner 1995; Vance et al. 2003). Plants developed several phosphate starvation responses to conserve phosphate levels including its remobilization, acquisition, and distribution (Raghothama 1999, Chiou and Lin 2011). Besides the transcriptional reprogramming under phosphate starvation, post-transcriptional regulation by small RNAs has emerged as a crucial module in past few decades. The regulation of P-homeostasis by small RNAs became evident with the discovery of miR399 induction in phosphate deficiency in *Arabidopsis* (Fujii et al. 2005). Under low phosphorus conditions, miR399 induction leads to the reduction in the level of its target transcript PHOSPHATE2 (PHO2) encoding an ubiquitin-conjugating E2 enzyme (UBC24) (Aung et al. 2006; Bari et al. 2006; Fujii et al. 2005). Similar miR399 regulation was recently identified in rice, where PHO2 ortholog LEAF TIP NECROSIS 1 (LTN1) is targeted by miR399 under phosphate starvation (Hu et al. 2011). Further, in *Arabidopsis* miR399 overexpressors accumulate more Pi in shoots with Pi toxicity symptom similar to the

loss-of-function mutants of UBC24 and *pho2* mutants (Fujii et al. 2005; Chiou et al. 2006; Aung et al. 2006; Bari et al. 2006). This accumulation was due to the increased root to shoot translocation and impaired remobilization of Pi within leaves (Aung et al. 2006). miR399 levels respond very rapidly depending upon the internal Pi levels, rises in deprived and decreases in replete conditions. Expression data indicates that PHO2 has another level of regulation besides miR399-mediated decay of its transcript. Two Pi starvation-induced genes AtIPS1 and AT4 are induced in both mutant background of PHO2 (*pho2*) and PHOSPHATE STARVATION RESPONSE 1 (*phr1*) suggesting that both *pho2* and *phr1* might be part of same signaling network (Rubio et al. 2001; Bari et al. 2006). miR399 induction was impaired in *phr1* mutant but not in *pho2*, which places PHR1 in upstream position to that of PHO2 and miR399. Further, miR399 acts as mobile signal to coordinate phosphate homeostasis between roots and shoots subsequent to "P" deprivation expression of miR399 starts first in shoots and then its movement to roots via phloem serves as a systemic signal to regulate phosphate homeostasis in the roots (Pant et al. 2008; Lin et al. 2008). Other phosphate limitation-responsive miRNAs, miR169, miR827, and miR2111 were also detected in the phloem sap of rape seed suggesting that the systemic movement of these small RNAs function in the regulation of phosphate homeostasis (Pant et al. 2009). Transcriptional activation of miR399 was regulated by MYB transcription factors, PHR1 (Phosphate starvation response 1), PHL1 (PHR1 like 1), and MYB2 (Bustos et al. 2010; Rubio et al. 2001; Baek et al. 2013). All miR399 genes present in *Arabidopsis* harbor PHR1-binding site P1BS in their promoters (Bari et al. 2006). Also several other P-responsive *cis*-regulatory elements were identified in the promoters of these phosphate-responsive miRNAs including PHO, PHO-like, W-box, NIT2, and MYB-binding sites (Zeng et al. 2010; Xu et al. 2013; Baek et al. 2013). Further findings also indicated that miR399-mediated regulation of PHO2 is under the control of target mimicry. The non-coding RNA IPS1 (INDUCED BY PHOSPHATE STARVATION 1) contains the binding site for miR399 but interrupted by mismatch loop at cleavage site enabling IPS1 to sequester miR399 (Franco-Zorrilla et al. 2007). Overexpression of IPS1 tends to accumulate miR399 target PHO2 and concomitantly reduces the shoot Pi content. Several reports suggest the conservation of this IPS1/miR399/PHO2 module (Branscheid et al. 2010; Liu et al. 2010; Hu et al. 2011; Huang et al. 2011; Valdés-López et al. 2008). PHO2, an E2 ubiquitin-conjugating enzyme, was recently shown to be involved in the degradation of PHO1 (PHOSPHATE1) (Liu et al. 2012a; Huang et al. 2013), which is implicated in Pi loading to the xylem, in the endomembrane to regulate phosphate homeostasis. Further in an iTRAQ-based quantitative membrane proteomics approach, Huang et al. (2013) identified PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1) and PHOSPHATE TRANSPORTER1.1 (PHT1.1) as the downstream targets of PHO2 in addition to PHO1.

Another well-explored miRNA-mediated regulation of phosphate homeostasis is through miR827, which is also specifically induced by phosphate starvation in several plant species (Kant et al. 2011; Secco et al. 2013; Lin et al. 2010, 2013). miR827 is involved in the regulation of nitrogen limitation adaptation (NLA) by

cleavage of its mRNA in phosphate starvation (Hsieh et al. 2009; Kant et al. 2011; Lin et al. 2013). Kant et al. (2011) demonstrated that in addition to the previously identified role of NLA in adaptive response to low nitrate conditions (Peng et al. 2007), this gene was also involved in phosphate homeostasis. They have identified two suppressors of *nla* mutants, PHF1 and PHT1.1, that are related to phosphate transport and able to restore the early senescence phenotype of *nla* due to Pi toxicity (Kant et al. 2011). Similar to *nla* mutant, the miR827 overexpression plants also displayed Pi toxicity phenotype. Moreover, *pho2* mutants showed nitrate concentration-dependent Pi toxicity phenotype, which suggests that PHO2 and NLA act in a similar pathway to regulate phosphate homeostasis (Kant et al. 2011). In summary, loss-of-function of both NLA and PHO2 leads to over accumulation of Pi under low nitrate and high phosphate conditions and Pi and nitrate have antagonistic interaction (Kant et al. 2011). Further support for this connection between PHO2 and NLA came from the fact that both NLA and PHO2 have common targets, i.e., PHT1.1 and PHF1 (Huang et al. 2013; Kant et al. 2011). While PHO2 localizes to ER and is involved in the degradation of PHT1.1 and PHO1 in the endomembranes (Liu et al. 2012a; Huang et al. 2013; Fig. 3.1), NLA directs the ubiquitination of PHT1s and regulates the protein abundance in the plasma membrane by endocytosis (Lin et al. 2013; Fig. 3.1). In rice also miR827 is highly induced under phosphate deficiency and target SPX-MFS1 and OsSPX-MFS2 involved in phosphate homeostasis and transport, instead of NLA (Lin et al. 2010). miR827/SPX-MFS module was found to be regulated by OsPHR2, ortholog of AtPHR1 (Wang et al. 2012a).

Expression analysis of PvPHO2 in phosphate deficiency tolerant (BAT477) and sensitive (DOR364) genotype of common bean revealed tolerant genotype-specific downregulation under phosphate starvation conditions (Ramírez et al. 2013). This difference was attributed to the less efficient cleavage of PvPHO2 mRNA by miR399 under phosphate starvation that leads to the increased PvPHO2-mediated degradation of proteins in sensitive genotype under phosphate limiting conditions. Interestingly, a new candidate involved in auxin signaling, ARF12 was recently identified as a novel regulator of phosphate homeostasis in rice (Wang et al. 2014). The transcript abundance of OsPHR2 and its downstream targets miR399j, OsPHO2, miR827, SPX-MFS1, and SPXMFS2 (Wang et al. 2014) are differentially regulated in *arf12* mutants. Further ARF12 is targeted by miR167 in rice (Liu et al. 2012c). Also miR167 family members are differentially regulated under low nitrogen conditions in rice (Nischal et al. 2012) suggesting the cross talk between nitrogen and phosphorus.

Besides the above well-studied miRNAs, several studies on phosphate starvation resulted into many candidate phosphate-responsive miRNAs in *Arabidopsis* (Hsieh et al. 2009), rice (Secco et al. 2013), soybean (Xu et al. 2013), and barley (Hackenberg et al. 2013). Overall data suggested that the antagonistic regulation of miR399/PHO2 and miR827/NLA in response to nitrate and phosphate levels derives the uptake and loading phosphate in plants and beside these two cross talks, ARF12-mediated regulation of phosphate starvation response highlights the involvement another nitrate-responsive miR167/ARF12 module in the plant nitrate-phosphate cross talk (Fig. 3.1).

3.3.1.3 Sulfate Homeostasis

During sulfate starvation, miR395 displayed strong induction in both *Arabidopsis* and rice (Jones-Rhoades and Bartel 2004; Jeong et al. 2011; Kawashima et al. 2009; Liang and Yu 2010; Hsieh et al. 2009; Jagadeeswaran et al. 2014) and expressed in the vascular system of roots and leaves (Kawashima et al. 2009). miR395 is involved in the regulation of LOW AFFINITY SULPHATE TRANSPORTER (SULTR2;1/AST68) and members of ATP SULPHURYLASES family (APS1, APS3, and APS4) (Kawashima et al. 2009; Liang and Yu 2010; Jagadeeswaran et al. 2014) and a known gene At2g28780 (Jagadeeswaran et al. 2014) is the validated targets through 5′ RLM RACE. Further, the sulfate deficiency-specific induction of miR395 is controlled by SLIM1 (SULPHUR LIMITATION 1) (Kawashima et al. 2009), the key regulator of sulfur homeostasis along with SULTR1;2 (Maruyama-Nakashita et al. 2006). Expression analysis confirmed the negative regulation of APS1 in seedlings (Jones-Rhoades and Bartel 2004) as well as APS4 in both root and shoot of *Arabidopsis* (Liang and Yu 2010) by miR395. On the other hand, SULTR2;1 shows anticorrelation of expression profiles upon sulfate starvation only in leaves (Takahashi et al. 2000) but not in roots, where both SULTR2;1 and miR395 are upregulated (Takahashi et al. 2000; Kawashima et al. 2009; Liang and Yu 2010). This relation was further confirmed by the overexpression of miR395 in *Arabidopsis* wherein except for SULTR2;1 in roots all target transcripts (APS1, APS3, and APS4) were decreased in roots and shoots (Liang and Yu 2010). This conflicting expression patterns of miR395-SULTR2;1 pair might be due to their spacial partition as miR395 is expressed in phloem companion cells (Kawashima et al. 2009) in contrast to xylem parenchymal localization of SULTR2;1 (Takahashi et al. 2000). Similar regulatory module is also reported in rice (Jeong et al. 2011) wherein miR395 overexpressor plants displayed sulfate deficiency symptoms with increased sulfate accumulation in shoots and low levels in roots (Liang and Yu 2010). Similar accumulation was observed for APS1 knockdown and APS4-RNAi plants and remobilization of sulfate was impaired in *sultr2;1* mutants similar to miR395 overexpression (Liang and Yu 2010). *Aps1-1 sultr2;1* APS4-RNAi triple mutant phenocopied miR395 overexpressors (Liang and Yu 2010). Both miR395 and SLIM function in the fine-tuning of the APS1 expression under sulfate starvation (Kawashima et al. 2011). Recent work indicates the inverse correlation between miR395 and its targets transcript level in rosette and cauline leaves even under sulfur-sufficient conditions (Jagadeeswaran et al. 2014). Constitutive expression of miR395 in *fou8* and *sultr1;2* mutants that are defective in sulphur accumulation suggests that internal sulfur levels drive the induction of miR395 (Matthewman et al. 2012). miR395 levels induced with the treatments of metabolites cysteine and O-acetylserine that accumulate during sulfate deficiency, while treatment with the inhibitor of glutathione synthesis results in decreased miR395 levels (Matthewman et al. 2012). Further, the miR395 levels correlate well with the glutathione levels under sulfate-sufficient conditions (Matthewman et al. 2012). Even under sulfate starvation, addition of glutathione (GSH) reduces the levels of

miR395 and its induction also with other heavy metals suggests the involvement of miR395 in redox signaling (Jagadeeswaran et al. 2014). Also mutants defective in glutaredoxin-dependent redox signaling, *cad2,* and mutants with defective thioredoxin-dependent redox signaling, *ntra/ntrb* have compromised induction of miR395 during sulfur starvation (Jagadeeswaran et al. 2014). Also the sulfate transporter genes, SULTR1;3, SULTR2;1, and SULTR3;4, are regulated by PHR1 in *Arabidopsis* (Rouached et al. 2011) suggesting the cross talk between phosphate and sulfate homeostasis (Fig. 3.1). PHR1 positively affect the expression of SULTR1;3 under phosphate starvation conditions, which is evident from the decreased root to shoot sulfate transport in the *phr1* mutant background (Rouached et al. 2011). Surprisingly PHR1 represses the expression of SULTR2;1 and SULTR3;4 in shoots only as evident from the increased expression of the above genes in *phr1* mutant background under phosphate starvation. So PHR1 is involved in the activation as well as repression of sulfate transporter genes (Rouached et al. 2011). Further, several sulfate-responsive miRNAs were also identified from Brassica (Huang et al. 2010).

3.3.1.4 Copper Homeostasis

Copper has very crucial role in plant growth and development by serving as an essential cofactor of many proteins involved in photosynthesis, electron transport, ethylene perception, ROS detoxification, and cell wall metabolism (Burkhead et al. 2009, Marschner 1995). As both its limitation and excess are harmful for survival, plants have adapted various strategies to maintain copper homeostasis (Burkhead et al. 2009). In plants, copper is primarily required for the proper functioning of plastocyanin, cytochrome c oxidase, Cu/Zn superoxide dismutases, ethylene receptors, laccases, ascorbate oxidase, amine oxidase, plantacyanins, and polyphenol oxidase (Burkhead et al. 2009). MicroRNAs have been shown to regulate copper homeostasis through cleavage of nonessential copper requiring proteins to save copper for the most important and abundant protein plastocyanin, which is required for plant autotrophic growth (Yamasaki et al. 2007). In *Arabidopsis*, several miRNAs are induced under copper limiting condition including miR398, miR408, miR857, and miR397 (Abdel-Ghany and Pilon 2008; Yamasaki et al. 2007). miR398 targets the Cu/Zn SODs (CSD1 and CSD2) and a subunit of mitochondrial cytochrome oxidase, COX5b-1 in *Arabidopsis* (Yamasaki et al. 2007, Sunkar et al. 2006, Beauclair et al. 2010). Three Cu/Zn SODs of *Arabidopsis* are partitioned in different compartments, CSD1 in cytosol, CSD2 in chloroplast, and CSD3 in peroxisomes (Kliebenstein et al. 1998). Under copper limiting conditions, while miR398 downregulates CSD1, CSD2, and COX5b, miR408, miR857, and miR397 downregulate the laccase family members and plantacyanin with no apparent change in the abundance of plastocyanin (Abdel-Ghany and Pilon 2008). Further, SPL7 (SQUAMOSA promoter-binding protein-like 7) act as a master switch to regulate copper homeostasis by regulating miR398, miR408, miR397, and miR857 expression by binding to the GTAC motifs in the promoters under copper limiting

conditions (Yamasaki et al. 2009). The reduced levels of many genes involved in copper homeostasis, COPT1, COPT2, ZIP2, FRO3, and CCH in *spl7* mutant suggest that SPL7 is the central regulator of copper homeostasis in *Arabidopsis* (Yamasaki et al. 2009). The miR398-mediated downregulation of CSD2 is compensated by the concomitant increase in Fe-SOD under limiting Cu as reported in *Arabidopsis*, rice, Brassica, tomato, and maize (Cohu and Pilon 2007). Interestingly, Fe deficiency downregulates miR398a, b, c, and miR397, thereby accumulates their target (Waters et al. 2012), indicating the cross talk of Fe and Cu homeostasis. Moreover during Fe deficiency, there is Cu accumulation in the rosette leaves. To test this cofactor economy model in populus, recent findings in populus revealed the expected upregulation of miR398, miR397, and miR408 and miR1444 resulted in the decreased transcript levels of Cu/Zn SODs, laccases, and polyphenol oxidases in response to copper limitation (Lu et al. 2011; Ravet et al. 2011). Surprisingly, they also reported decreased plastocyanin levels and stable COXII levels, which leads to Cu economy model where under low copper, preference is first for mitochondrial electron transport and second for plastocyanin of chloroplast electron transport activities (Ravet et al. 2011; Fig. 3.1). Further, the well-known upregulation of FSD1 in low copper was also not observed in populus that shows the variation in the plant species in response to particular condition.

3.3.2 Extreme Temperature Stress

Extreme temperature, either cold or heat affects the plant development and productivity. Appropriate temperature conditions are critical for the development of plant at different growth stages. For example, in rice, there is higher impact of cold stress at the germination and reproductive (anthesis) stages compared to the vegetative stage; similarly, high temperature adversely affects the reproductive (anthesis) and seed ripening stages, leading to grain loss (Nguyen 2004).

Earlier studies in *Arabidopsis* revealed the induced expression of miR319c primarily under cold stress while miR393, miR397b, and miR402 responded to cold as well as dehydration, NaCl, and ABA treatment (Sunkar and Zhu 2004). Cold stress-regulated miRNAs have been reported based on the analysis of microarray data in *Arabidopsis* (Zhou et al. 2008; Liu et al. 2008) and *Populus* (Lu et al. 2008b) as well as deep sequencing analysis of small RNA libraries in *Brachypodium* (Zhang et al. 2009a). Cold stress-induced miRNAs in *Arabidopsis* includes miR165/166, miR393, miR396, and miR408 (Sunkar and Zhu 2004, Liu et al. 2008). In *Populus*, miR168a, miR168b, miR477a, and miR477b were upregulated while miR156g–j, miR475a,b, and miR476a were downregulated under cold stress. In *Brachypodium*, the expression of miR169e and miR172b showed more than fivefold increase in the cold-treated small RNA library as compared to control. miR172a (about threefold) was also induced, though not as significant as miR172b (Zhang et al. 2009a). Cold-responsive miRNAs in *Oryza sativa* include miR319, miR166, miR169, miR156, miR167, miR168, miR171 (Jian et al. 2010; Lv et al. 2010), miR1425, and miR812q

(Jeong et al. 2011). miR167 and miR319 were downregulated by cold stress in rice seedlings with the inverse correlation of its targets, i.e., TCP and MYB transcription factors, which gets up-regulated after cold treatment (Lv et al. 2010). Contrastingly, both miR167 and miR319 were reported to be up-regulated by cold stress in *Arabidopsis* (Sunkar and Zhu 2004; Zhou et al. 2008) indicating a possibility of species-specific expression in cold stress. Functional characterization of miR319 in rice further elaborated its role in cold stress tolerance (Yang et al. 2013). In rice, it targets members of TEOSINTE BRANCHED CYCLOIDEA/PCF (TCP) family viz. PCF5, 6, 7, 8, and 21. Overexpression and concomitant decreased expression of its target transcripts of MIR319a and MIR319b resulted in plants with wider leaf blades and enhanced cold tolerance. Similarly, RNAi lines of miR319 targets, i.e., PCF5 and PCF8 phenocopies cold tolerance of miR319 overexpression (Yang et al. 2013). The cold inducible expression pattern of miR319 and decreased target transcript, PCF5, PCF6, and GAMyb was also observed in sugarcane (Thiebaut et al. 2012). miR1425 is a rice-specific miRNA (Lu et al. 2008a) that targets Rf-1 (Fertility restorer gene) and PPR (Pentatricopeptide repeat) protein known to increase cold tolerance in hybrid rice at the booting stage by increasing the number of potentially fertile pollen grains (Komori and Imaseki 2005). Rf-1 was up-regulated during cold stress when miR1425 is downregulated in rice panicle tissues, implying that miR1425 plays role in modulating the expression of Rf-1 under cold conditions (Jeong et al. 2011). PPR proteins are RNA-binding proteins having a role in RNA splicing, RNA editing, RNA stability, and translation in organelles such as mitochondria and chloroplasts (Delannoy et al. 2007; Schmitz-Linneweber and Small 2008). Another miRNA that is significantly upregulated by cold stress at the booting stage of rice plants is miR812q (Jeong et al. 2011). It is a 24-nt miRNA that originates from a stem-loop precursor of MITE Stowaway1 and has unique sequence among its family members.

Heat stress-responsive small RNAs have not been studied much as compared to other abiotic stresses so far. Few reports are there in *Arabidopsis*, *Panax*, *Triticum*, and *Brassica* (Guan et al. 2013, Wu et al. 2012, Yu et al. 2012). One well-explored miRNA responsive to heat stress is miR398, which is rapidly induced by heat stress in *Arabidopsis* while its target gene transcripts CSD1, CSD2, and CCS are reduced (Guan et al. 2013). Transgenic plants expressing modified coding sequence of CSD1, CSD2, and CCS resistant to miR398 are more sensitive to heat stress than those expressing normal coding sequences under the control of their native promoters (Guan et al. 2013; Fig. 3.2). Whereas the *csd1*, *csd2*, and *ccs* loss-of-function mutants are comparatively more tolerant to heat stress. Further HSFA1b and HSFA7b were found to derive the miR398 heat-induced expression (Guan et al. 2013). *Panax ginseng*, an economically important medicinal plant has miR6135e.2/j, miR6135i, miR6138, miR6140a, and miR6143b-3p responsive to heat stress only, whereas miR6136b, miR6135k, miR6139, miR6140d, and miR6141 were responsive to both dehydration and heat stresses (Wu et al. 2012). Heat stress-responsive miRNAs have been studied in wheat using NGS followed by cloning the small RNAs from heat stress treated wheat leaves. miR156, miR159, miR160, miR166, miR168, miR169, miR393, and miR827 were upregulated

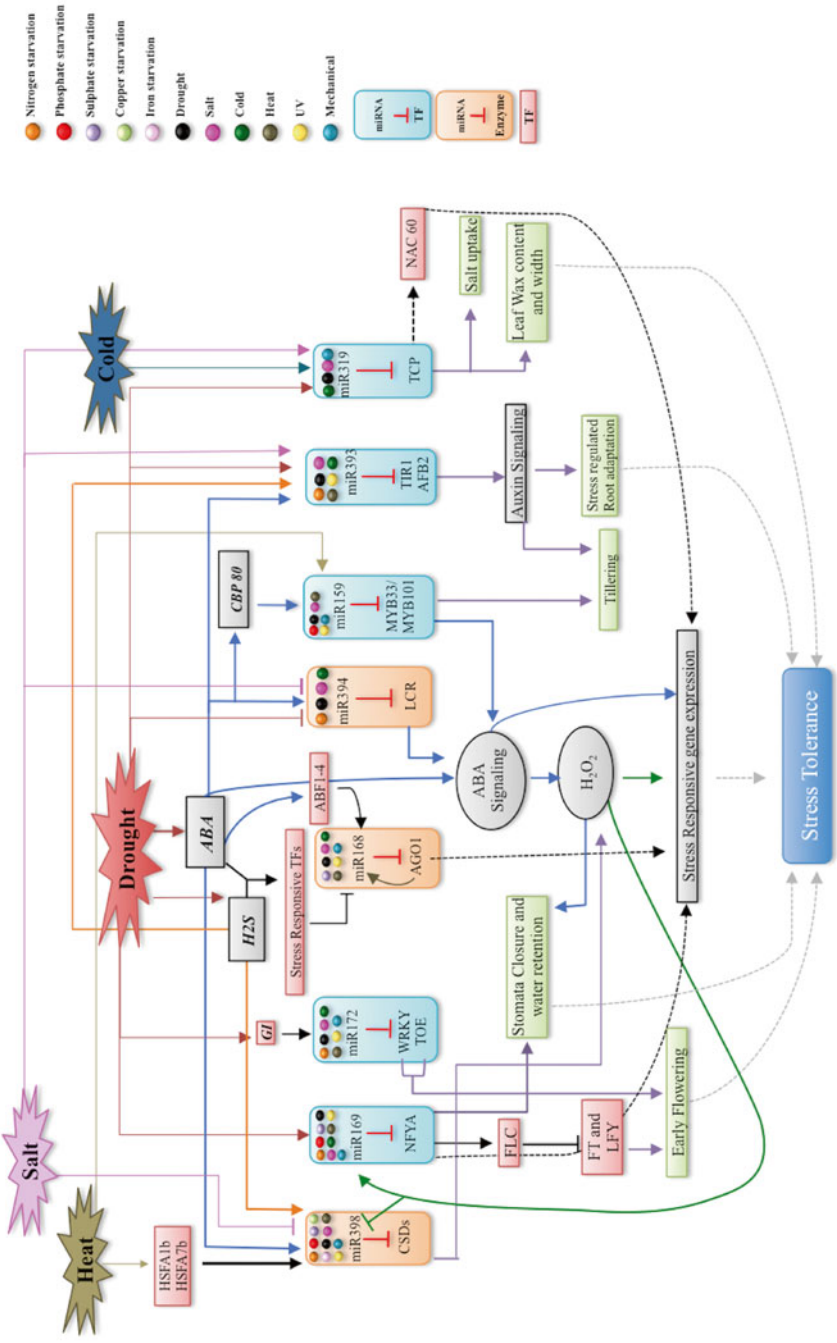
Nitrogen starvation
Phosphate starvation
Sulphate starvation
Copper starvation
Iron starvation
Drought
Salt
Cold
Heat
UV
Mechanical
miRNA
TF
miRNA
Enzyme
TF
Cold
Salt
Heat
Drought
ABA
H2S
CBP 80
GI
HSFA1b
HSFA7b
miR398
CSDs
miR169
NFYA
miR172
WRKY
TOE
miR168
AGO1
ABF1-4
Stress Responsive TFs
miR394
LCR
miR159
MYB33/
MYB101
miR393
TIR1
AFB2
miR319
TCP
NAC 60
Salt uptake
Leaf Wax content
and width
Auxin Signaling
Stress regulated
Root adaptation
Tillering
ABA
Signaling
H2O2
Stress Responsive gene expression
Stomata Closure and
water retention
FLC
FT and
LFY
Early Flowering
Stress Tolerance

Fig. 3.2 Model summarizing the miRNA-target nodes involved in drought, salt, and temperature-mediated stress signaling. miR398/CSD module is important for maintaining the redox homeostasis by post-transcriptionally regulating CSD genes. It is also reported to be differentially regulated in almost every abiotic stress suggesting the crucial role of redox balance in the overall stress tolerance mechanism. The miR394/LCR module also contributes to the redox pool in an ABA-dependent manner. Further, H_2O_2 is involved in the activation of several stress-responsive genes including miRNAs. The miR169/NFYA node regulates the stomatal closure and water retention. miRNA nodes miR169/NFYA, miR172/WRKY-TOE, and miR393/TIR1/AFB2 function in the stress mediated early flowering to cope with severe situation. Further, the differential regulation of miRNAs in response to ABA, H_2S, and H_2O_2 has also been highlighted wherever information was available. The model is based on well-studied miRNA genes and does not include data of all stress-responsive miRNAs reported in NGS-/microarray-based studies. *Solid* and *dotted black lines* represent the transcriptional regulation; *red lines* indicate the post-transcriptional regulation; *blue*, *orange*, and *green lines* represent ABA, H_2S, and H_2O_2-mediated signaling

under heat stress while miR172 was significantly decreased in heat-tolerant variety (TAM107) (Xin et al. 2010). While in sensitive variety (Chinese spring), miR159 was downregulated under heat stress suggesting that differential regulation of miR159/GAMYB module under heat stress in two varieties is crucial for heat tolerance in wheat (Xin et al. 2010) (Fig. 3.2). TamiR159 overexpression rice plants are more sensitive to heat stress (Wang et al. 2012b). Deep sequencing of small RNA libraries from heat-stressed *Brassica rapa* seedlings (46 °C, 1 h) showed that miR398a and miR398b were downregulated while miR156g and miR156h were heat induced. The study also identified novel miRNAs that were heat responsive (Yu et al. 2012). Recently, a very interesting example demonstrating the role of stress-induced alternative splicing in the regulation of miRNA expression further extended the current understanding of small RNA-mediated regulatory networks (Yan et al. 2012). Intronic miR400 co-transcribes with its host gene under control conditions. Heat stress-induced alternative splicing event leads to the accumulation of its primary transcript but downregulation of mature miR400 (Yan et al. 2012).

3.3.3 Salt Stress

Salt stress affects many aspects of the cellular processes including transcription and translational alterations (reviewed in Ji et al. 2013). Mutant allele of DCL1, *dcl1-11*, is hypersensitive to salt and other abiotic stresses including ABA. Similarly mutants of other biogenesis factors such as *hen1–16* (allele of HEN1), *hsty* (allele of HASTY), *hyl1*, and *se-1* (allele of SE) also show hypersensitivity to salinity stress (Zhang et al. 2008a) indicating that miRNAs play important role in regulating plant response to salinity.

Analysis in *Arabidopsis* indicated that miR393, miR397, and miR402 were induced while miR389a.1 was repressed under salt stress (Sunkar and Zhu 2004). Subsequently, miR417 was also shown to play a negative role in salinity response as transgenic overexpressing miR417 had a decreased seed germination and growth in the presence of NaCl (Jung and Kang 2007). Apart from high-throughput sequencing, microarray analysis identified miR156h, miR167a,c,d, miR168, miR171b, and miR396a to be upregulated in response to salt stress in *Arabidopsis* (Liu et al. 2008). The study also showed the presence of various stress related *cis*-elements in the 1,000 bp upstream promoter region of these miRNAs including ABRE (ABA response elements), ARE (anaerobic response element), MBS (MYB binding site), HSE (Heat stress response elements), and LTRs (low-temperature response elements). In *Arabidopsis*, miR398 is downregulated upon salt treatment, after 12 h of 200 mM NaCl treatment (Jagadeeswaran et al. 2009). The expression of its target CSD1 correspondingly increases in sync with miRNA suppression, but the CSD2 induction could be seen only after 48 h of stress. Thus, miR398 displays a preferential downregulation among its target genes. Deep sequencing studies in rice identified many salt-regulated miRNAs (Sunkar et al. 2008) such as members of families

miR167, miR168, miR169, miR171, mir172, miR319, miR393, miR396, miR398, miR530, miR535, miR806, miR818, and miR820. This study also identified several new miRNAs such as miR1436 that may be regulated by salt stress. Detailed study regarding the miR169 family showed that among all the members, only mir169g,n,o responded to 150 mM NaCl stress persistent for up to 48 h (Zhao et al. 2009). OsHAP2E or NF-YA-8 was identified as a valid target of miR169 by 5′ RACE. This transcription factor is a member of the NF-Y complex that binds to CCAAT-box and regulated the transcription of several genes (Mantovani 1998). This CCAAT *cis-element* is found very commonly among protein coding genes including histone proteins in wheat (Yang et al. 1995) and heat shock proteins in tobacco (Rieping and Sehfffl 1992). miR393 (and not miR393b) was also shown to be differentially regulated by 150 mM NaCl and 75 mM NaHCO3 (alkali treatment) (Gao et al. 2011). Rice as well as *Arabidopsis* transgenic lines overexpressing osa-miR393 showed decreased tolerance to salt and alkali treatments thus implying that the miR393 is a positive regulator of salt sensitivity. Recently, the role of rice miR319a was elucidated through transgenic plants overexpressing the miRNA in creeping bentgrass (*Agrostis Stolonifera*) (Zhou et al. 2013). The transgenics displayed greater tolerance over a prolonged and severe salinity and drought stress. The mechanism elucidated responsible for this phenomenon turned out to be greater retention of water and less accumulation of Na^+ ions even under a severe salt stress (300 mM NaCl). The transgenics were shown to have lesser cell electrolyte leakage under various concentration of NaCl. Besides the transgenics accumulated more of K^+, PO_4^{3-}, Ca^{2+}, and Mg^{2+} ions in the cell when they were grown in soil and treated with 200 mM NaCl.

Similarly, studies in maize roots identified members of 13 families to be responsive to 200 mM NaCl. These families included the well-conserved miRNAs present in rice and *Arabidopsis* such as miR156, miR159, miR160, miR162, miR164, miR166, miR167, miR168, miR171, miR319, miR395, miR396, and miR399 (Ding et al. 2009). The dynamics of miR398 regulation by salt stress was also studied in *Populus* (Jia et al. 2009a). A persistent stress of 300 mM NaCl up to 72 h showed an induction (although to a lower level) in the miR398 expression and a due downregulation of the target gene CSD1. The same group studied and found that the regulation of miR395 under similar stress conditions is different as miR395 steadily increases up to 72 h. Accordingly, the target gene APS1 keeps declining in abundance. An independent group also studied various miRNAs under salt stress but could only identify mir1446a–e being downregulated to a significant level by salinity in *P. trichocarpa* (Lu et al. 2008b). The hydro-halophyte species of *Populus*, *P. euphratica*, is considered to be ideal for studying salt stress response. A global screen for both known and novel miRNAs that respond to short-term and long-term salt stress identified a total of 59 miRNAs, out of which 20 showed consistent upregulation, 35 show consistent downregulation while 4 show downregulation under short-term but up-regulation under long-term stress (Li et al. 2013b). Also out of these 59, 14 were novel to *P. euphratica* and 45 were conserved between *P. euphratica* and *P. trichocarpa*.

Similarly, miR2118, miR159.2, and miR393 were upregulated in the presence of 200 mM NaCl in *Phaseolus vulgaris* (Arenas-Huertero et al. 2009). In tobacco

(*N. tabacum*), miR159, miR167, and miR169 were downregulated under various concentration of NaCl stress although this inhibition of expression was relieved as the concentration of the salt increased. On the other hand, miR172 and miR396 were consistently upregulated in a dose-dependent manner, while miR398 displayed a consistent moderate downregulation (Frazier et al. 2011). miR395 and miR399 were initially downregulated but as the salt concentration increased, the abundance increased. A comparative study between salt-tolerant wild species of tomato (*Solanum penelli*) and a salt-sensitive elite variety M82 showed that the expression of miR395 that target ATP sulfarylase was higher in the tolerant variety than the sensitive cultivar (Shivaprasad et al. 2012). Similarly analysis of introgression lines generated by crossing the two also indicated that higher expression of miR395 correlated positively with greater salt tolerance. In barley (*Hordeum vulgare*), miR156d and miR396d are downregulated while miR399b is upregulated in salinity (Lv et al. 2012). In another genus of Fabaceae family, *Caragana intermedia* (Zhu et al. 2013) levels of miR157a, miR159a, miR165a, miR167b, miR172b, miR390a, and miR396a peaked at 1, 3, or 6 h post-treatment. On the other hand, miR398 rapidly decreased within 1 h of treatment and the fall continued thereafter. The conserved target genes like *Ci*SPL16 (miR157a), *Ci*HD-ZIPIII (miR165a), *Ci*AP2 (miR172b), and *Ci*FGRF (miR396a) also showed an anticorrelation by decreasing in abundance under salinity stress (Zhu et al. 201). A search for salinity responsive known and novel miRNAs in soybean (*Glycine max* cv Houzimao) also led to identification of 13 upregulated and 13 downregulated known miRNAs along with several novel candidate miRNAs (Dong et al. 2013). Once again members of miR171 family like miR171g,j,o,p,u, miR166a,b, miR482, miR1520b, miR390a-3p, miR408a,c, and miR395b,c were upregulated in the nodules. The ones that were downregulated are miR159a,b, miR169b,c, miR160, miR319a,b, miR5559, miR1517, miR1520c, miR1523, miR4416b, miR1521, and miR1519. An analysis to observe the effect of mild and severe salinity stress on the sugarcane (*Saccharum sp.* Cv SP70-1143) shoots and roots identified several salinity regulated miRNAs such as miR166III, miR168III, miR396II, miR398II, and miR528I (Carnavale Bottino et al. 2013).

3.3.4 Drought

The first direct evidence that miRNA was involved in the stress response came in 2006, where the repression of miR398 in response to oxidative stress leads to the upregulation of its targets CSD1 and CSD2 mRNA (Sunkar et al. 2006). Later, microarray-based approach by Liu et al. (2008) identified several drought-responsive miRNAs (miR396, miR168, miR167, miR408, miR171, miR157, and miR393) in *Arabidopsis* and thereafter several studies in different species, e.g., rice (Zhou et al. 2010b; Zhao et al. 2007; Jeong et al. 2011); *Arabidopsis* (Reyes and Chua 2007); maize (Wei et al. 2009); wheat (Kantar et al. 2011); Medicago (Wang et al. 2011; Trindade et al. 2010); and Populus (Li et al. 2011a; Shuai et al. 2013) identified several drought-responsive miRNAs. Nevertheless, only few have been

characterized in detail for their role in drought stress response. One such module is miR168/*AGO1*, where miR168 is induced under drought in *Arabidopsis* (Liu et al. 2008) but downregulated in rice (Zhou et al. 2010b), and involved in the regulation of AGO1. Its mutant *mir168a-2* showed narrow and twisted leaves with early flowering phenotype under abiotic stress conditions (Vaucheret 2009), suggesting the crucial role of miR168/AGO1 in stress response. Further, miR168a overexpression and *AGO1* loss-of-function mutants in *Arabidopsis* resulted in the hypersensitivity to ABA and drought. Conversely, miR168 mutants, *mir168a-2* displayed ABA and drought hyposensitivity (Liu et al. 2008), under drought in *Arabidopsis* (Li et al. 2012a). As *AGO1* is a crucial protein for both miRNA- and siRNA-mediated regulation, this miR168/*AGO1* feedback regulatory module highlights not only the role of miR168 but also the other small RNAs in the stress-mediated regulatory responses. Another report links the miRNA and drought tolerance via downregulation of CBP80 gene in potato (Pieczynski et al. 2013). Consistent with the findings in *Arabidopsis*, artificial miRNA-mediated silencing of CBP80 gene in potato renders plants drought tolerant and ABA hypersensitive. Also the downregulation of CBP80 leads to the decreased accumulation of miR159 and increased accumulation of MYB33 and MYB101 in the potato transgenic plants and *Arabidopsis cbp80* mutants (Pieczynski et al. 2013). Recently, comparative profiling of sRNA between control and TaDREB3 overexpressing drought-tolerant barley plants revealed the decrease in the miR168 levels (Hackenberg et al. 2012). Another miRNA family probably involved in drought stress response is miR169, which is upregulated in rice (Zhao et al. 2007) and tomato (Zhang et al. 2011b) while downregulated in *Arabidopsis* (Li et al. 2008) and Medicago (Wang et al. 2011). In tomato, upregulation of miR169 under drought leads to the downregulation of its targets NF-YA1/2/3. Tomato miR169 overexpression plants displayed enhanced drought tolerance with reduced stomatal opening, transpiration, and leaf water loss (Zhang et al. 2011b). Surprisingly, this module follows opposite drought-mediated regulation in *Arabidopsis*. NFYA5 was strongly upregulated while miR169 was downregulated in response to drought in *Arabidopsis*. Consistent with that *nfya5* knockdown and miR169 overexpression resulted in the increased water loss and are sensitive to drought (Li et al. 2008). Further studies have delineated the possible underneath mechanism involved in the miR169-mediated stress response. Generally, MIR169 family members exhibit upregulation under abiotic stress in both monocots and dicots except for few cases where downregulation is also reported (Xu et al. 2014). Since overexpression of miR169d displayed early flowering phenotype, while overexpression of miR169d resistant form of AtNF-YA2 leads to the late flowering. It is speculated that under stress conditions the upregulation of miR169 regulates AtNF-YA, which in turn decreases the FLC (Flowering locus C) allowing its downstream targets FT (Flowering locus T) and LFY (Leafy) to regulate early flowering (Xu et al. 2014). Another study linking the miRNA to early flowering under drought conditions (drought escape) in *Arabidopsis* suggested the involvement of GIGANTIA (GI)-miR172-WRKY44 in the regulation of drought escape (Han et al. 2013). While miR172e is upregulated under drought stress in a GI-dependent manner, *gi* mutants are sensitive and miR172 overexpressors are tolerant to drought (Han et al. 2013).

Drought escape strategy enables plants to shorten their lifecycle by early flowering. Recently, it was proposed that in *Arabidopsis* drought conditions stimulates drought escape response in an ABA-dependent manner under long day conditions only when the activation of GI by photoperiod facilitates the ABA-dependent upregulation of FT/TSF. These findings speculate the roles of miRNAs in drought escape via regulation of their targets. One more conserved miRNA miR394 act as positive regulator of drought stress in *Arabidopsis* and interestingly as a negative regulator for salt stress (Song et al. 2013). miR394 is involved in the post-transcriptional regulation of its target LCR (Leaf curling responsiveness), which encode for an F-box protein (SKP1-Cullin/CDC53-F-box) (Jones-Rhoades and Bartel 2004; Song et al. 2012). This module has been shown to play role in the regulation of leaf curling-related morphology of *Arabidopsis* (Song et al. 2012). Moreover, miR394 act as mobile signal from the protoderm to the distal meristem by reducing LCR, thereby conferring stem cell competence (Knauer et al. 2013). Both the MIR394 overexpression and loss-of-function of LCR confers drought tolerance through ABA sensitivity. MIR394 overexpression further leads to more ABA-induced hydrogen peroxide and superoxide anions with upregulation of several ABA and stress-responsive genes (Song et al. 2013). Drought-mediated upregulation of miR394 was also reported in soybean and overexpression of gma-MIR394a in *Arabidopsis* resulted in the drought tolerance (Ni et al. 2012). Another well characterized and conserved module in plant development is miR319/TCP (Teosinte branched/cycloidea/proliferating cell factors {PCF}) (Palatnik et al. 2003; Ori et al. 2007; Nag et al. 2009), which is involved in drought response in monocots. Overexpression of osa-miR319 in creeping bentgrass resulted in the enhanced salt and drought tolerance by increasing leaf wax content and greater water retention with wider and thick leaves, less tillering, and bigger stem (Zhou et al. 2013). Interestingly, miR393 is the only miRNA that is commonly upregulated in drought stress in *Arabidopsis* (Sunkar and Zhu 2004; Liu et al. 2008), rice (Zhao et al. 2007), and sugarcane (Ferreira et al. 2012). In *Arabidopsis*, the miR393 guided cleavage of TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and AUXIN SIGNALING F-BOX 2 (AFB2) is required for the ABA and osmotic stress-regulated root growth inhibition (Chen et al. 2012). In rice, miR393 was characterized as a negative regulator of drought and salt stress by fine-tuning their targets OsTIR1 and OsAFB2 (Xia et al. 2012b). Further in rice, miR164 target six NAC genes (OMTN1–6) and this recognition site was shown to be indispensable for the transactivation of these NAC genes (Fang et al. 2014). Overexpression of four miR164-targeted NAC genes (OMTN2, 3, 4, and 6) lead to high drought sensitivity at reproductive stages, suggesting that miR164 act as a positive regulator and its target NAC genes as negative regulators of drought stress (Fang et al. 2014). Recently, a study shown that the mutation of ER associated integral membrane protein associated AMP1 (Altered Meristem Program 1), which is involved in the miRNA-mediated translational repression, exhibit decreased water loss and stomata aperture leading to enhanced drought tolerance in *Arabidopsis* (Yao et al. 2014). Also these mutants accumulate higher ABA due to the induction of AtNCED1 and showed high seed germination under mannitol stress (Yao et al. 2014). The above roles of miRNAs in stress tolerance are summarized in Fig. 3.2.

3.3.5 UV

Excessive UV-B radiations (280–320 nm) due to the thinning of the protective stratosphere surrounding the earth are a potential threat to the healthy survival of living forms on earth. In *Arabidopsis*, UV-B radiations are perceived by a receptor, UVR8 protein characterized by a beta-propeller with a total of 14 tryptophan residues acting as its chromophore (Kliebenstein et al. 2002; Christie et al. 2012). UVR8 interacts with COP1 (Favory et al. 2009), and subsequent signaling involves HY5 and HYH (Brown and Jenkins 2008), thereby strengthening the link between UV perception and blue light signaling (Ulm et al. 2004). Calcium (Long and Jenkins 1998) and ROS (Mackerness A-H et al. 2001) have also been implicated in UV-B signaling and suggested as the second messengers involved. The changes that UV-B irradiation brings about in the plant cell include metabolic changes such as accumulation of flavonoids and phenylpropanoids that stand as major protectants from UV radiation and intracellular signal transduction components, chloroplast localized proteins, and protein turnover enzymes (Brosche et al. 2003). About half of the genes differentially expressed in response to UV-B treatment belong to light signaling pathways (Tohge et al. 2011).

Probable UV-B-responsive miRNAs were first suggested in *Arabidopsis* based on computational analysis of gene co-expression and co-regulation data (Zhou et al. 2007). A set of 21 miRNAs upregulated under UV-B treatment with anticorrelation with their target genes were identified. These include members of miRNA families like miR156, miR159, miR160, miR165, miR166, miR167, miR169, miR170, miR171, miR172, miR393, miR398, and miR401.

Subsequently, UV-B-responsive miRNAs were discovered in *Populus tremula* (Jia et al. 2009a). A total of 24 miRNAs were identified among which 13 were upregulated while 11 were downregulated by UV-B treatment. Some of the upregulated miRNAs included members of miR156, miR160, miR165/166, miR167, and miR398 while on the other hand; members of miR159, miR169, miR390, miR393, miR395, miR399, and miR472 were downregulated. The target genes included transcription factors such as SPB proteins, Myb, NAC domain proteins, and HD-ZIP factors; signal transduction factors such as ARFs and homologs of IAA receptors; metabolic components such as ATP sulfurylases (APSs), Cu^{2+}/Zn^{2+} superoxide dismutases (CSDs), as well as some disease resistance proteins.

Similarly, 6 UV-B-responsive miRNAs were identified in wheat (*Triticum aestivum* cvar Suwon11) (Wang et al. 2013c). Out of the six, three were shown to be downregulated (miR156, miR164, and miR395) while three were found to be induced (miR159, miR167, and miR171) by northern analysis.

One of the common aspects found in all three studies was the presence of light-responsive elements in the miRNA promoters. Most frequently found elements were the G-box, GT-1 site, and I-box, which thereby strengthens the hypothesis of possible interactions between white light and UV signaling. Interestingly, the promoters also contained elements for other abiotic responses such as Myb Binding site (MBS), Anaerobic response element (ARE), and ABA response element

(ABRE). Interestingly, several miRNAs show species-specific response to UV-B radiation. mir156 showed upregulation in both *Arabidopsis* and *P. tremula* but downregulation in *T. aestivum*. miR159 is upregulated in *Arabidopsis* and *T. aestivum* but downregulated in *P. tremula*.

In addition, recently it was shown that the inhibition of cell proliferation in the leaves of *Arabidopsis* by UV-B is mediated by miR396 and repression of its target GRF genes (Casadevall et al. 2013). It was experimentally proven that miR396 was induced by UV-B treatment, which in turn downregulated the levels of GRF1, GRF2, GRF3, and GRF9. GRFs are known to be involved in regulating cell proliferation and adjusting the leaf size in *Arabidopsis* (Kim et al. 2003). Triple knockout mutants of *grf123* have smaller leaves with fused cotyledons. miR396 mediates the inhibition of cell proliferation via MPK3 as *mpk3* mutants showed significant increase in the levels of GRF1, GRF2, GRF3 as well as the leaf size. Even the induction of miR396 in *mpk3* was not as pronounced as in the wild type. The other signaling components that were checked but found to have no role in this process were UVR8, ATR (Ataxia telangiectasia-mutated and Rad3-related) and MPK6. The miR396-GRF-MPK3 signaling mechanism is for a transient repression of the GRFs as it was shown that after a prolonged treatment by UV-B, miR396 levels become stable as compared to the unstressed plants but the levels of GRFs are lowered. Thus, the repression of GRFs is at the transcriptional level in the later stages but probably for an instant initial response, the MPK3-miRNA pathway mediates it.

3.3.6 Mechanical Stress

Gravitropism is a major mechanical force triggering tension and compression of wood development in trees. A developmental feature specific to angiosperm trees is the formation of tension wood (TW) and opposite wood (OW) that are considered to play a role in stress-sensing mechanism leading to increased mechanical support as against the loads generated by wind and/or gravity (Wu et al. 2000). *Populus trichocarpa* is a deciduous broad-leaf tree that represents a model species for trees. Study on expression of miRNAs in developing xylem and/or phloem of *Populus trichocarpa* suggests their functional association with cambium differentiation activities. miR156 and miR472 are highly expressed in leaves while miR160, miR164, miR171, miR473, miR477, miR478, miR479, and miR480 are more xylem or woody tissue specific. The transcript levels of miR156, miR162, miR164, miR475, miR480, and miR481 are similarly reduced under tension and compression stresses. However, miR408 expression is upregulated in both tension- and compression-stressed tissues. On the other hand, miR159, miR476, and miR479 exhibit preferential up-regulation in compression tissues. The expression of miR160 and miR172 is reduced only in compression tissue, whereas only tension stress appears to induce an attenuated expression of miR168. These miRNAs are possibly associated with more specialized regulations that may lead to a preferential development of either TW or OW (Lu et al. 2005). Further study on this revealed that

during the formation of TW and OW in the *Populus trichocarpa* stem suffering from mechanical stress, the expression levels of miR530a (reduced in OW), miR827 (reduced in OW), miR1444a (strongly upregulated in both TW and OW), miR1446a–e, miR1447, and miR1450 (reduced in OW) were altered (Lu et al. 2008b).

Wounding is another common form of mechanical stress in plants, which need not be tree specific. Study in *Ipomoea batatas* found that miR828 was detected only in leaves and is induced by wounding, signaling molecules such as ethylene, hydrogen peroxide (H_2O_2), methyl jasmonate, or nitric oxide (NO), which are known to induce wounding responsive genes do not induce miR828. However, cyclic guanosine monophosphate (cGMP) was necessary for miR828 accumulation in leaves on wounding (Lin et al. 2012). Wounding is believed to induce defensive response in tobacco as well. miR164 and miR168 were significantly induced in roots by both topping and wounding treatments while miR172 and miR390 were significantly induced only in roots treated by wounding. Furthermore, miR159, miR319, and miR2911 were upregulated only in topping treated sample while were downregulated or remained unchanged in wounding treated sample. In addition, seven conserved miRNA families (miR169, miR395, miR397, miR398, miR399, miR408, and miR827) were not detected in the untreated tobacco root sample but were detected in at least one damage-treated sample. Nine newly identified miRNA families (Nta-miR15, Nta-miR21-22, Nta-miR29, Nta-miR31–32, and Nta-miR35–37) that did not express in the untreated roots were detected in damage-treated samples. Different members of the same miRNA family could behave differently upon damage treatment; for example, Nta-miR4a.2 was highly induced by damage treatment, whereas Nta-miR4a.1 and miR4b were not induced. Nta-miR1, Nta-miR4c, and Nta-miR25 were highly induced in roots by both wounding and topping treatments; Nta-miR26 was highly induced only in topping treated sample. These results indicate different miRNA families behave differently upon damage treatment, suggesting a different role of these miRNAs in response to damage treatment in tobacco (Tang et al. 2012).

3.4 Role of Other Small RNAs in Abiotic Stress

Apart from miRNAs, other small RNAs have been shown to play role in plant stress-mediated response as well. Several reports confirm the role of other small RNAs excluding miRNAs in P homeostasis. smRPi1^{LTR}, DCL1-dependent sRNA generated from the LTR of Copia95 retrotransposons, accumulated specifically under phosphate starvation conditions in *Arabidopsis* roots (Hsieh et al. 2009). In addition, upregulation under phosphate starvation was also observed for the ta-siRNA (TAS4-siR81) derived from TAS4 and miR828 in shoots and targets PAP1/MYB75, PAP2/MYB90, and MYB113 involved in anthocyanin biosynthesis (Hsieh et al. 2009). As per their model under phosphate-deprived conditions, upregulation of PAP1/MYB75 results in the production of TAS4-si81, which counteracts its own expression and other MYBs. In rice, phosphate starvation-induced upregulation of

cis-$NAT_{PHO1;2}$ that positively regulate the levels of its cognate PHO1;2 protein at the level of translation and thereby contribute to the additional layer of regulation of phosphate homeostasis (Jabnoune et al. 2013). Significant amount of small RNAs belonging to sense and antisense, rasi RNA, t-RNA-derived sRNA, and chloroplast-derived sRNAs have been found to differentially regulate under phosphate starvation (Hackenberg et al. 2013).

In *Arabidopsis*, a 24 nt-nat-siRNA (natural antisense small interfering RNA) reported to be originating from two overlapping transcripts encoding P5CDH and SRO5 are generated exclusively in response to salt stress (Borsani et al. 2005). The nat-siRNA cleaves its originator mRNA of P5CDH, a crucial enzyme for catabolism of proline, thereby conferring salt tolerance to the plant. This was experimentally proven by measuring the levels of proline in the knockout mutants of NUCLEAR RNA POLYMERASE D 1A (*nrpd1a*) and Suppressor of Gene Silencing 3 (*sgs3*) that are impaired in the formation of the nat-siRNA. Subsequently, several nat-siRNAs were expressed in rice in response to drought as well as salt stress (Zhou et al. 2009). Salt stress also regulates the expression of long non-protein coding RNAs in *Arabidopsis* (Ben Amor et al. 2009). While npc60, npc536 (upregulation), and npc82 (downregulation) show a stable regulation up to 24 h by 150 mM NaCl, npc72 shows a transient downregulation and then recovery within 24 h. The transgenic lines overexpressing npc536 displayed longer root growth as compared to the wild type under salt stress (100 and 125 mM). However, its probable target transcript (AT1G67930) remains unaltered in the transgenic lines.

Alteration in the expression of four siRNAs under heat stress has been seen in *Triticum aestivum* seedlings wherein heat stress downregulates *siRNA002061_0636_3054.1*, *siRNA 005047_0654_1904.1*, and *siRNA080621_1340_0098.1* but not *siRNA007927_0100_2975.1*. Salt and drought stress downregulates *siRNA002061_0636_3054.1*, *siRNA 005047_0654_1904.1*, and *siRNA007927_0100_2975.1* but not *siRNA080621_1340_0098.1*. *siRNA 005047_0654_1904.1* and *siRNA080621_1340_0098.1* are upregulated in cold stress but *siRNA007927_0100_2975.1* is downregulated (Yao et al. 2010; Khraiwesh et al. 2012).

3.5 Small RNA-Mediated Signaling in Response to Abiotic Stress

From the above-discussed roles of small RNA especially miRNAs, it is evident that small RNA reside at the core of plant regulatory mechanisms. Several of miRNA families are differentially regulated by variety of stresses with miR398 and miR169 leading the list, suggesting their role in some common stress-responsive regulatory module to cope with adverse situations (Fig. 3.2). Also these evidences clearly indicate the cross talk between different abiotic stresses, which are linked by miRNAs/small RNAs. Phytohormone, ABA is a crucial and central player in the plant abiotic stress-mediated signaling responses. In response to stress, ABA binds to the PYR1/

PYL family of ABA receptors and promotes the interaction of PYR1/PYL with and inactivate PP2C, which release and activate the Sucrose nonfermenting 1 (SNF1)-related protein kinase 2s (SnRK2s) to phosphorylate its downstream targets to mediate ABA response (Fujii et al. 2009, Ma et al. 2009, Park et al. 2009). Its association with the small RNA-mediated stress responses became evident with the finding that the mutation in *hyl1* makes *Arabidopsis* plants hypersensitive to ABA at germination stage (Lu and Fedoroff 2000). Subsequently, several reports confirmed the hypersensitivity of different miRNA biogenesis-deficient mutants (*serrate*, *dcl1*, *hen1*, *cbp20*/*cbp80*, *cpl1*) to ABA (Hugouvieux et al. 2001, Kim et al. 2008, Zhang et al. 2008a, Manavella et al. 2012). Further mutant deficient in miRNA-mediated translational repression, *suo1* and *amp1* also displayed ABA hypersensitivity and drought resistant (Yang et al. 2012, Yao et al. 2014). These facts clearly demonstrate the involvement of miRNA-mediated regulation of transcriptome in ABA-dependent stress response. During seed germination, ABA induces the expression of miR159, which in turn regulated two positive regulators of ABA response, MYB101 and MYB33, in ABI3-dependent manner (Reyes and Chua 2007). In addition to that MIR168, overexpression and mutation in AGO1 (*ago1–27*) leads to the increased sensitivity to ABA in contrast to *mir168a-2* mutant with drought hypersensitivity and ABA hyposensitivity (Li et al. 2012a). Further several studies reported the differential regulation of miRNAs by exogenous ABA treatment (Sunkar and Zhu 2004). Recently, consistent with the above findings, quantitative phosphoproteomics based global comparison of WT and snrk2.2/2.3/2.6 triple mutant identified several proteins involved miRNA biogenesis including Exo-ribonuclease 2 (XRN2) and XRN3, SERRATE and importin β-like protein as the downstream effectors of SnRK2s (Wang et al. 2013d).

A recently identified endogenous gasotransmitter, hydrogen sulfide (H_2S), plays important roles in seed germination and root formation (Zhang et al. 2008b, 2009b), flower senescence (Zhang et al. 2011c) and abiotic stress regulation (Jin et al. 2011, 2013, Li et al. 2012b). Evidence of involvement of H_2S in ABA signaling mechanism yielded a new perspective, where H_2S interaction with ABA control the stomata movement under drought stress conditions (Jin et al. 2013). Mutants with reduced H_2S productions are more sensitive to drought due to enlarged stomatal aperture, decreased expression of Ca^{2+} channel, outward-rectifying K^+ channel, increased inward-rectifying K^+ and ABA receptors. Also stomata closure by ABA is partially dependent on the H_2S (Jin et al. 2013). Drought stimulated by PEG triggered H_2S production, which in turn regulate drought-associated miRNAs (Shen et al. 2013). As per the proposed model drought stress induce H_2S producing enzymes to produce more H_2S, which in turn interacts with ABA to regulate stomata closure. On the other hand, H_2S is involved in the modulation of expression of DREB2A, DREB2B, RD29A, and CBF4. It also regulates drought-associated miRs such as miR167, miR393, miR396, and miR398 (Shen et al. 2013).

Recently, seven miRNAs were identified to be H_2O_2 responsive in rice seedlings (Li et al. 2011b). miR169, miR397, miR827, miR408-5p, and miR1425 were upregulated while miR528 and miR319a.2 were downregulated upon oxidative stress. Further in rice oxidative stress leads to the up regulation of genes involved in the redox homeostasis (Mittal et al. 2012). The miR398-CSD module presents an excel-

lent example of abiotic stress signaling. Studies have clearly shown that miR398-copper-zinc SODs module is conserved in diverse plant species. Also it is differentially regulated in almost all abiotic stress condition. Furthermore, miR398 demonstrates dynamic regulation in populus and decreased in *Arabidopsis* in response to ABA (Jia et al. 2009b). The miR398 expression levels modulate the Cu/Zn SOD levels, which in turn contribute to the ROS homeostasis in response to stress.

The above fact that miRNA forms a central core in the stress-mediated regulatory networks further strengthened by the presence of several stress-responsive elements in the promoter regions of miRNAs. In soybean, analysis of promoters of miRNAs demonstrates the presence of light-responsive element (LRE) in promoters of nearly all analyzed miRNAs, which suggest their circadian regulation of miRNA transcription. In addition, about 87 % of miRNAs also contain stress-responsive elements in the upstream regulatory region including anaerobic induction elements (AREs), defense SREs (TC-rich repeats), ABA response elements (ABREs), low-temperature-responsive elements (LTRs), MYB binding sites (MBSs), salicylic acid responsiveness elements (SAEs), and heat stress-responsive elements (HSEs and LREs) (Han et al. 2014). Several abiotic stress-associated transcription factor-binding motifs are identified in the regulatory region of stress-responsive miRNAs in rice (Devi et al. 2013) and in *Arabidopsis* (Megraw et al. 2006). Several individual miRNA studies also identified the *cis*-acting elements in their upstream region, e.g., ABA-mediated response of AtMIR168a is due to the presence of two ABRE in upstream region (Li et al. 2012a), the presence of PHR1-binding site and other phosphate-responsive *cis* regulatory elements in the promoter of phosphate-responsive miRNAs (Bari et al. 2006, Zeng et al. 2010, Xu et al. 2013, Baek et al. 2013) and core GTAC motif as AtSPL7-binding site in the upstream regulatory region of copper-responsive miRNAs (Yamasaki et al. 2009). Also the LONG HYPOCOTYL 5 (AtHY5) acting downstream of many families of photoreceptors, binds to the upstream region of several miRNAs (miR156d, miR172b, miR402, miR408, miR775, miR858, miR869, and miR1888) (Zhang et al. 2011a).

In yet another instance that was proven recently, it was shown that siR441 and siR446 relay the signal from various abiotic stresses to a negative regulator of ABA signaling and thus positively regulate it (Yan et al. 2011). It was earlier shown that miRNA regulated and abiotic stress-induced F-box gene, MAIF1, is induced by drought, salt, and low-temperature stress (Jain et al. 2007). The transgenic plants overexpressing MAIF1 show reduced tolerance to drought, salinity, and low temperature as well as they were rendered ABA hyposensitive during seed germination and root growth phenomena (Yan et al. 2010). The gene was also shown to be upregulated by sucrose, auxin, cytokinin, and ABA (Yan et al. 2010). A cross talk between sugar and all these phytohormone signaling has been demonstrated (reviewed in Gibson 2004), and thus the gene is said to be involved in multiple signaling pathways and a positive regulator of root growth phenotype (Yan et al. 2010). But the precise mechanism of this abiotic regulation displayed by MAIF1 was unknown until the functional elucidation of siR441 and siR446 (Yan et al. 2011). It was shown that stresses like drought, salt, low temperature, and ABA accumulate the precursor intermediate of siR441 and siR446, which causes inter-

molecular base pairing of this processing intermediate. Due to this base pairing, processing of precursor into mature siRNA is impaired and thus the abundance of siR441 and siR446 is reduced. These two siRNAs target MAIF1 for degradation via cleavage of its transcript thereby regulating it. Thus, the siR441 and siR446 become positive regulators of ABA signaling by regulating the negative regulator MAIF1. These siRNAs were also shown to be repressed by drought, salinity, and low temperature thus showing that ABA senses the stress signal and carries it to the siRNAs that get repressed, which in turn up-regulates the MAIF1 that functions to negatively regulate ABA signaling.

3.6 Small RNA-Mediated Epigenetic Regulation in Abiotic Stress

To maintain the genome stability and integrity from the transposable element activity, plants have strategies to inhibit TE proliferation and maintain them in silenced state via epigenetic repression by small RNAs (Slotkin and Martienssen 2007). Several studies demonstrated that plant stress response involves the epigenetic reprogramming, which is intimately connected to the stress-induced transcriptional reprogramming (Bonasio et al. 2010; Chinnusamy and Zhu 2009). This stress-induced epigenetic memory inheritance allows plant to cope with adverse environmental conditions (Chinnusamy and Zhu 2009). Because abiotic stress-induced methylation changes are not global in nature, small RNAs may mediate sequence-specific change in DNA methylation/demethylation. In plants, siRNA controls locus-specific de novo methylation through the process of RNA-directed DNA methylation (RdDM) (Chinnusamy and Zhu 2009). siRNA guides methylation at CHH sites in a process called RNA-directed DNA methylation (RdDM) (Chinnusamy and Zhu 2009), which is involved in several processes like transposon silencing, gene regulation, plant development, and stress responses (Matzke et al. 2007). Compared to DNA that does not change upon stress, the expression of RNA is influenced by environmental cues; thereby suggesting the role of RdDM in stress-induced epigenetic modification (Bond and Baulcombe 2013).

One such regulatory module is miR820 and its target DMR2 (DNA methyltransferase) (Lu et al. 2008a) that catalyze the sRNA-directed de novo DNA methylation (Pang et al. 2013). In rice, miR820 is downregulated by drought stress (Jeong et al. 2011). As both miR820 and OsDMR2 behaves similarly under drought stress suggesting that miR820 is involved in the spatiotemporal regulation of OsDMR2 (Lu et al. 2008a; Jeong et al. 2011). Further, it was shown that the pri-miR820a/b/c cleaved by DCL3 to generate the pre-miR820, which is processed, by the action of DCL1 and DCL3 to release canonical miR820.1 (21 nt) and long mi820.2 (24 nt), respectively (Wu et al. 2010). The 21 nt miR820.1 is involved in the DMR2 cleavage by AGO1 while 24 nt miR820.2 is predominantly loaded into the AGO4b to mediate DNA methylation of its loci as well as target, OsDRM2 in *trans* (Wu et al. 2010). Another study demonstrated the transposable element-derived small RNA in

the stress response in *Arabidopsis* (McCue et al. 2012). siRNA854 (previously annotated as miRNA854) is produced from Athila family of LTR retrotransposons and is involved in the regulation of UBP1b gene in *trans*. In wild type plants, siRNA854 is produced as a 24 nt siRNA in a PolIV and RDR2-dependent manner. But in the DNA methylation mutants, *ddm1* and *met1*, i.e., when the loss of TE epigenetic silencing occurs, this siRNA accumulates as 21 nt form in a DCL2, DCL4, and RDR6-dependent manner and loaded into the AGO1 and thereby regulate the UBP1b transcript at post-transcriptional as well as translational level. Similar to its mammalian homolog, UBP1b is an RNA-binding protein, which upon stress moves out of the nucleus and aggregates to form stress granule in cytoplasm. Further *ubp1b* mutants are sensitive to ionic and osmotic stress regimens (McCue et al. 2012). These finding highlights the TE epigenetic activity-mediated modulation of stress response.

Recent analysis on the heat tolerance of DNA methylation-defective mutants revealed that the *nrpd2* and *hda6* mutant plants are hypersensitive to heat stress (Popova et al. 2013). In addition to that other RdDM mutants, *rdr2*, *dcl3*, and *ago4* also showed mild sensitivity towards heat stress (Popova et al. 2013). NRPD2, the second largest subunit of PolIV and PolV, is crucial for sRNA biogenesis and RdDM (Haag and Pikaard 2011; Zhang and Zhu 2011). One more report links the warm temperature and transgenerational epigenetic through siRNA-mediated regulation (Zhong et al. 2013) wherein increase in ambient temperature from 22 to 30 °C leads to the reduction in the protein levels of SGS3, which inhibit the dsRNA formation step in the post-transcriptional gene silencing pathways induced by antisense sequences, small RNA and tasiRNA (Zhong et al. 2013). Conversely, the overexpression of SGS3 abolished the warmth-induced inhibition of siRNA formation. Further, this moderate increase also resulted in the altered DNA methylation status of transgenes. Interestingly, this phenomenon exhibited transgenerational epigenetic memory, which persisted for three generations (Zhong et al. 2013).

3.7 Cultivar Biased Regulation of Small RNA in Response to Stress

Development of stress tolerant and high yielding cultivars requires identification of candidate genes involved in the stress tolerance. Also within the cultivated pool of a particular cereal crop, some traditional cultivars are more tolerant to several abiotic and biotic stress conditions than the elite high yielding ones. Comparative genomic, transcriptomic, or proteomic approaches are instrumental in identifying such candidate genes involved in the natural stress tolerance molecular networks of tolerant cultivar (Walia et al. 2005; Zahaf et al. 2012; Guo et al. 2009; Cotsaftis et al. 2011). Several reports have highlighted the importance of small RNA in abiotic stress regulatory mechanisms by comparing the genome-wide miRNA profiles of stress-sensitive and -tolerant genotypes upon stress (Barrera-Figueroa et al. 2011; Nischal et al. 2012; Wang et al. 2013a). Most crop species have huge genetic diversity and harbor robust alleles, which are instrumental in defining the molecular basis

of natural stress tolerance capacity. In soybean, a set of 8 miRNAs (miR166a-5p, miR169f-3p, miR397ab, miR1513c, miR4415b, miR-seq11, miR-seq13, and miR-seq15) showed upregulation in drought-sensitive cultivar (BR 16) while the same set of miRNAs have higher basal levels in tolerant cultivar (Embrapa) were downregulated under drought (Kulcheski et al. 2011). In contrast, miR166f had similar basal level in both cultivars but under drought regimens, its transcript levels showed upregulation in sensitive cultivar and while downregulation in tolerant cultivar (Kulcheski et al. 2011). Similarly, in rice comparison of drought stress response in tolerant and susceptible indica rice varieties identified differential regulation of miR408-3p at different growth stages of rice (Mutum et al. 2013). miR408-3p level decreases upon stress in sensitive cultivars (PB1 and IR64) but remains elevated in drought-tolerant cultivars/varieties (Vandana and N22) upon drought. This trend was also reflected in the expression of its target gene transcripts. miR408-3p targets plantacyanin family members, which are upregulated in PB1 but downregulated in N22. Further, the upstream transcription factor OsSPL9 also showed a similar variety-specific expression in drought stress (Mutum et al. 2013). Further, Barrera-Figueroa et al. (2011) identified 11 miRNAs that were differentially regulated only in one genotype while comparing the small RNA data of drought-tolerant (IT93K503-1) and -sensitive cowpea (CB46) cultivars. miR160a, miR160b, and miR171e with four novel molecules were only regulated in IT93K503-1 while miR171d, miR2111b, miR390b, and miR393 were differentially regulated in CB46 (Barrera-Figueroa et al. 2011). miRNA profiles demonstrate unique to common behavior upon waterlogging stress in maize depending upon the tolerance of inbred lines. A cluster of miRNAs (miR156, miR164, miR168, miR169, miR171, miR319, miR390, miR393, and miR529) upregulated only in the tolerant line but decreases in mid-tolerant and sensitive maize lines in response to hypoxia (Liu et al. 2012b). Interestingly, another cluster of miRNAs (miR159{h/i}, miR172, miR398, miR408, miR528, and miR1432) is upregulated in tolerant and mid-tolerant lines but downregulated in the sensitive cultivar. Similar comparative analysis was also performed in soybean under nitrogen starvation (Wang et al. 2013a) and in maize under low phosphorus conditions (Pei et al. 2013). While such studies are instrumental in identifying agronomically important miRNA genes they also highlight the evolution of regulation of miRNA genes in different plant species.

Conclusions and Future Prospects

In this review, we have summarized the roles of small RNAs, especially miRNAs, in regulating plant response to abiotic stress conditions such as nutrient starvation (nitrogen, phosphorus, sulfur, and copper), extreme temperature, salinity, drought, and UV. Significant data implicates a global miRNA-mediated regulation in abiotic stress response, however, understanding about the other classes of small RNA in abiotic stress is still limiting. It is clear from the data discussed above that miRNAs occupy nodal position in the stress-mediated regulatory circuits. The differential regulation of common miRNAs in different environmental conditions reflects a

significant cross talk between different stress-induced signaling networks. The fact that miRNAs act as mobile signals further expands the dimension of miRNA-mediated abiotic stress regulatory networks. Further, the dynamic regulation of conserved miRNA-target nodes, such as miR398/CSD, miR399/PHO2, and miR167/miR160/ARF, in response to abiotic stress highlights the evolutionarily conserved or universal stress-mediated regulatory strategy to cope with stress. On the other hand, the responsiveness of some of these conserved miRNA-target pair is distinctly variable in different cultivars of the same species. While more studies are required to estimate the impact of such regulatory dynamism in different cultivars, it nevertheless indicates that miRNAs themselves are under a very dynamic regulation. Indeed, studies have shown stress-mediated regulation of miRNA transcription via specific transcription factors or alternate splicing as well as modulation of miRNA biogenesis machinery. In addition to the well-characterized miRNA-target nodes, data also indicates several potential miRNA-target nodes that might participate in miRNA-mediated stress regulatory networks and thus need to be further explored. Majority of the reports identify candidate stress-responsive miRNAs through NGS data analysis while only few are functionally characterized by gain-of-function or loss-of-function studies. Further, very few studies demonstrate the spatiotemporal regulation of these stress-regulated molecules. Another limitation is the correct identification of targets for miRNA since in-silico target prediction is not accurate while the PARE/degradome data is available for only few species and that too in one or two tissue.

Thus, while miRNAs and other small RNAs are clearly involved in the regulation of plant abiotic stress response, more precise data is required regarding their own regulation as well as their association with their target genes before they can be effectively used in molecular breeding programs for crop improvement. A global comparative study of small RNA populations in related yet contrasting cultivars/varieties would prove highly effective in identifying target small RNA molecules.

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Chapter 4
The Role of Long Non-coding RNAs in Abiotic Stress Tolerance in Plants

Swati Megha, Urmila Basu, Muhammad H. Rahman, and Nat N.V. Kav

Abstract Non-coding RNAs (ncRNAs) are a family of regulatory RNAs, which do not encode mRNA, rRNA or tRNA, found in a variety of organisms including plants. Different classes of ncRNAs have been identified based on their length and their position in the genome, including small ncRNAs (microRNAs and small-interfering RNAs), natural antisense transcripts (NATs), and long intronic/intergenic ncRNAs (lncRNAs, 200nt or longer). Recent advances in next-generation sequencing technologies and computational analysis for transcriptome profiling have led to the genome-wide identification of ncRNAs. Functional characterization of these ncRNAs has implicated them to play a role in a wide range of cellular functions, such as epigenetic silencing, transcriptional regulation, and RNA metabolism. Emerging evidence suggest that several lncRNAs play important roles in many fundamental biological processes including growth and development as well as abiotic stress responses. Recent findings on the roles of lncRNAs in the aforementioned plant processes are summarized in this chapter.

Keywords lncRNAs • Abiotic stress • Plant development • Chromatin modifier • Target mimicry

4.1 Introduction

Plants are sessile organisms and, therefore, being unable to evade stress, require avoidance or tolerance mechanisms to survive when exposed to such conditions. Abiotic stresses including limitation of water, exposure to salt, or temperature extremes are major factors limiting crop productivity. Increasing world population, reduction of available arable land due to urbanization, changes in soil quality (increased drought/salinization), and climate change are all expected to further exacerbate challenges associated with food security (Mittler 2006). A thorough understanding of plant responses to environmental stresses, at the organismal,

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cellular, and molecular levels, will aid in the development of rational strategies for crop improvement.

When exposed to adverse abiotic or biotic conditions, plants detect and respond by preserving resources for growth and reproduction, while curtailing damage due to the imposed stress (Atkinson and Urwin 2012). These responses include changes in physiological and cellular processes aimed at limiting stress-induced damage, through the modulation of gene expression brought about by the imposed stress. Such regulation of gene expression can be achieved through transcriptional, post-transcriptional, translational and post-translational means (Ingram and Bartels 1996; Sunkar and Zhu 2004; Brodersen et al. 2008; Bartel 2009).

Although proteins such as transcription factors (TFs) have been classically considered as the main regulators of gene expression, recent research have indicated important roles of various non-coding RNAs (ncRNAs) in the regulation of gene expression and consequently cellular functions through various other pathways including transcriptional gene silencing (Knowling and Morris 2011). For example, in the human genome, only 2 % of RNAs actually encode functional proteins, although almost 90 % of the genome is transcribed (Wilhelm et al. 2008). RNA molecules consist of transfer (t)RNA and ribosomal (r)RNA, which have functions in protein translation, while short ncRNAs (small nucleolar, snoRNA; small-interfering, siRNA; micro, miRNA), natural antisense transcripts (NATs), and long non-coding RNA (lncRNA) have possible regulatory roles in gene expression (Matsui et al. 2010; Lei et al. 2012; Jian et al. 2013). Examples of such regulation may include transcriptional gene silencing, post-transcriptional gene silencing, or mRNA degradation. This chapter focuses exclusively on the role of lncRNAs in mediating plant responses to stress, and for a review of miRNA and siRNA function and their role in abiotic stress, readers are referred to Bartel (2004), Jones-Rhoades et al. (2006), Sunkar (2010), Sunkar et al. (2012), de Alba et al. (2013), and Verma et al. (2014).

4.2 Biogenesis of lncRNAs

Similar to protein-coding mRNAs and sncRNAs, majority of the lncRNAs are transcribed by RNA polymerase II (Geisler and Coller 2013; Zhang and Chen 2013), although some are transcribed by RNA polymerase III (Dieci et al. 2007). Similar to mRNAs, majority of lncRNAs are spliced, polyadenylated, and 5′-capped (Nie et al. 2012). LncRNAs can originate from intronic, exonic, intergenic, intragenic, promoter regions, 3′- and 5′-UTR or enhancer sequences, and can be bidirectional transcripts (Geisler and Coller 2013; Zhang and Chen 2013). In particular, a large group of lncRNAs is antisense to known protein-coding transcripts that have also been referred to as NATs, which could be categorized as *cis*-NATs, trans-NATs, and pseudogenes (Nie et al. 2012). They can also be classified as sense or antisense lncRNAs based on their strand of origin (Nam and Bartel 2012), divergent, or convergent based on their orientation of transcription, and as intronic or intergenic lncRNAs based on their location (Derrien et al. 2012). The majority of lncRNAs, unlike mRNA and structural ncRNAs, are confined to the nucleus, although some of

them are found in both cytoplasm and nucleus, and several lncRNAs are specifically distributed in the cytoplasm (Nie et al. 2012). Widespread occurrence of NATs have been reported in eukaryotes (Lapidot and Pilpel 2006), including humans (Conley and Jordan 2012) and *Arabidopsis* (Henz et al. 2007).

The identification of lncRNAs involves the determination of all transcripts using either genomics techniques including microarrays (Michelhaugh et al. 2011), serial expression of gene expression, SAGE (Gibb et al. 2011), expressed sequence tag, EST (Huang et al. 2012), or RNA-Seq (Li et al. 2012; Sigova et al. 2013). The data is processed to filter RefSeq-annotated protein-coding transcripts, pseudogenes, miRNA, tRNA, rRNA, and snRNA (Sigova et al. 2013). These transcripts are then compared to genomic sequences to rule out the ones overlapping with protein-coding genes, followed by Open Reading Frame (ORF) prediction of the remaining sequences (Zhu and Wang 2012). The threshold ORF length for an RNA to be considered as lncRNA is usually 70–100 amino acids (Zhu and Wang 2012). Further analysis of the data is performed to select for transcripts with correct 5′ ends that meet minimal read coverage threshold, remove transcripts with positive coding potential, select for long transcripts, and remove repetitive elements (Sigova et al. 2013). ORF length strategy (Ma et al. 2012), conservation of coding potential (Pauli et al. 2012), including codon substitution frequency (Guttman et al. 2009) and reading frame conservation (Clamp et al. 2007) are some of the parameters, which are employed by available bioinformatics tools such as ORF-Predictor (Jia et al. 2010); Coding Potential Calculator (Kong et al. 2007); integrated ncRNA finder (Lu et al. 2011); and RNAcode (Washietl et al. 2011). Using whole-genome tiling array for high-resolution transcriptome analysis, rice (Li et al. 2006) and *Arabidopsis* (Rehrauer et al. 2010) lncRNAs have thus been identified. Recently, a strand-specific RNA-Seq approach has been used to identify a number of lncNAT and long intergenic non-coding RNAs (lincRNAs) induced in *Arabidopsis* in response to *Fusarium oxysporum* (Zhu et al. 2014).

4.3 Molecular Mechanisms of lncRNAs

In plants, the molecular basis of how lncRNAs function and mediate gene regulation is poorly understood. In animals, some of lncRNAs have been shown to be involved in the regulation of gene expression at various levels (Wilusz et al. 2009; Geisler and Coller 2013). For a comprehensive study on molecular mechanisms of lncRNAs, readers can refer to an excellent review by Wilusz and coworkers (Wilusz et al. 2009). In this chapter, we focus on the mechanisms, which have been characterized thus far in plants (Fig. 4.1). In plants, lncRNAs have been shown to recruit chromatin modifiers such as polycomb repressive complex (e.g., PRC2); be natural target mimics of miRNA and act as cargo for protein re-localization (Zhu and Wang 2012).

Chromatin modification has been found to be important for tissue-specific gene expression in both plants and animals and can be instigated by recruitment of chromatin-modifying complexes at specific sites (Ho and Crabtree 2010; Pfluger and Wagner 2007). Several lncRNAs in animal systems (e.g., *Air*, *HOTAIR*, and

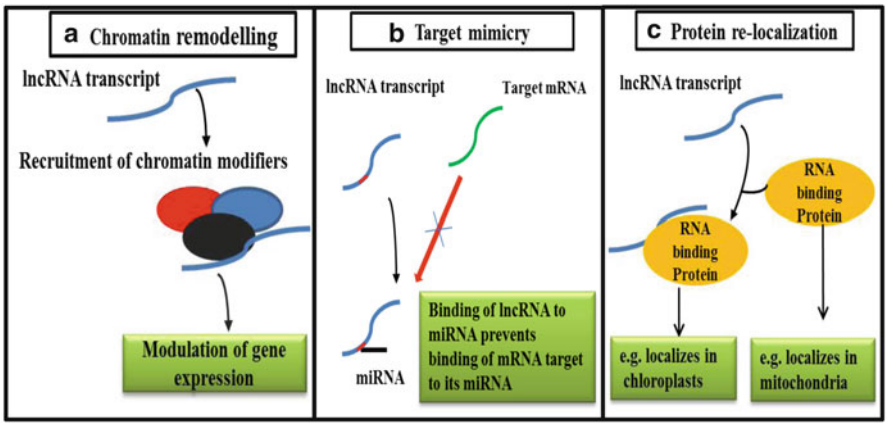

Fig. 4.1 Three possible mechanisms of lncRNA-regulated gene expression in plants. (**a**) Recruitment and direct association of chromatin-modifying complexes by lncRNAs results in histone modifications, modulating the expression of protein coding genes. (**b**) Presence of complementary sites on lncRNA transcripts bind miRNAs, competitively preventing the binding of target mRNA, also termed as target mimicry. (**c**) Some lncRNAs have been found to bind specific RNA-binding proteins, causing them to localize to a different organelles

Xist) have been shown to be responsible for epigenetic gene silencing through interaction with specific chromatin domains (Nagano et al. 2008; Pandey et al. 2008; Zhao et al. 2008). In plants, two lncRNAs (*COOLAIR* and *COLDAIR*) have been reported to function by guiding chromatin-modifying complexes to their target in *Arabidopsis* (Fig. 4.1a) (Heo and Sung 2011). Some lncRNAs can base pair with other small RNAs such as miRNA thereby modulating their activity (Wilusz et al. 2009). This process has been referred to as target mimicry (Franco-Zorrilla et al. 2007) (Fig. 4.1b) since lncRNA, mimicking mRNA, binds miRNAs reducing their availability for binding with intended targets (Ebert et al. 2007). This mechanism of action has been reported to be employed by the lncRNA *IPS1* (*Induced by Phosphate Starvation 1*) in *Arabidopsis* (Franco-Zorrilla et al. 2007) and will be further discussed in subsequent sections.

As shown in Fig. 4.1c, lncRNAs in plants have also been shown to act as molecular cargo for re-localization of proteins (Campalans et al. 2004). The transcript of an early nodulin gene (*Enod40*, identified in soybean and *Medicago truncatula*) lacks long ORFs, but code for two short peptides (<28 amino acids) and thus have been categorized as lncRNA (Yang et al. 1993; Crespi et al. 1994; Sousa et al. 2001; Rohrig et al. 2002). This *Enod40* transcript has been found to act as a molecular cargo for MtRBP1 (*M. truncatula* RNA-binding protein 1) by re-localizing this protein from nuclear speckles into cytoplasmic granules during nodulation (Campalans et al. 2004). The mode of action of lncRNAs in plants has not been extensively studied yet, but with the advent of new techniques and growing interests, this situation is sure to change in the near future.

4.4 Role of lncRNAs in Abiotic Stress Tolerance

A number of studies have identified and established the role of other small ncRNAs such as miRNAs and siRNAs in mediating abiotic stress tolerance in plants through sequence-dependent gene silencing at transcriptional and post-transcriptional level (Voinnet 2009; Sunkar et al. 2012; Meng et al. 2013). In contrast to small ncRNAs, only a few lncRNAs have been identified and functionally characterized further to implicate their involvement in abiotic stress response in plants (Ben Amor et al. 2009; Contreras-Cubas et al. 2012).

A genome-wide analysis of ncRNAs was performed in *Arabidopsis* to identify lncRNAs transcribed during abiotic stress by Ben Amor et al. (2009). From the 76 ncRNAs identified by bioinformatic analysis of full-length cDNA database, 14 were thought to have a *cis*-regulatory role because of their antisense orientation similarities with respect to their respective protein-coding mRNAs. Analysis of the expression of lncRNAs under abiotic stress conditions (phosphate starvation, salt or water stress) in the same study showed altered expression of 26 lncRNAs. Phosphate starvation stress resulted in up-regulation of npc43 and npc536 and the downregulation of npc33, as confirmed by RT-PCR. Moreover, a 100-fold increase in the expression of npc60 was observed under salt treatment. Several lncRNAs were differentially expressed in roots and leaves of the stressed plants. These data suggest a possible role of lncRNAs in plant stress response (Ben Amor et al. 2009).

Xin and coworkers (2011) attempted to identify lncRNAs responsive to heat stress in the heat-tolerant wheat genotype TAM107 by microarray analysis and Solexa sequencing. They identified 54 heat stress-responsive lncRNAs, which were not evolutionarily conserved suggesting that wheat has developed its own set of specific lncRNAs for regulating gene expression and cell activity. Furthermore, the expression of two heat responsive lncRNAs, TahlnRNA27, and TahlnRNA5, was found to be up-regulated after 1 h of heat treatment. The results of this study provide a good starting point towards understanding the role of lncRNAs in regulation of abiotic stress tolerance (Xin et al. 2011).

Phosphate is an essential nutrient, vital for plant growth and development. When exposed to low concentrations of phosphate (inorganic phosphate, Pi) in the soil, plants employ several strategies to enhance the uptake of Pi from the soil, through alteration of root structure and function, as well as modification of the rhizosphere (Grennan 2008). A lncRNA well studied for its role is *IPS1*, which plays an important role in phosphate homeostasis (Fig. 4.2). *IPS1* is a member of TPS family and has been identified in tomato (*TPS11*), *M. truncatula* (*Mt4*) and *Arabidopsis* (*At4*, *At4-1*, *At4-2*, and *At4-3*) (Liu et al. 1997; Burleigh and Harrison 1999; Martin et al. 2000; Wasaki et al. 2003). A 23-nt long sequence motif is conserved among the members from different plant species including *IPS1*, *At4*, and other members of this family. An ORF that has the potential to encode a peptide comprising of four amino acids, Met-Ala-Ile-Pro, is shared between *IPS1* and its close paralog *At4* (Martin et al. 2000; Wasaki et al. 2003). In plants, phosphate homeostasis is controlled by the expression of miR399, which is up-regulated by Pi deprivation, resulting in

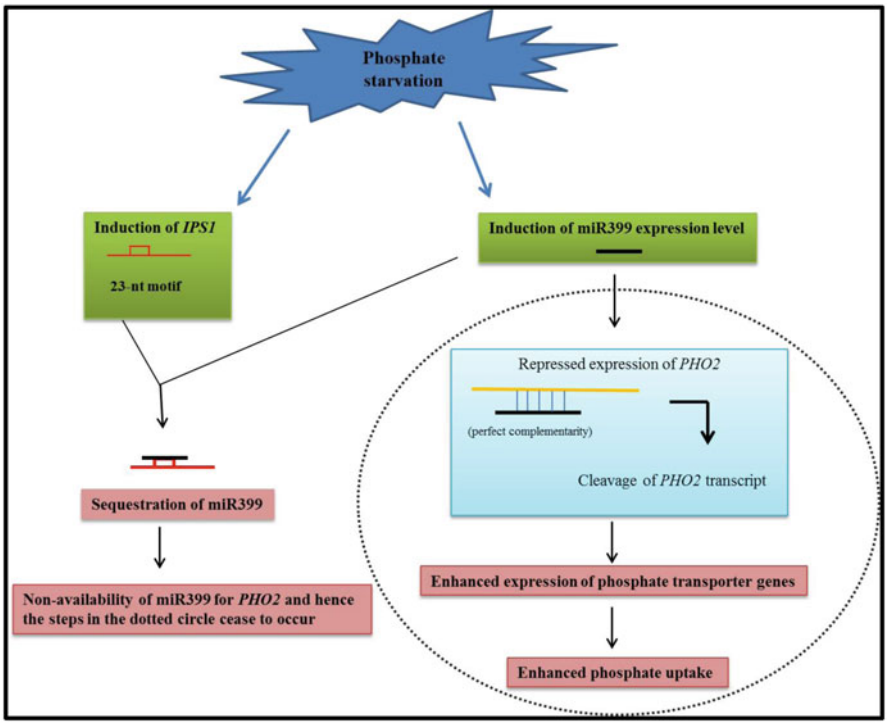

Fig. 4.2 Regulation of phosphate homeostasis by *IPS1* through the mechanism of target mimicry. Under phosphate stress conditions expression of both *IPS1* and miR399 is up-regulated. *IPS1* possesses a 23-nt motif with partial complementarity to miR399 and hence competes with *PHO2*; the target of miR399. The sequestration of miR399 by *IPS1* helps plant to maintain the balance of functional miR399 in phosphate absorption

reduced *PHO2* (phosphate2) mRNA level (Fujii et al. 2005; Chiou et al. 2006; Chiou 2007; Lin et al. 2008). *PHO2* encodes an E2 ubiquitin conjugase-related protein that negatively affects shoot Pi content and Pi remobilization (Chiou et al. 2006; Chiou 2007). The reduced expression of *PHO2* results in enhanced expression of two phosphate transporter genes, *Pht1*;*8* and *Pht1*;*9*, culminating in increased phosphate uptake (Aung et al. 2006; Bari et al. 2006). Functional characterization of lncRNA (*IPS1*) has shown that this lncRNA employs target mimicry mechanism (by binding to the miRNAs, thereby reducing their availability for binding with their respective target genes) (Ebert et al. 2007; Franco-Zorrilla et al. 2007; Wu et al. 2013). *IPS1* is also induced by phosphate starvation and its conserved 23-nt motif has been found to be partially complementary to miR399 with a central mismatch (Franco-Zorrilla et al. 2007; Rymarquis et al. 2008). Owing to this partial complementarity, *IPS1* forms a non-cleavable RNA duplex with miR399, thereby preventing the binding of miR399 to its actual target, i.e., *PHO2* (Franco-Zorrilla et al. 2007; Wu et al. 2013). The sequestration of miR399 by *IPS1* under phosphate starvation (Fig 4.3) thus leads to regulation of *PHO2* expression and modulates phosphate uptake (Rymarquis et al. 2008).

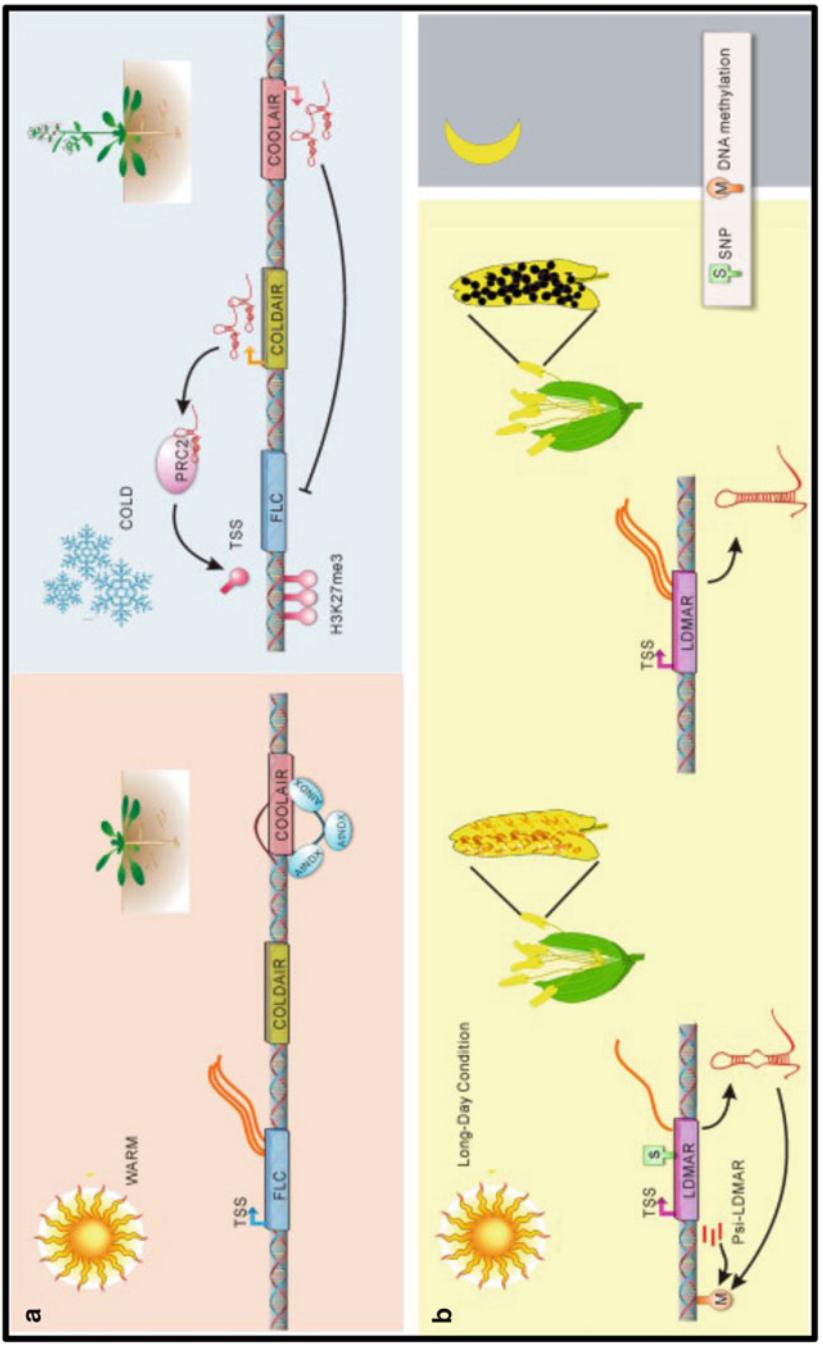

Fig. 4.3 Function of lncRNAs in response to cold stress/during plant development. (**a**) Schematic representation of how lncRNAs *COOLAIR* and *COLDAIR* regulate the expression of *FLC* during the process of vernalization in *Arabidopsis*. *COOLAIR* results in early, cold-dependent repression of FLC, while *COLDAIR* recruits chromatin modifier, PRC2 and is expressed later during vernalization. (**b**) Regulation of anther development through LDMAR during long-day conditions (Zhang and Chen 2013, Biochem Biophys Res Commun. 436:111, with permission)

4.5 Regulatory Role of LncRNAs in Plant Development

Long ncRNAs have been recognized to be involved in numerous regulatory functions (Table 4.1) including nutrient metabolism, flowering, and male sterility through modulation of transcriptional patterns of target loci. These transcriptional changes are mediated through mechanisms discussed in the above section such as recruitment of chromatin modifiers, target mimicry, and protein relocation (Campalans et al. 2004; Orom et al. 2010; Wierzbicki 2012; Rinn and Chang 2012). Multiple studies have indicated that changes in expression levels of many lncRNAs are tissue dependent (Xin et al. 2011) and are correlated with developmental processes (Kim and Sung 2012; Zhang and Chen 2013). In maize and rice, 38 lncRNAs were found to be imprinted in developing endosperms, which were transcribed from intronic regions of normal protein-coding genes or from intergenic regions (Zhang et al. 2011; Luo et al. 2011).

Table 4.1 List of known plant lncRNAs, their targets, mode of action, and possible functions

lncRNA	Plant	Target	Mode of action	Function	Reference
GmEnod40	*Glycine max*	[a]	[a]	Nodule formation	Yang et al. (1993)
MtEnod40	*M. truncatula*	MtRBP1	[a]	Nodule formation	Crespi et al. (1994)
OsPI1	Rice			Phosphate uptake	Wasaki et al. (2003)
Csm10	Cucumber	[a]	[a]	Male sex differentiation	Cho et al. (2005)
IPS1	*Arabidopsis*	miR399	Target mimicry	Involved in phosphate homeostasis	Franco-Zorrilla et al. (2007)
zm401	Maize	[a]	[a]	Male sex differentiation	Ma et al. (2008)
COOLAIR	*Arabidopsis*	*FLC*	Promoter interference	Represses *FLC* early during vernalization	Swiezewski et al. (2009)
npc43	Wheat	[a]	[a]	Responsive to phosphate stress	Ben Amor et al. (2009)
npc536	Wheat	[a]	[a]	Responsive to phosphate stress	Ben Amor et al. (2009)
COLDAIR	*Arabidopsis*	*FLC*	Recruiter of chromatin remodelling complex	Represses *FLC* at later points during vernalization	Heo and Sung (2011)
LDMAR	Rice	[a]	Recruiter of chromatin remodelling complex	Regulation of photoperiod-sensitive male sterility	Ding et al. (2012)

[a]Not yet determined

The role of two lncRNAs, *COOLAIR* and *COLDAIR*, in response to cold stress in *Arabidopsis* have been extensively studied (Fig. 4.3a). Both of these are transcribed from the flower repressor FLOWERING LOCUS C (*FLC*), which is repressed by prolonged cold during the process of vernalization (Michaels and Amasino 1999). During vernalization, association of VERNALIZATION INSENSITIVE 3 (VIN3) protein with polycomb repressive complex (PRC2) represses the expression of *FLC* (de Lucia et al. 2008; Wood et al. 2006). In animals, association between lncRNAs and chromatin-modifying complexes is considered as a general mechanism for epigenetic gene silencing (Nagano et al. 2008). As PRC2 is also a repressive chromatin modifier, studies were undertaken to investigate if *FLC* locus generates lncRNA transcripts and elucidate their role in repression of *FLC* expression. The lncRNA *COOLAIR* was found to be transcribed in antisense orientation relative to the *FLC* locus by a promoter downstream of *FLC* and also expression level of *COOLAIR* increased during vernalization process. Induction of *COOLAIR* occurs earlier than the onset of expression of other vernalization markers, VIN3; and has been suggested to be involved in early, cold-dependent silencing of *FLC* (Swiezewski et al. 2009). In contrast, *COLDAIR* is transcribed in sense orientation relative to *FLC* by the first intron of *FLC* (Heo and Sung 2011). The expression of *COLDAIR* is induced at later time points when compared to *COOLAIR* during vernalization and its transcripts are also found to interact directly with a component of PRC2 (i.e., CURLY LEAF, CLF). This interaction between *COLDAIR* and PRC2 indicates its role in recruiting PRC2 at *FLC* locus during vernalization (Heo and Sung 2011). The vernalization process was found to be compromised in *COLDAIR* knockdown lines. The return of *COLDAIR* knockdown plants to warm conditions was not sufficient to maintain the vernalization-induced repression of *FLC*. These results suggested the crucial role of *COLDAIR* in establishing and sustaining the silencing of *FLC* during cold (Heo and Sung 2011).

Additional evidence for the role of lncRNA in plant development has been established in a recent study (Ding et al. 2012) on long-day-specific male-fertility-associated lncRNA (*LDMAR*) in rice. *LDMAR*, 1,236 bases in length, has been found to regulate the photoperiod-sensitive male sterility (PSMS) (Fig. 4.3b). For normal pollen development of plants grown under long-day conditions, sufficient amount of the *LDMAR* transcript is required (Ding et al. 2012). In mutant rice lines, single nucleotide polymorphism caused increased methylation in the putative promoter region of *LDMAR*, with a consequent reduction in the transcription of *LDMAR*. Reduced *LDMAR* activity led to premature programmed cell death in developing anthers, resulting in PSMS under long-day conditions (Ding et al. 2012) indicating the important regulatory role of lncRNAs in reproductive development of rice.

Other putative lncRNAs such as *CsM10* from cucumber (Cho et al. 2005) and *zm401* from maize (Dai et al. 2004; Ma et al. 2008) have been found to be expressed in somatic tapetal cells, developing male gametophyte and might function in male reproductive development. Transgenic plants with downregulated *zm401* transcripts exhibited abnormal development of the tapetum, leading to the production of infertile pollen grains (Grant-Downton and Rodriguez-Enriquez 2012). Also, Zhou et al.

(2012) demonstrated that a point mutation in the cytoplasmic male sterility locus *P/TMS12-1* (encoding an lncRNA) resulted in cold- or photoperiod-sensitive sterility in rice. Interestingly enough, *P/TMS12-1* generates a small RNA (21nt long), but does not possess typical microRNA secondary structure. All these studies clearly suggest roles of different lncRNAs in important developmental processes.

4.6 Conclusion and Future Studies

Breakthroughs over the past few years have revealed that lncRNAs ubiquitously exist in different plant species including *Arabidopsis*, wheat, maize, and rice indicating that lncRNAs have conserved roles. Several recent studies have begun to establish that lncRNAs can play an important role in several gene regulatory networks involved in various biological processes of plants development and stress response. A limited number of lncRNAs have been shown to perform targeting functions through chromatin modification complexes, co-activation or co-suppression of trans-acting RNAs. Molecular elucidation of PSMS mutant and the DNA polymorphism has been exploited for the development of male sterile germplasms, PSMS lines, for hybrid crop breeding programs in rice. Further studies are necessary to understand in detail the functional motifs of lncRNAs, and how specific lncRNAs seek out selective sites in the genome for interaction, for improved understanding of the possible functional roles of lncRNA in plant biology. Genome-wide screening by strand-specific high-throughput RNA sequencing and computational approaches (Jin et al. 2013) for comprehensive identification of lncRNAs will be useful for detailed mapping and structural studies to understand the RNA–protein and RNA–DNA interactions. These studies would provide more insight into the regulatory role of more and more lncRNAs in plant stress responses as well as growth and development. Such information can be used in the rational improvement of crop plants.

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Part II
Diverse Stress Signaling Networks

Chapter 5
Molecular Physiology of Heat Stress Responses in Plants

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Abstract Heat stress is one of the major abiotic stresses that plants encounter. Heat stress causes billions of dollars in losses of agricultural crops worldwide. Here, we summarize the molecular and whole genome responses due to heat stress in plants. It has been reported that there are cascades of biochemical reactions that lead to heat stress. In most cases, heat stress is coupled with drought stress response. With the advancements in genomic tools, we have more information on genes, which are up- or down-regulated in plants due to heat stress. The heat-stressed plants may exhibit various physiological responses, including stomatal closure, suppressed photosynthesis, stunted growth, etc. Microarray and transcriptome sequencing gave us the tools to perform genome-wide expression profiling in heat-stressed plants. Understanding how gene expression in heat-stressed plants works will help us to discover novel heat stress-tolerant genes. These genes could be overexpressed in crop plants to make transgenic heat-tolerant agricultural crops. Climate change and global warming are major concerns for us and production of thermotolerant plants could address the issue of global crop loss due to heat and drought stresses.

Keywords Abiotic stress • Adaptation • Genetic transformations • Heat shock proteins • Heat stress response • Membrane fluidity • Metabolites • Osmolytes • ROS scavenging system • Small non-coding RNA • Thermotolerance • Transcriptome

5.1 Introduction

Plants are sessile organisms, and they are constantly exposed to environmental changes. Any change in the nonliving factors that can adversely affect the growth and development of the plant is known as abiotic stress. Extreme temperature is one of the major detrimental abiotic factors for the plant, causing heat stress (Yeh et al. 2012; Źróbek-Sokolnik 2012). The effects of high temperature can manifest through modification of membrane properties, stability of the protein structures, the rate of

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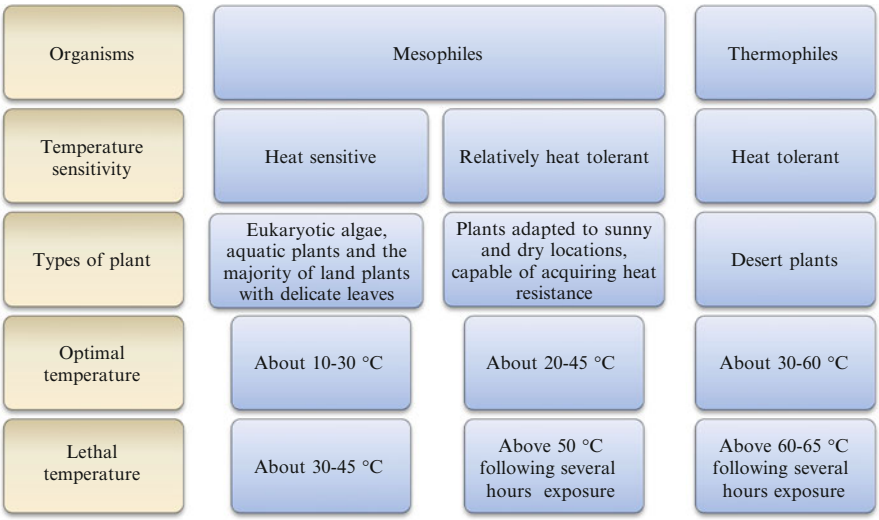

Fig. 5.1 Classification of the plants, based on their sensitivity to high temperature (partly adapted from Źróbek-Sokolnik 2012)

metabolic reactions, and the liquid viscosity inside plant cell organelles (Źróbek-Sokolnik 2012).

Generally, living organisms can be classified into three groups according to their temperature preferences: psychrophilic organisms (psychrophiles), mesophilic organisms (mesophiles), and thermophilic organisms (thermophiles) (Fig. 5.1). Most higher plants are classified as mesophilic organisms preferring temperatures between 10 and 30 °C (Źróbek-Sokolnik 2012). Although optimal plant growth can occur in a certain temperature range, mesophilic plants encounter a wide range of temperature fluctuations. As temperature deviates from the optimal level, cellular and molecular changes occur within the plant in order to maintain growth and cellular homeostasis. Despite the ability to adjust to temperature fluctuation, prolonged plant exposure to temperature above the threshold level may cause irreversible damage to plant growth and productivity, which is defined as heat stress (Willits and Peet 1998). According to the scientific standards, temperatures higher than the optimal level of 10–15 °C are denoted as heat stress (Larkindale et al. 2007). Heat stress has varying effects on plants due to intensity, duration, and the rate of temperature change. The duration of the plant exposure to high temperature is the main determinant of the lethal temperature range (Fig. 5.1) (Sung et al. 2003; Źróbek-Sokolnik 2012).

Plants need to adapt to any abiotic changes by exhibiting appropriate response; this is necessary in order for plants to survive. The ability of plants to grow and remain productive under high temperatures is defined as heat tolerance (Huang and Xu 2008). Plants can be classified into three categories according to their tolerance levels to high temperature: heat sensitive, relatively heat tolerant, and heat tolerant (Fig. 5.1) (Hasanuzzaman et al. 2013; Źróbek-Sokolnik 2012). The form

and magnitude of the heat tolerance depends on the plant, tissue, and cell type (Sung et al. 2003).

An increase in ambient temperature could affect crop productivity. In recent decades, human activities have caused an exponential increase in greenhouse gas emission especially carbon dioxide. It has been reported by the Intergovernmental Panel on Climate Change (IPCC) that the global mean temperature will increase by 0.3 °C per decade (Wheeler and von Braun 2013). This temperature increase may also affect distribution pattern of plant species. There are already losses of billions of dollars in agricultural crops worldwide due to heat stress. In addition, human demand for food is growing along with the world population growth. Any negative environmental effect on crop productivity can directly affect food security (Saidi et al. 2011).

Numerous studies have been performed that aimed to understanding plant heat stress responses (HSRs) and to minimize the detrimental effects of heat stress on plant productivity. It is believed that better understanding of the molecular mechanisms activated by heat stress may help in the development of more efficient crop thermotolerance (Yeh et al. 2012). Microarray and transcriptome sequencing gave scientists the tools to perform genome-wide expression profiling in heat-stressed plants. Understanding the gene expression profile in heat-stressed plants will help researchers discover novel heat stress-tolerant genes. These genes could be overexpressed in crop plants to make transgenic heat-tolerant agricultural crops. This chapter elaborates upon the impact of high temperatures on plant growth and development, and it emphasizes on physiological and molecular response of plants to heat stress.

5.2 Plant Responses to Heat Stress

Heat stress causes a series of biochemical, morphological, physiological, and molecular changes that adversely affect plant development (Fig. 5.2). The growth and productivity of the plants rely on numerous different biochemical reactions that are powered with temperature-sensitive proteins. At high temperatures, the enzyme functions can be disrupted with irreversible denaturation of proteins (Howarth 2005). If the temperature rises to extreme levels, severe cellular injuries may occur followed by immediate cell death within a few minutes; however, at moderately high temperatures, only long-term exposure may cause injury or death (Howarth 2005; Schoffl et al. 1999). The increased fluidity of membrane lipids, and protein denaturation and aggregation are immediate injuries occurring after exposure to high temperature (Howarth 2005). Slower heat injuries include loss of membrane integrity, inhibition of protein synthesis, enzyme inactivation in chloroplasts and mitochondria, and protein degradation (Essemine et al. 2010; Howarth 2005). Heat stress also affects cell cycle and cell division through changing the microtubules organization, elongation of phragmoplast microtubules, and formation of microtubule asters in mitotic cells (Smertenko et al. 1997). All these injuries together ultimately cause starvation, growth inhibition, decreased ion flux, accumulation of toxic compounds and reactive oxygen species (ROS) (Howarth 2005; Schoffl et al. 1999).

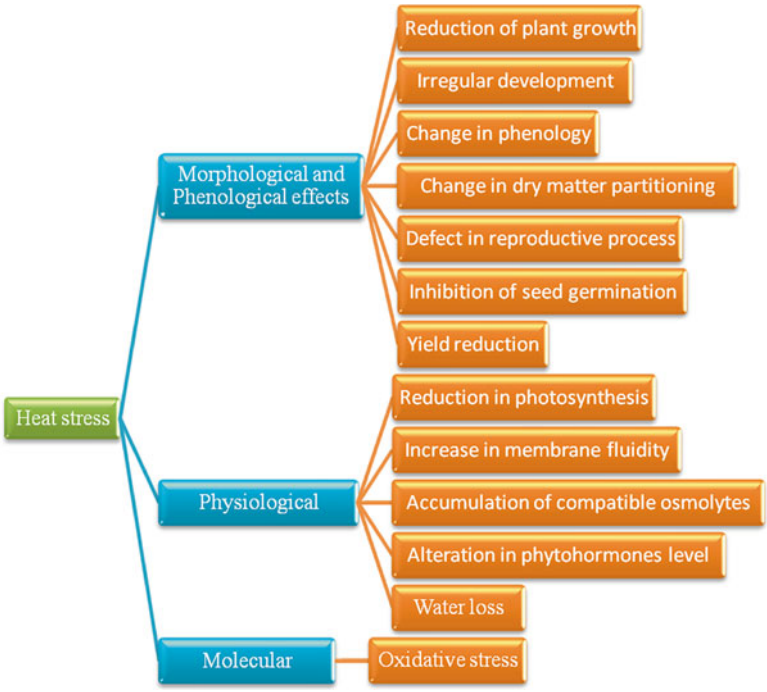

Fig. 5.2 Effects of heat stress on plant physiology

5.2.1 Morphological and Phenological Responses

Temperature plays an important role in controlling the rate, timing, and pattern of plant development. Therefore, growth retardation is the most prominent effect of heat stress on plants. In higher plants, cell division and cell elongation rates are significantly impaired under heat stress, which, in turn, can affect leaf size and weight. Plants' exposure to severe heat stress reduces the stem growth, causing lower plant height (Prasad et al. 2006; Źróbek-Sokolnik 2012). Exposure to high temperatures during sowing time negatively affects the plant height and number of tillers (Ahamed et al. 2010).

The impact of high temperatures on plants may vary depending upon the developmental stages of the plant. During the reproductive stage, even a short exposure to high temperatures can cause significant abortion of floral buds and opened flowers; however, during the vegetative stage, high day temperature can only affect leaf gas exchange processes (Young et al. 2004). Long-term exposure of developing seeds to high temperatures can delay germination and diminish vigor, resulting in lower emergence and seedling establishment. Inhibition of seed germination under heat stress is often induced by abscisic acid (ABA) (Essemine et al. 2010). In developing shoots, high temperatures cause severe declines in the first internode length, shoot dry mass, and relative growth rate, leading to premature death of plants or early senescence

(Patel and Franklin 2009). High temperature during growth stage can cause elongated stems and leaf hyponasty in some plant species (Patel and Franklin 2009). Moreover, heat stress may alter the total phenological duration by reducing the plant lifetime (Zhang et al. 2006). The temperature effects on phenological stages may vary based on the species and genotype due to great genetic variations (Hasanuzzaman et al. 2013).

Other morphological injuries derived from heat stress include scorching of leaves and twigs, sunburn of stem, branches and leaves, growth inhibition of shoot and root, leaf senescence and abscission, fruit discoloration and damage, along with a reduction in yield and dry matter production (Guilioni et al. 1997; Ismail and Hall 1999; Vollenweider and Günthardt-Goerg 2005). At the tissue and cellular level, water loss, stomata closure, cell size reduction, and increased number of xylem vessels in root and shoot are observed. The subcellular level damages include modifications in the thylakoid structure and swelling or loss of grana stacking (Karim et al. 1997; Wahid et al. 2007).

In tropical and temperate regions, heat stress is one of the most important causes of yield loss. Reproductive processes are significantly sensitive to high temperatures in most plants. Heat stress may adversely affect meiosis in both male and female reproductive organs, pollen germination, pollen tube growth, ovule survival, fertilization and post-fertilization processes, and growth of the endosperm and embryo (Foolad 2005). High temperatures also affect fruit quality, causing reduction in the levels of growth regulators and carbohydrates concentrations, leading to poor fruit set (Foolad 2005).

5.2.2 Physiological Responses

5.2.2.1 Water Relations

The most important variable affected by heat stress is plant water status. Plants are able to maintain the tissue water status in stable levels in humid environment regardless of temperature (Tsukaguchi et al. 2003). Heat-induced elevation in transpiration and water transportation are necessary tools for plant survival under high temperatures. It has been shown that in *Pinus ponderosa* seedlings, water transport through stems helps to cool plant by heat transferring mechanisms (Kolb and Robberecht 1996). Rapid water flow through seedling stems reduced the stem temperature by 30 °C during peak sunlight hours (Kolb and Robberecht 1996). However, heat stress is often linked with reduced water availability. Water deficit is significantly higher under the combined heat and drought stress rather than each single condition (Rampino et al. 2012).

Water loss most likely to occur more during daytime than nighttime under high temperatures. During the daytime, intensive leaf transpiration leads to water loss and reduction in water potential (Tsukaguchi et al. 2003). Since water is essential for any metabolic reaction, water deficiency causes disturbance of many physiological processes in plants. Significant water loss due to high temperatures can negatively affect both growth and biomass production (Simões-Araújo et al. 2003).

5.2.2.2 Osmotic Adjustment

Different plant species may accumulate certain organic compounds, which are generally known as compatible osmolytes (Sakamoto and Murata 2002). Some examples of the compatible osmolytes are sugars, sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds, and tertiary sulfonium compounds (Sairam and Tyagi 2004). It is thought that the accumulation of such compounds may be associated with enhanced stress tolerance in plants. For example, glycinebetaine (GB), an amphoteric quaternary amine, acts as a compatible solute in some plant species under heat stress. It has been suggested that GB synthesis may protect cellular redox potential under heat stress (Li et al. 2011). Maize plants (Quan et al. 2004) and sugar cane (Wahid and Close 2007) are able to accumulate high levels of GB due to water deficiency or high temperature while rice or mustard naturally don't produce GB. Nowadays, genetic engineering approaches have provided researchers with a tool to introduce GB-biosynthetic pathway into GB-deficit strains. Heat tolerance in plants can be improved through use of these tools, through enhancing the production of such compatible osmolyte (Li et al. 2011; Quan et al. 2004).

5.2.2.3 Cellular Membranes

Cellular membranes play an important role in both photosynthesis and transpiration processes. High temperatures increase the membrane fluidity through either denaturation of proteins or through intensifying unsaturated fatty acids (Savchenko et al. 2002). Heat stress alters the tertiary and quaternary structures of the proteins within the membranes, enhancing their membrane permeability. Increased electrolyte leakage through membranes indicates a reduction in cell membrane thermostability (CMT) (Wahid et al. 2007). Different factors can affect solute leakage, including type of organ, degree of hardening, developmental stage, plant age, growing season, and plant species (Karim et al. 1997, 1999). It has been shown that adverse effects of heat stress are more severe on the mature leaves than the developing ones due to an enhanced number of unsaturated fatty acids (Karim et al. 1997, 1999). It has been suggested that alteration in membrane fluidity triggered by low or high temperatures influences temperature perception and gene expression (Saidi et al. 2009, 2010).

5.2.2.4 Photosynthesis

One of the most heat-sensitive physiological processes in green plants is photosynthesis (Crafts-Brandner and Salvucci 2002). Although there is a positive correlation between temperature changes and photosynthesis in the normal growing range of plants (15–45 °C), high temperatures disrupt the functionality of photosynthetic enzymes (Larkindale et al. 2008). The impact of high temperature on photosynthetic functionality is more enhanced in the C3 plants than in C4 plants. Any injuries on the photosynthetic apparatus that are inflicted by heat stress directly restrict plant growth. High temperatures affect photosynthetic capacity through disruption of enzyme

activity in the electron transport chain, carbon metabolism, and oxygen-evolving complex (OEC) of PSII (Salvucci and Crafts-Brandner 2004). The photochemical reactions in thylakoid lamellae and carbon metabolism in the stroma of chloroplast are the most sensitive sites to injury under high temperatures (Wise et al. 2004). An increase in temperature can induce phase change in lipids incorporated in the thylakoid structure, which can eventually lead to lipid separation (Krumova et al. 2008).

Photosystem II (PSII), which is located in the thylakoid lamellae, is particularly sensitive to high temperatures; this causes its activity to be significantly decreased or even partially stopped. This is mainly due to the properties of the PSII location on the thylakoid membranes (Camejo et al. 2005). The damage to PSII is usually irreversible and it can cause a disassociation of the OEC (Sharkey and Zhang 2010). OEC is a water-oxidizing enzyme, and its dysfunctionality under heat stress causes an imbalance between an electron donor from OEC toward an electron acceptor of PSII. On the other hand, PSI stromal enzymes are thermostable in which PSI can drive cyclic electron pathway and associate with the thylakoid proton gradient (De Ronde et al. 2004).

The effect of heat stress on photosynthesis is also exhibited through a decline in soluble proteins, RuBisCO binding proteins (RBP), large subunits (LS), and small subunits (SS) of RuBisCO in darkness, and enhancement of them in light (Demirevska-Kepova et al. 2005). As temperature increases, photosynthesis declines due to increases in photorespiration that are faster than the subsequent increases in photosynthesis. The low affinity of RuBisCO, and its dual nature as an oxygenase and a carboxylase, is the main reason that limits the enhancement of net photosynthesis at higher temperatures. RuBisCO deactivation is the primary constraint for photosynthesis since it occurs at temperatures well below those that damage PSII (Salvucci and Crafts-Brandner 2004; Sharkey and Zhang 2010).

Another consequence of heat stress is degradation of chlorophyll *a* and *b*, which tends to be more significant in the mature leaves compared to the developing one (Karim et al. 1999). Such effects on chlorophyll may be associated with the production of active oxygen species (Camejo et al. 2005). Altered structural organization of thylakoids, grana swelling, and loss of grana stacking are among major alterations that occur in chloroplasts under heat stress (Djanaguiraman et al. 2010; Marchand et al. 2005). High temperatures also decrease the amount of photosynthetic pigments as a result of the lipid peroxidation of both chloroplast and thylakoid membranes (Djanaguiraman et al. 2010; Marchand et al. 2005).

Furthermore, high temperatures can greatly impact starch and sucrose synthesis by reducing the activity of sucrose phosphate synthase, ADP glucose pyrophosphorylase, and invertase (Chaitanya et al. 2001; Vu et al. 2001).

Photosynthetic responses of the thylakoid to heat stress significantly correlates with CO_2 concentration (Sharkey and Zhang 2010). In addition, plant ability to sustain leaf gas exchange and CO_2 assimilation rates under heat stress is directly associated with heat tolerance. High temperatures can significantly reduce the leaf CO_2 assimilation rates during the vegetative stage (Hall 1992). Stomata closure under heat stress is the main reason of decline in the intercellular CO_2 concentration, which can lead to impaired photosynthesis (Hasanuzzaman et al. 2013). Limitation of RuBisCO availability is another factor involved in the low CO_2 assimilation rate (Sharkey and Zhang 2010).

5.2.2.5 Plant Hormones

Following heat stress, the level of selected phytohormones, such as ethylene, ABA, and salicylic acid (SA), is rapidly increased, while others, such as cytokine, auxin, and gibberellin, will be decreased (Larkindale and Huang 2005). The alterations in the level of these phytohormones accelerate plant aging (Larkindale and Huang 2005). Different abiotic stresses, including heat and drought stresses, result in increased levels of ABA (Larkindale and Huang 2005). ABA is involved in the biochemical pathways that are necessary for survival under heat-induced desiccation stress, and it is also responsible for stomatal closure under osmotic stress. Furthermore, it mediates the adaptation of plants to desiccation through modification of the expression status of numerous genes. It is also related to ROS generation in guard cells through Rboh regulation (Miller et al. 2008). It may also play a role in induction of several heat shock proteins (HSPs) to confer thermotolerance to plants (Miller et al. 2008; Wahid et al. 2007). Ethylene is a gaseous hormone, which is involved in regulation of plant growth and development by controlling seed germination, flowering, fruiting, and stress tolerance (Munné-Bosch et al. 2002). The effect of heat stress on ethylene production may vary in different plant species (Arshad and Frankenberger 2002). For example, in wheat leaves, the ethylene production is inhibited when temperature reaches to 40 °C, but in soybean plants, the ethylene production in hypocotyls is increased by increasing temperature up to 40 °C (Tan et al. 1988; Wahid et al. 2007). SA is also involved in HSRs since it functions in plant growth and development. SA prevents oxidative damage to membranes through detoxification of superoxide radicals. SA correlates in signaling pathways in response to hypersensitive response (HR) and systemic acquired resistance (SAR). SA can induce long-term thermotolerance in plants through association with the expression of HSP genes, Ca^{2+} homeostasis, antioxidant mechanisms, improved fertility, and increased yield (Larkindale and Knight 2002; Wang and Li 2006). Among other hormones, gibberellins and cytokinins have been suggested to be involved in heat tolerance, where their effects are opposite to those of ABA. Under high temperature, the concentrations of these hormones begin to decrease, which correlates with the decline in root and shoot growth and dry matter production (Liu and Huang 2005). Also, it has been reported that the amount of endogenous auxin is reduced under heat stress, particularly in developing anthers (Teale et al. 2006).

5.2.3 Molecular Responses

5.2.3.1 Oxidative Responses

Heat stress can alter the function of many enzymes involved in different metabolic pathways. These alterations in enzymatic activity may lead to the accumulation of harmful ROS including singlet oxygen, superoxide radical $\left(O^{2-}\right)$, hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Liu and Huang 2000). High temperatures

induce generation of ROS, leading to oxidative stress, which ultimately can cause cellular injury. ROS disrupts membrane semi-permeability function by enhancing autocatalytic peroxidation of membrane lipids and pigments (Xu et al. 2006). The ROS generation can occur in peroxisomes, mitochondria, and chloroplasts, particularly in reaction centers of PSI and PSII (Apel and Hirt 2004; Sharma et al. 2012). Heat stress can dramatically increase the level of ROS, which may disturb cell homeostasis (Mittler et al. 2004). Disruption of the homeostasis balance may occur either through enhancement of ROS production or decline in antioxidant activity in the cell (Bowler et al. 1992).

Oxygen radicals are constantly produced in chloroplast and mitochondria. Singlet oxygen, which is formed during photoinhibition, and PSII electron transfer reactions, can directly oxidize DNA, proteins, and polyunsaturated fatty acids (Karuppanapandian et al. 2011). Superoxide radicals are formed in many photo-oxidation reactions in chloroplasts, electron transport chain reactions in mitochondria, and other reactions in the plasma membrane (Halliwell 2006). The function of superoxide dismutase (SOD) is to scavenge the superoxide to hydrogen peroxide, which is removed by ascorbate peroxidase (APX) or catalase (CAT) (free radical scavenging enzymes). In the presence of Fe^{2+} and Fe^{3+}, the reaction of superoxide with hydrogen peroxide can form hydroxyl radicals (Haber–Weiss reaction). The toxicity of OH^{-} is significantly higher than O^{2-} and H_2O_2, because it can damage DNA, proteins, lipids, and other essential macromolecules. Plants are able to protect their metabolism and growth from the harmful impacts of ROS through detoxification systems (Sairam and Tyagi 2004). For example, induction of SOD or APX expression and activation is associated with the appearance of physiological injuries in plants under heat stress (Mazorra et al. 2002). However, in most plant species, extreme conditions induce higher production of ROS, which overwhelms the scavenging activity of the antioxidant system, leading to severe cellular injury (Fadzillah et al. 1996). It has been shown that under heat stress, plants accumulate higher amounts of non-enzymatic antioxidant and up-regulate the expression of antioxidant enzymes (Almeselmani et al. 2009). However in many plant species, these increased activities are not sufficient for development of stress tolerance (Almeselmani et al. 2009). Under stress conditions, protection against oxidative stress is an important factor in the determination of plant stress tolerance (Xu et al. 2006).

5.2.3.2 Stress-Related Proteins

Plants' response to heat stress is composed of several integrated circuits including multiple pathways, specific cellular organelles, special cofactors, and signaling molecules coordinating an appropriate feedback. The high temperature signal is first perceived with the specific heat receptors on the membrane of the plant cells. The stimulus information is then transduced downstream leading to the activation of various heat stress-responsive genes. The products of these genes eventually result in the heat tolerance response or plant adaptation to survive during the harsh

conditions (Iba 2002). The HSPs are known as the most important products of these genes for heat stress adaptation. It is believed that the HSPs are universal heat-protective agents against heat stress that maintain homeostasis in organisms. This is mainly based on the fact that the primary protein structure for HSPs is well conserved among prokaryotes and eukaryotes including higher plants. The heat shock response occurs after exposure of the plant tissue or cells to sudden high temperature stress, resulting in transient expression of the HSPs. The molecular masses of HSPs can vary but often ranges from about 10 to 200 kDa. Despite their different sizes and weights, they all have the ability of binding to structurally unstable proteins, having the chaperon-like function, and being involved in signal transduction during heat stress (Al-Whaibi 2011; Schoffl et al. 1998). The heat shock domain is the main characteristic of all HSPs, which is recognized by the presence of a conserved carboxylic terminal (Helm et al. 1993). HSPs are expressed in different locations, including the cytoplasm, ribosomes, endoplasmic reticulum, chloroplasts, mitochondria, and membranes (Vierling 1991). Certain HSPs can also be expressed in the absence of environmental stress during some stages of plant development, such as the development of pollen grains, embryogenesis, germination, and fruit ripening (Sun et al. 2002).

In plants, HSPs can be classified into five classes according to their molecular weight, amino acid sequence homologies, and functions: HSP100, HSP90, HSP70, HSP60, and the small heat shock proteins (sHSPs) (with molecular weight between 15 and 30 kDa) (Gupta et al. 2010; Kotak et al. 2007; Schoffl et al. 1998). HSPs production in plants can vary greatly according to the expression level and their type. Higher plants often contain about 20 types of sHSPs, whereas some species can express about 40 types of these proteins. These sHSPs show unusual abundance and diversity, in which it is suggested that these proteins can proffer adaptive tolerance to plants under heat stress (Korotaeva et al. 2001). In a study, wheat, rye, and maize seedling were subjected to 42 °C; the results show expression of five sHSPs in maize mitochondria while only one of them was expressed in rye and wheat (Korotaeva et al. 2001). These results suggest that higher expression of sHSPs is the reason for higher heat tolerance in maize than in wheat and rye (Korotaeva et al. 2001). In plants, six nuclear gene families encode all sHSPs, where each gene family is related to proteins present in distinct cellular compartments (Waters et al. 1996).

Under heat stress, HSPs aggregate and assemble into heat shock granules (HSGs), usually in the cytoplasm. It seems that HSGs function in protecting the protein synthesis machinery, which is critical for survival of plant cells under continuous heat stress. The aggregation state of HSPs can be essential for their role in prevention of protein denaturation caused by high temperatures (Miroshnichenko et al. 2005). The ability of plants for HSPs production, the intensity and duration of synthesis, is significantly different depending upon species and tissue types. Fast accumulation of HSPs under the stress is another key factor for developing heat tolerance in plants (Nieto-Sotelo et al. 2002).

Accumulation of HSPs is regulated by heat shock transcription factors (HSFs), which bind specifically to *cis*-acting sequences known as heat shock elements

(HSEs) (Akerfelt et al. 2010). HSFs are main components in heat stress signaling that are sensitive to changes in temperature. Like many other transcription factors, they have a modular structure composed of a highly conserved N-terminal DNA binding domain and an adjacent oligomerization domain (OD). The DNA binding domain is characterized by an HLH motif and the oligomerization domain with a hydrophobic heptad repeat pattern (Baniwal et al. 2004). These structural domains are important in heat stress-dependent activation that switch inactive HSF monomers to the active trimeric form, which specifically bind to HSE in the promoter of HSF-responsive genes (Scharf et al. 2012).

Plants and vertebrates contain multiple families of HSF genes while invertebrates including yeast and *Drosophila* have a single HSF (Akerfelt et al. 2010). For example, heat shock treatment induces about 21 HSFs in *Arabidopsis thaliana* (Swindell et al. 2007) and at least 15 HSFs in tomato (von Koskull-Döring et al. 2007). Based on the peculiarities of the oligomerization domains, plant HSFs are classified into three conserved evolutionary classes: A, B, and C (Scharf et al. 2012). Among these three groups, class A has shown to form a regulatory network during response to heat stress, while class B or C has not shown any evident activities of transcription activators of their own (Czarnecka-Verner et al. 2004; Kotak et al. 2004).

Although low- and high-molecular-weight HSPs are the most important stress proteins toward heat stress tolerance, there are a number of other proteins that are involved in the HSR. Among those ubiquitin, cytosolic Cu/Zn-SOD, Mn-POD, Pir proteins, and dehydrins are well known. These proteins play roles in minimizing dehydration and oxidative damages, chloroplast stability, and prevention of protein degradation (Iba 2002; Khanna-Chopra and Sabarinath 2004; Schoffl et al. 1999; Yun et al. 1997). They act as chaperones to fold and unfold cellular proteins in order to inhibit adverse effects of high temperatures on the functional sites. The expression of these proteins can be specified to organelles and tissues (Wahid et al. 2007). A main function of these proteins is protection of cellular and subcellular structures against dehydration and oxidative damages (Schoffl et al. 1999).

5.2.3.3 Heat Stress Signaling

There are multiple signaling pathways involved in the HSR, in which some of them control expression and synthesis of HSPs, whereas others regulate the production or activation of different effector constituents (Fig. 5.3). Due to the complexity of multigenic traits, the molecular pathways involved in the HSR in plants are not fully understood. Generally, several signal transduction cascades are triggered by high temperature perception, which all together contribute in the activation of several transcription factors, including HSFs, that bind to the HSE and induce expression of HSPs, regulatory proteins, and proteins involved in metabolism and redox homeostasis (Hu et al. 2009).

A primary signaling for induction of the HSR is the role of calcium-mediated signaling. Increases in temperature are sensed at the plasma membrane (PM) by a

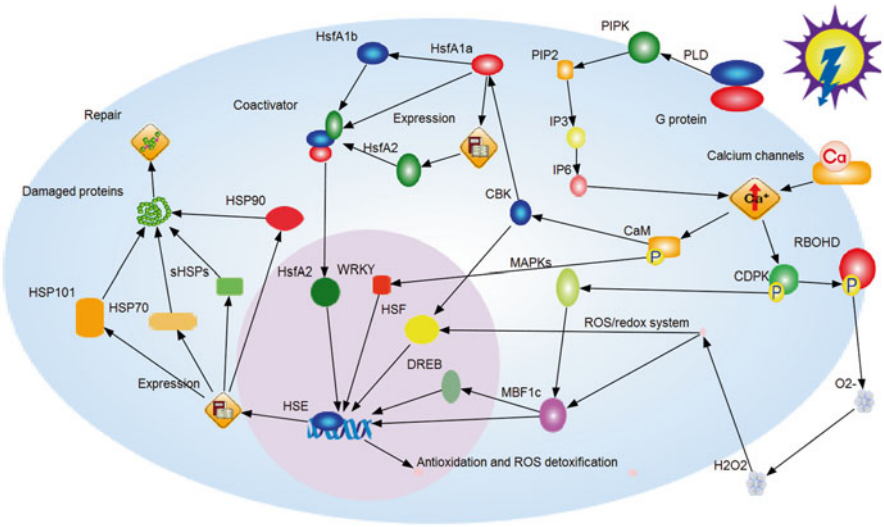

Fig. 5.3 Overview of signaling pathways and factors involved in heat shock response. High temperatures affect PM and change membrane stability, which in turn causes activation of both lipid signaling and calcium channel located in PM. These activations result in an influx of calcium into the cytosol. Calcium can bind to calmodulin and activate multiple kinases and transcriptional factors, such as CBK, HSFs, and WRKY. Calcium can also lead to phosphorylation of CDPK, which activates the MAPKs signaling pathway. Another protein activated by calcium is ROS-generating NADPH oxidase (Respiratory burst oxidase homolog D-RBOHD) located in the PM (*middle right*). RBOHD-derived ROS triggers the ROS/redox signaling system, which in turn activates MBF1c and HSFs. The most-characterized part of the network contains heat stress transcription factors (HSFs) that regulate genes encoding heat stress proteins (HSPs), which act as molecular chaperones and repair damaged proteins (*left*) (Mittler et al. 2012; Qu et al. 2013; Sung et al. 2003)

specific calcium channel that serves as one of the primary heat sensors in plants. Activation of this membrane protein, which triggers the influx of calcium into the cytoplasm, may be due to heat-induced increase in fluidity of the PM (Saidi et al. 2011). Under heat stress, changes in membrane fluidity trigger the activation of phospholipase D (PLD) and phosphatidylinositol-4-phosphate 5-kinase (PIPK), which results in the accumulation of different lipid signaling molecules, including phosphatidylinositol-4,5-biphosphate (PIP_2), myo-inositol-1,4,5-trisphosphate (IP_3), and phosphatidic acid (PA). A G protein associating with PM perceives the heat signal, and leads to accumulation of PIP_2 and PA. PIP_2 and PA act as key mediators in the lipid signaling pathway, cytoskeletal organization, and membrane dynamics (Mishkind et al. 2009; Zheng et al. 2012). IP_3, a product of PIP_2 hydrolysis, is rapidly converted to IP_6, which is responsible for the opening of calcium channels and stimulates the Ca^{2+} influx into the cell (Mishkind et al. 2009; Zheng et al. 2012).

The heat-induced influx of Ca^{2+} can trigger multiple signaling pathways in plants (Larkindale and Knight 2002; Saidi et al. 2009). Under high temperatures, the cytosolic Ca^{2+} is sharply increased, which ultimately leads to protein phosphorylation and activation of various heat stress transcriptional factors (Saidi et al. 2011). One of the targets that cytosolic Ca^{2+} can bind to is calcium-dependent protein kinase (CDPK), which can in turn, activate mitogen-activated protein kinase (MAPK), or the ROS-producing enzyme NADPH oxidase (RBOH). MAPK signaling pathways are highly conserved module involved in many responses to external signals, such as different abiotic stresses (Ichimura et al. 2000). CDPK can also lead to the activation of multiprotein-bridging factor 1c (MBF1c), a transcriptional regulator of the dehydration-responsive element-binding (DREB) transcription activator and several HSFs (Suzuki et al. 2011). High temperatures induce the production of DREB2A and DREB2B, which function during both heat and drought stresses. It has been shown that overexpression of DREB2A in Arabidopsis enhances thermotolerance (Qin et al. 2011). Calmodulin (CaM) is another calcium sensor protein that mediates Ca^{2+} signal transduction. In Arabidopsis, AtCaM3 increases thermotolerance by activation of several transcription factors such as WRKY39 and HSFs. AtCaM3 also activates calmodulin-binding protein kinase (CBK), which phosphorylates HSFA1a (Liu et al. 2008; Zhang et al. 2009).

It has been shown that obstruction of Ca^{2+} signaling by calcium channel blockers intensifies heat-induced oxidative damage in Arabidopsis (Larkindale and Knight 2002; Saidi et al. 2009). Heat stress is often accompanied by different degrees of oxidative stress, representing the presence of a cross-talk between heat and oxidative stress pathways. A short exposure to high temperatures can induce a significant increase in H_2O_2 levels due to NADPH oxidase activity. The histidine kinases sense the ROS signal and transduce it to HSFA4a, which in turn, activates downstream transcription factors by correlating with a MAPK signaling pathway. The transcription factors involved in the oxidative pathway are Zat, WRKY, MBF1c, and RBOH. Zat is necessary for the expression of APX and WRKY (Baniwal et al. 2007). MBF1c regulates the expression of SA and trehalose, which are important factors in the plant defense response (Suzuki et al. 2008). RBOH enhances the production of ROS signaling through the oxidation of NADPH (Miller et al. 2008). MAPK signaling pathways activate redox-sensitive transcription factors, which bind to the oxidative-sensitive *cis* elements in the gene promoter. These transcription factors enhance expression of antioxidants, including APX and catalase (CAT) that act as ROS scavengers under heat stress (Gill and Tuteja 2010).

Moreover, production of H_2O_2 stimulates the expression of heat shock response genes through activation of HSFs. Ultimately, HSPs' production can achieve heat stress tolerance by acting as chaperons to protect protein synthesis (Kotak et al. 2007). Studies on H_2O_2 pretreatment, NADPH oxidase (Larkindale et al. 2005), and mitochondrial respiratory mutants have confirmed the relationship between ROS generation and induction of HSP synthesis; however, ROS roles in regulating HSPs expression under heat stress is poorly understood (Kotak et al. 2007; Kuzmin et al. 2004).

5.2.3.4 The Molecular and Genomic Responses to Heat Stress

Genetic studies on heat-tolerant crop plants have shown that tolerance to heat stress is a multigenic feature. Different sets of genes are involved in control of different heat tolerance components in various tissues at diverse developmental stages (Howarth 2005). Due to this complexity, many biochemical reactions involved in the molecular pathways toward heat tolerance are not yet understood completely. However, newly developed techniques in biotechnology have bestowed a better understanding of molecular and genetic basis of heat tolerance. Microarray technology, one of recent techniques, provides the possibility of simultaneous analysis of numerous genes. This approach has been widely used for identification of genes encoding Hsps and Hsfs in some model plants. For example, in *Arabidopsis*, 18,7, 8, 27, and 21 genes have been discovered for Hsp70, Hsp90, Hsp100, sHsp, and Hsf, respectively (Guo et al. 2008; Hu et al. 2009).

Whole-genome microarray studies on heat-stressed plants have revealed fast and global effect of heat stress on the transcriptome. In *Arabidopsis*, approximately 11 % of the screened genes show differential expression after 1 h of exposure to high temperatures (Busch et al. 2005). The hallmark of HSR is up-regulation of well-characterized HSPs such as Hsp70, Hsp101, and sHSPs as well as HSF (Larkindale and Vierling 2008). Additional genes have also been identified, and their expression is elevated under heat stress and are linked with stress-protective responses; these include cell respiration and oxidative stress response (Table 5.1). For example, elevated expression of genes encoding mitochondrial proteins, such as cytochrome c oxidase and subunits of NADH dehydrogenase, is associated with enhanced respiratory activity and was detected in heat-stressed plants along with elevated expression of APX, a defense enzyme regulated by HSFs, correlates with cell protection against reactive oxygen intermediate (Rizhsky et al. 2004). In other genes, their expression increases dramatically with high temperatures, and include galactinol synthase, peptidyl prolyl isomerases, enzymes in the raffinose oligosaccharide pathway and energy metabolism particularly in glycolysis, and members of DREB family of transcription factors. In addition, cluster analysis of gene expression has shown up-regulation of many genes with HSE (GAAnnTTC) (Larkindale and Vierling 2008). The HSEs located in the promoter of heat shock genes play a key role in the expression of a group of heat-inducible genes through interaction with HSFs (Hua 2009). Other common promoter motifs associated with up-regulated genes are site II motif, DRE, and ABRE (ABA response element, ACGTG). The oxidative stress-related genes, endoglucan transferase and xyloglucan endotransglycolsylases are among up-regulated genes containing ABRE sequence.

During heat stress, expression of many genes is also down-regulated. The disease resistance genes, expansions, cytochrome P450s, auxin-induced genes, and genes involved in cell detoxification (mainly glutathione *S*-transferases) are some of the genes whose expressions are greatly reduced under high temperature (Larkindale and Vierling 2008). The cluster analysis shows the presence of W-box and TATATA

Table 5.1 Representative genes that are up-regulated under heat stress (partly adapted from Lim et al. 2006; Rizhsky et al. 2004)

Gene symbol	GenBank Accession No.	Gene description
HSP18.2	At5g59720	Heat shock protein 18
HSP23.6-MITO	At4g25200	Mitochondrion-small heat shock protein
HSP17.4	At3g46230	Heat shock protein 17.4
HSP21	At4g27670	Heat shock protein 21
HSP17.6	At1g53540	Heat shock protein 17.6
HSP17.6II	At5g12020	Heat shock protein 17.6-II
HSP17.6A	At5g12030	Heat shock protein 17.6A
HSP70	At3g12580	Heat shock protein 70
HSP101	At1g74310	Heat shock protein 101
ATHSP22.0	At4g10250	Heat shock protein 22.0
HSP20	AT2G29500	HSP20 family protein
Hsp89.1	At3g07770	Heat shock protein 89.1
HSP90.1	At5g52640	Heat shock protein 90.1
HSP15.7	At5g37670	Cytosolic class I small heat shock protein
DREB2B	At3g11020	Dehydration-responsive element-binding protein 2B
MBF1C	At3g24500	Multiprotein-bridging factor 1c
NF-X1	AT1G10170	Nuclear transcription factor, X-box binding 1
bZIP28	AT3G10800	Putative bZIP transcription factor
BIP2	AT5G42020	Luminal-binding protein 2
RBL14	At3g17611	Rhomboid family protein
BAG6	At2g46240	BAG domain-containing protein
ROF2	At5g48570	Peptidylprolyl isomerase
MATR	AtMg00520	Maturase
APX2	At3g09640	Putative ascorbate peroxidase
NAD5C	AtMg00513	NADH dehydrogenase subunit 5
NAD6	AtMg00270	NADH dehydrogenase subunit 6
NAD4L	AtMg00650	NADH dehydrogenase subunit 4L
GolS1	At2g47180	Galactinol synthase
HAI1	At5g59220	ABA-induced protein phosphatase 2C
GSTF8	At2g47730	Glutathione S-transferase phi 8
MXC9.7	AT5G12110	Elongation factor 1B alpha-subunit
TIC20-IV	At4g03320	Putative chloroplast import component
F14G24.14	At1g52870	Peroxisomal membrane protein-related
T3P18.7	At1g62510	Similar to 14-kDa Pro-rich protein
COX1	AtMg01360	Cytochrome c oxidase subunit 1
COX2	AtMg00160	Cytochrome c oxidase subunit 2
F28P22.15	AT1G72660	Developmentally regulated G-protein 2
HSP70T-2	At2g32120	70-kDa Heat shock protein
CDC48D	At3g53230	Cell division control protein 48-D
T13C7.15	AT2G20560	Putative heat shock protein

(continued)

Table 5.1 (continued)

Gene symbol	GenBank Accession No.	Gene description
T9A21.130	AT4G18280	Gly-rich cell wall protein
CCMFN2	AtMg00960	Cytochrome c biogenesis
F22G5.31	AT1G07350	Transformer serine/arginine-rich ribonucleoprotein
QCR7-2	AT5G25450	Ubiquinol-cytochrome-c reductase
AHP4	At3g16360	Putative two-component phosphorelay mediator
SMP1	At1g65660	Step II splicing factor SLU7
HSFC1	At3g24520	Heat shock transcription factor HSF1
CYP94B3	At3g48520	Cytochrome P450-like protein
T1K7.5	At1g26580	Putative MYB family transcription factor
MAPKKK19	At5g67080	Mitogen-activated protein kinase kinase kinase 19
ATSRP30	At1g09140	Serine-arginine rich RNA binding protein
RANBP1	At5g58590	Ran binding protein 1 homolog
rpl16	ArthCp060	Ribosomal protein L16
CPN60B2	AT3G13470	Chaperonin 60 beta
GI	At1g22770	Putative gigantea protein
AT4G22590	At4g22590	Trehalose-6-phosphate phosphatase
RAS1	At1g09950	Response to ABA and salt 1
T11I18.11	At3g04000	Short-chain type dehydrogenase/reductase
ATP9	AtMg01080	ATP synthase subunit 9
T19K4.140	At4g36010	Pathogenesis-related thaumatin family protein
AT3G59350	At3g59350	PTI1-like tyrosine-protein kinase 3
P5CR	At5g14800	Pyrroline-5-carboxylate reductase
TIL	At5g58070	Outer membrane lipoprotein
BIP1	At5g28540	Luminal binding protein
CRT1a	At1g56340	Calreticulin (crt1)
CCMFC	AtMg00180	Cytochrome c biogenesis
COR47	AT1G20440	Dehydrin (COR47)
LGT8	At1g70090	Putative galacturonosyltransferase-like 9
T3K9.7	AT2G41160	Ubiquitin-associated protein
SAY1	AT4G11740	Ara4-interacting protein, putative
PRO5	At5g56600	Profilin 5

motifs in the promoter of down-regulated genes indicating their similar patterns of regulation (Molina and Grotewold 2005; Raffaele et al. 2006).

Expression data suggest that HSFs play important roles in regulating HSR, although the function of each one of these components is not yet fully understood (Guo et al. 2008). Previous studies have shown that the expression of certain HSF genes in plants is induced by environmental stresses, while others display constitutive expression. Among all 21 HSF genes in *Arabidopsis*, only six of them show significant increases in expression level under heat stress. Furthermore, it has been demonstrated that HSFs control the expression of HSPs, genes involved in

protective environmental stress response as well as other HSFs (Busch et al. 2005; Rizhsky et al. 2004). Genome-wide transcriptome analysis of different HSF knockout mutants has been used to elaborate the details of HSF functions. Such studies on *Arabidopsis* suggest that HSFA1a and HSFA1b play key roles in the initial phase of HSR (Busch et al. 2005) while HSFA2 functions in the later recovery phase and under prolonged heat stress condition (Schramm et al. 2006). In tomato, HSFA1a gene is constitutively expressed and induces expression of HAFA2 and HSFB1 under heat stress (Mishra et al. 2002). Although in tomato HSFA1a acts as a nuclear retention factor and a coactivator of HSFA2 by forming HSFA1a–HAFA2 heterooligomeric complexes, in *Arabidopsis* the heat stress-induced expression of HSFA2 is not influenced by either HAFA1a or HSFA1b (Bharti et al. 2004; Busch et al. 2005). Moreover, it has been shown that under high light intensity, HSFA2 regulates the expression of ascorbate peroxidase 2 (APX2), which encodes an important enzyme in oxidative stress response (Sakuma et al. 2006). These findings indicate that HSFA2 plays different roles under diverse abiotic stresses. It has been shown that the expression of *Arabidopsis* HSFA3 under heat stress is directly regulated by DREB2A, a transcription factor that interacts with *cis*-acting dehydration-responsive element (DRE) in drought and salt stress responses (Sakuma et al. 2006). In addition, the presence of DRE in the promoter of a cluster of heat-inducible genes has been demonstrated (Larkindale and Vierling 2008).

There are numerous genes and key regulators involved in heat stress tolerance, which remain to be discovered. Several biotechnological techniques, including transcriptome analysis, proffer the capability for discovering novel genes involved in the stress responses. Through these studies, additional heat-inducible transcription factors have been found that are related to thermotolerance. One of them is NF-X1 (nuclear transcription factor x-box binding 1) gene that promotes tolerance to salt and heat stress and shows a similar induction pattern to genes with DREs in their promoter (Larkindale and Vierling 2008). Heat tolerance may also be accelerated with induction of bZIP28 gene (a putative membrane-tethered transcription factor), since *Arabidopsis* plants harboring mutations in this gene exhibit a heat-sensitive phenotype (Gao et al. 2008). The transcriptional coactivator MBF1c, which accumulates rapidly after heat stress, is involved in several stress responses. MBF1c promotes thermotolerance by regulating several signaling pathways including salicylic acid (SA), ethylene, and trehalose during heat stress (Suzuki et al. 2008).

Among environmental stresses, high temperatures and water deficits are two main factors causing severe yield loss. Furthermore, simultaneous occurrence of different stresses is common in the field, such as high temperatures and drought periods particularly in semi-arid and arid areas. In order to develop multiple stress-tolerant crops through genetic manipulation, it is necessary to understand the molecular mechanism underlying the response of crop plants to the combination of abiotic stresses. Studies on tobacco, Arabidopsis, and wheat suggest that the response to the combination of stresses is very different from each individual stress (Rampino et al. 2012; Rizhsky et al. 2002, 2004). For example, a comparison of the effects between heat, drought, and combined stress on wheat plants showed a higher number of up-regulated genes in combined stressed plants with respect to each individual.

These results indicate that combined stress induces a separate set of genes, which are not activated by each individual stress (Rampino et al. 2012; Rizhsky et al. 2004). It has been suggested that combined stress induces the activation of a specific genetic program that is mediated by key regulators (Rizhsky et al. 2002).

In the last decade, results obtained from different microarray experiments have been gathered in several genome-wide microarray datasets. These resources can be utilized in analyzing the response of Hsf and Hsp expression under different abiotic stresses. Many studies have focused on such responses to unlock the relation between different genes activated by diverse abiotic stresses (Hu et al. 2009; Rampino et al. 2012; Rizhsky et al. 2004). The results have shown that, while there are extensive overlapping response of Hsp and Hsf under different stresses, some genes show specific response to distinct stresses. For example, in a study on rice, the number of genes expressed under heat, cold, salt and drought stresses were 1,054, 276, 1,200, and 2,742 while the number of overlapped genes between heat and each one of cold, salt and drought were 33, 127, and 240 (Hu et al. 2009). Although the expression pattern is different under all these stresses, the overlapping response of Hsfs and Hsps implies their importance in cross-talk of stress signal transduction networks. Due to the fact that there is an urgent need for developing multiple stress-resistant crops to combat adverse effects of global warming, it is important to detect co-regulators of these overlapped genes (Hu et al. 2009). These results indicate that activation of similar HSFs during different environmental stresses leads to induction of similar responses. Although this fact has formed the fundamentals of many trials for developing multiple stress-resistant plants, these attempts have not yet reached to produce fruitful results due to the complexity of signal transduction in different environmental stresses.

5.3 Plant Adaptation to Heat Stress

The adaptation to heat stress or "heat tolerance" is commonly defined as plant ability to grow and produce economic yields under high temperatures. During evolution, plants have adapted to harsh environmental conditions by developing different stress-tolerance mechanisms. These mechanisms can be divided into long-term changes, such as phenological and morphological adaptations, or short-term acclimation, such as transpiration cooling, changing membrane lipid compositions, or leaf orientation (Adams et al. 2001). For example, plants growing in hot climate avoid heat stress by changing the orientation of leaf blades away from light, completing the entire reproductive cycle during cooler months, or developing small hairs (tomentose), small leaves, and heat-resistant buds (Fitter and Hay 2001). In well-hydrated plants, intensive transpiration keeps leaf temperature below ambient level and prevent heat stress. Such phenological and morphological adaptations are commonly linked with biochemical adaptations, such as net photosynthesis (Fitter and Hay 2001). Heat tolerance can also be induced in plants by prior treatment with high temperatures. This heat acclimation also lead to activation of the heat

responsive molecular mechanisms, particularly the accumulation of HSPs (Hua 2009; Kotak et al. 2007).

Since plants are sessile organisms, their behavioral responses to abiotic stresses are strongly dependent on cellular and physiological mechanisms of adaptation. The adaptation mechanisms may differ based on different environmental stresses, developmental stages, or tissue types (Queitsch et al. 2000). Changes in temperature stimulate downstream signal transduction pathways to activate stress tolerance mechanisms to restore homeostasis and repair damaged proteins and membranes (Bohnert et al. 2006b; Vinocur and Altman 2005). Some major adaptive mechanisms activated under heat stress include HSPs, free-radical scavengers, osmoprotectants, and ion transporters (Wang et al. 2004). Heat stress manifests its initial effects on plasma membrane by inducing more fluidity of lipid bilayer. This stimulates the induction of calcium influx and cytoskeletal rearrangement, leading to upregulation of mitogen MAPKs and CDPK. These proteins activate other mediators in the pathway, resulting in activation of tolerance responses, including production of antioxidant to cope with ROS produced in the organelles (such as mitochondria and chloroplast), or osmotic adjustment by production of compatible osmolytes for cell water balance (Bohnert et al. 2006b; Saidi et al. 2011; Sung et al. 2003). The antioxidant defense mechanisms play an essential role in the heat stress adaptation, and its strength associates with acquisition of thermotolerance (Hasanuzzaman et al. 2013). Also, higher ascorbic acid content or activities of CAT and SOD correlate with the capacity to acquire thermotolerance (Ara et al. 2013; Sairam and Tyagi 2004).

Heat tolerance also associates with higher degree of membrane lipid saturation, which increases the lipid phase transition (melting) temperatures, and inhibits membrane's liquidity due to heat stress (Kotak et al. 2007). Under heat stress, some plant species may accumulate different types of osmolytes, including proline, sugars, sugar alcohols (polyols), tertiary sulfonium compounds, and tertiary and quaternary ammonium compounds, which may enhance heat tolerance (Singh and Grover 2008). For example, proline may buffer the cellular redox potential under environmental stresses including heat stress (Wahid and Close 2007).

Another mechanism of thermotolerance is induction of HSPs and other heat-induced proteins, such as dehydrins, LEA, Pir proteins, and ubiquitin. These proteins are involved in protein degradation pathway, oxidative stress, and protection against adverse effects of dehydration (Arora et al. 1998; Goyal et al. 2005; Schoffl et al. 1999; Yun et al. 1997). HSPs act as molecular chaperones, which facilitate removing of misfolded proteins and refolding of denatured proteins. As main components in the network of chaperon machinery, they also interact with other stress-response mechanisms, such as production of antioxidant and osmolytes. They also play a role in stress signal transduction, gene activation, and regulation of cellular redox state. HSPs confer heat tolerance to plant by improving physiological mechanisms, such as membrane stability, efficiency of assimilate partitioning, photosynthesis, and water and nutrient utilization (Al-Whaibi 2011; Kotak et al. 2007).

Alteration of gene expression is an important factor in acquisition of thermotolerance. Heat stress rapidly alters the pattern of gene expression by induction of HSPs expression, and inhibition of non-heat-induced genes through destabilization of their mRNA. Induction of HSPs expression is regulated at the transcriptional level by HSFs, which specifically bind to the HSE existing in the promoter of HSP genes (Scharf et al. 2012). Although the mechanisms leading to preferential up-regulation of the stress-responsive genes, such as HSPs are still unclear, it has been suggested that HSFs are central component in heat stress signaling (Scharf et al. 2012).

5.4 Biotechnological Approaches in Developing Thermotolerant Plants

Due to gradual increase in atmospheric greenhouse gases, global warming is expected to have significant impact on ecological system in the coming years. A report on region and emission scenarios (B1, A1B, and A2) has speculated that the extreme annual daily maximum temperature will likely increase by about 1–3 °C by mid-twenty-first century and by about 2–5 °C by the late twenty-first century (IPCC 2012). This rising temperature will have adverse effect on plant growth and vegetation. The growing population and subsequent increase in food demand also creates pressing needs to develop crop varieties with higher yields.

Unlike animals, plants are static and are very susceptible to different environmental stresses like drought, temperature, salinity, etc. However, some plants growing in adverse environment had evolved several strategies to cope with it. For example, expression of HSPs and osmolytes, changes in lipid membrane permeability, and production of super radicals detoxifying enzymes (Hasanuzzaman et al. 2013; Wahid et al. 2007). Understanding the molecular mechanism of thermal tolerance in plants will help to identify key genes, proteins, and different metabolites to develop heat stress-tolerant crop plants (Hasanuzzaman et al. 2013).

HSR is a complex phenomenon and current advances in X-omics (genomics, transcriptomics, epigenomics, proteomics, and metabolomics) studies have shown the involvement of different genes, proteins, and metabolites in thermotolerance as reviewed in Bokszczanin et al. (2013). Interestingly, small non-coding RNA has also been found to regulate plant stress response (Ruiz-Ferrer and Voinnet 2009; Sunkar et al. 2007). The difference in quantity of small RNAs in *A. thaliana* in response to various abiotic stresses was also reported (Sunkar and Zhu 2004).

The negative impact of high temperature on agriculture has been already observed (Hatfield et al. 2011; Lobell et al. 2011) and further loss may be more to global warming trends. The knowledge of conventional breeding and biotechnological strategies like marker-assisted selection (MAS) and genetic transformation is crucial to generate thermotolerant plant today than before. Realizing the potential of heat-tolerant plants to sustain the food demand, great efforts are being made to develop heat resistance plants.

5.4.1 Genetic Transformations

Several genetic and biochemical studies have revealed proteins encoded by certain genes or biomolecules governing thermotolerance properties of plants in certain developmental stage or plant parts. With the advent of new genetic transformations techniques, production of transgenic plants with enhanced thermotolerance was made possible. Heat stress affects almost all system of plants including its morphology, anatomy, physiology, growth, and reproduction as reviewed before (Bokszczanin et al. 2013; Wahid et al. 2007). In a similar way to any other organism, plants do also have the ability to adapt to environmental heat stress (Larkindale et al. 2005). Plants have known to use different strategies to cope with this stress by producing different chaperones, osmolytes, and secondary metabolites as reviewed (Bokszczanin et al. 2013; Wahid et al. 2007). Knowledge of genes or metabolites involved in abiotic stress response or tolerance is very crucial to develop a stress tolerance plant. Systematic study of such correlations has been made possible through advancement in different techniques including all kind of omics and high-throughput next generation sequencing. A general overview of genetic analysis of HSR and its application to develop a thermotolerant crop plant is represented in Fig. 5.4.

In addition to genetic transformation, some other strategies to develop thermotolerant plants are also known. One of them is pretreatment of seeds and plants for

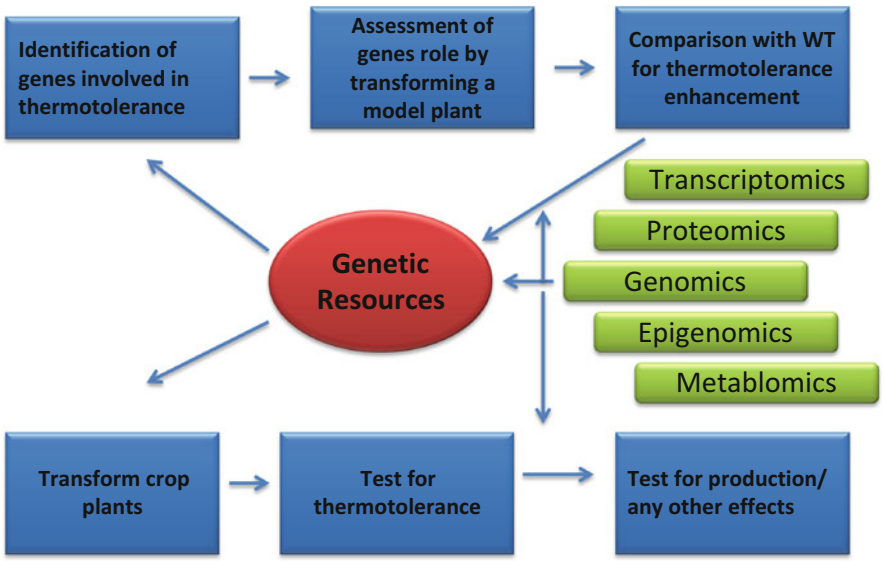

Fig. 5.4 General process for heat-tolerant gene identification and to generate thermotolerant crop plant

heat tolerance. Pretreatment of seeds and plants is a simple and fast approach to induce heat tolerance in plants. Despite the fact that genetic approaches tend to increase heat tolerance in plants, it may also suffer from low product yield. Pre-exposure to heat stress or addition of osmolytes can be used to increase thermotolerance in already high-yielding plant cultivars (Wahid et al. 2007). While genetic engineering and cross-breeding approaches may take years to produce high yielding heat-tolerant cultivars, preconditioning of plants could address immediate need to increase thermotolerance. For example, pre-sowing heat treatment of black spruce seedlings resulted in increased thermotolerance (Colclough et al. 1990). Similarly, barley seeds pre-treated with glycinebetaine, a low-molecular-weight osmolyte, demonstrated reduced membrane damage and higher photosynthetic rate alleviating effect of heat stress compared to control seeds (Wahid and Shabbir 2005). Under abiotic stress, Ca^{2+} acts as antioxidant in plants. Thus exogenous application of $CaCl_2$ prior to heat treatment has shown to increase activity of super-radical scavenging enzyme including catalase, SOD, guaiacol peroxidase, that help to induce heat tolerance in plants (Kolupaev et al. 2005). Exogenous application of some of the hormones and hormone precursors was also found to induce heat stress tolerance. So far, use of ABA, salicylic acid (SA), and ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was reported to improve the thermotolerance of *A. thaliana* by providing protection against oxidative damage caused from heat stress (Larkindale and Knight 2002).

Conventional breeding and MAS are other commonly used techniques for developing thermotolerant plants. These techniques have their own advantages, however, they are limited only to similar or related plant species. Heat tolerance in plants is a multigenic trait involving synergistic effect of multiple gene and its products (Wahid et al. 2007). This multigenetic nature of the process is one of the main challenges to generate a thermotolerant plant by genetic engineering. Furthermore, a plant may be more susceptible to a particular developmental stage and a specific tissue may be more prone to damage due to heat stress. So, a tissue or developmental stage-specific thermotolerance strategy could play crucial role for plant thermotolerance (Bohnert et al. 2006a; Howarth 2005). Understanding genetic backgrounds conferring plant heat tolerance would be keys to develop heat-tolerant crop cultivars with horticulture or agronomic importance. MAS is a traditional breeding technique. This technique is considered as another valuable way to develop plant with enhanced thermotolerance (Foolad 2005). Some of the quantitative trait loci (QTL) associated with heat tolerance has been identified in different plant species (Maestri et al. 2002). Four QTLs related to thermotolerance were characterized in *Arabidopsis* by creating heat-sensitive mutant library (Hong and Vierling 2000).

Genetic transformation has its own advantages over simplicity of above methods. Genetic engineering is specific and wide open in terms of genetic resources to prokaryotic to eukaryotic organisms. Some of the successful genetic transformation to improve thermotolerance in plant is represented in Table 5.2. Some of the most used strategies in developing a thermotolerant plant are described below.

Table 5.2 Examples of production of transgenic plants for thermotolerance

Genes	Sources	Transformed plant	Role	References
Athsf1	*A. thaliana*	*A. thaliana*	Heat shock factor	Lee et al. (1995)
AtHsfA2	*A. thaliana*	*A. thaliana*	Heat shock factor	Li et al. (2005)
OsHSFA2e	*O. sativa*	*A. thaliana*	Heat shock factor	Yokotani et al. (2008)
HsfA1	*S. lycopersicon*	*S. lycopersicon*	Heat shock factor	Mishra et al. (2002)
mtHsp70	*O. sativa*	*O. sativa*	Heat shock protein	Qi et al. (2011)
TLHS1	*N. tabacum*	*N. tabacum*	Chaperone	Park and Hong (2002)
sHSP	*S. lycopersicon*	*N. tabacum*	Chaperone	Sanmiya et al. (2004)
hsp101	*A. thaliana*	*O. sativa*	Chaperone	Katiyar-Agarwal et al. (2003)
AtP5CR	*A. thaliana*	*G. max*	Proline synthesis	De Ronde et al. (2004)
HvaPX1	*Hordeum vulgare*	*A. thaliana*	ROS-scavenging enzyme	Shi et al. (2001)
Cu/Zn SOD	*Manihot esculenta*	*S. tuberosum*	ROS-scavenging enzyme	Tang et al. (2006)
APX	*Pisum sativum*			
GASA4	*Zea mays*	*A. thaliana*	ROS scavenging	Ko et al. (2007)
OsAKR1	*O. sativa*	*N. tabacum*	Oxidative stress	Turóczy et al. (2011)
AtGRXS17	*A. thaliana*	*S. lycopersicon*	Oxidative stress	Wu et al. (2012)
badh	*Spinacia oleracea*	*N. tabacum*	Osmolyte production	Yang et al. (2005)
pan D	*E. coli*	*N. tabacum*	Osmolyte production	Fouad and Rathinasabapathi (2006)
fad 7	*A. thaliana*	*N. tabacum*	Lipid metabolism	Murakami et al. (2000)
fad 7	*A. thaliana*	*O. sativa*	Lipid metabolism	Sohn and Back (2007)
BnTR1	*B. napus*	*O. sativa*	Membrane protein	Liu et al. (2014)
OsAREB1	*O. sativa*	*A. thaliana*	Transcriptional factor	Jin et al. (2010)
GmGBP1	*Glycine max*	*N. tabacum*	Transcriptional factor	Zhao et al. (2013)
ZFP177	*O. sativa*	*N. tabacum*	Stress-associated protein	Huang et al. (2008)
OsMYB55	*O. sativa*		Transcriptional factor	El-Kereamy et al. (2012)
Dnak I	*A. halophytica*	*N. tabacum*	Salt tolerance	Ono et al. (2001)

5.4.2 *Genetic Transformation Using Genes for HSPs and Heat Shock Factors*

Initiation of HSPs production is one of the first lines of response to heat stress in most organisms ranging from prokaryotes to eukaryotes. Both sudden or a gradual raise in temperature enhanced the production of HSPs in plants (Nakamoto

and Hiyama 1999; Schoffl et al. 1999). HSP's production and accumulation in plants and its thermotolerance are clearly evident from numerous research works as reviewed in Bokszczanin et al. (2013), Singh and Grover (2008), and Wahid et al. (2007). Similarly, transcription factors regulating the expression of different HSPs (heat shock factors, HSFs) are also directly involved in thermotolerance. To date, different genetic engineering work to increase the production of different HSFs and HSPs to improve thermotolerance in plant has been reported as reviewed in Bokszczanin et al. (2013), Singh and Grover (2008), and Wahid et al. (2007).

Genetic transformation of *A. thaliana* with different HSFs such as Athsf1, Athsf3, and AtHsfA2 under CaMV35S promoter was reported to improve thermotolerance (Lee et al. 1995; Li et al. 2005; Prändl et al. 1998). Similarly enhanced thermotolerance was reported in *A. thaliana* expressing transcription factor gene OsHSFA2e from *O. sativa* (Yokotani et al. 2008). Constitutive expression of hsp101 gene in *A. thaliana* was shown to respond better to sudden changes in high temperature than controls (Queitsch et al. 2000). Also, when *Arabidopsis* hsp101 gene was expressed in *Oryza sativa* under maize Ubi 1 promoter, improved thermotolerance was observed (Katiyar-Agarwal et al. 2003).

5.4.3 Genetic Transformation Using Genes Involved in ROS Scavenging System

ROS produced in response to heat stress mainly causes oxidative damage to cellular components. So, different ROS scavenging enzyme systems have been used to reduce ROS and to produce thermotolerant plant. For instance, transgenic potato (*Solanum tuberosum*) plants expressing APX and Cu/Zn superoxide dismutase under oxidative inducible promoter (SWPA2) showed increased heat tolerance (Tang et al. 2006). Similarly, expression of HvAPX1 gene encoding APX under constitutive CaMV35S promoter also resulted in significant heat tolerance in *A. thaliana* compared to control plants (Shi et al. 2001).

5.4.4 Genetic Transformation Using Genes Involved in Synthesis of Different Osmolytes

Plants accumulate compatible solutes, low-molecular-weight organic compounds, which includes amino acids, quaternary amines, sugars, and polyols under stress conditions, especially to osmotic stresses (Gepstein et al. 2005). These osmolytes have been also known to involve and improve thermotolerance in different ways in plants. Therefore, overproduction of such a molecule is another strategy to develop plants with improved thermotolerance. For instance, expression of a bacterial

choline oxidase (CodA) gene in *A. thaliana*, which increase internal pool (biosynthesis) of glycinebetaine, a quaternary ammonium compound, was reported to have more thermotolerance than wild type plant (Alia et al. 1998). Betaine aldehyde dehydrogenase (BADH) is an enzyme involved in glycinebetaine biosynthesis. Tobacco plant with higher level of glycinebetaine after overexpression of BADH gene from spinach showed better thermotolerance during young seedlings growth (Yang et al. 2005).

But, the role of such molecules as protecting agent might represent just for one species-specific adaption and might not be similar for all (Bokszczanin et al. 2013). For instance, accumulation of proline in response to heat stress has been identified as a protective mechanism in some plant types but not all as reviewed by Bokszczanin et al. (2013). In soybean, heat stress and drought tolerance were improved using overexpression of *A. thaliana* pyrroline-5-carboxylate reductase (AtP5CR) gene. However, overexpression of the Δ(1)-pyrroline-5-carboxylate synthetase 1 (AtP5CS1) gene which improves the biosynthesis of proline was found to reduce the thermotolerance in case of *A. thaliana* (Lv et al. 2011).

5.4.5 Genetic Transformation Using Genes Involved in Membrane Fluidity

A major effect of heat stress in plants is change in membrane fluidity. Protein denaturation as well as elevated level of unsaturated fatty acids in lipid bilayer of biological system cause increase in membrane fluidity (Savchenko et al. 2002). Elevated level of enzyme fatty acid desaturases was found to involve in increasing membrane fluidity by catalyzing unsaturation of membrane lipid as reported before (Murata 1983) in response to low temperature. Similarly, membrane rigidity was reported to increase with increase in saturation of membrane lipids as observed in response to high temperature (Thomas et al. 1986). The information presented above shows the possibility of developing a thermotolerant plant by altering the lipid membrane saturation. Lowering of unsaturation by lowering the amount of trienoic fatty acids than dienoic fatty acids in chloroplast membrane of a transgenic tobacco resulted in better photosynthesis and growth under heat stress (Murakami et al. 2000). In the above study, they achieved the lower unsaturation of chloroplast by silencing gene for chloroplast omega-3 fatty acid desaturase.

Plants growth as well as its yield is directly correlated with rate of photosynthesis, which in turn is severely affected by heat stress. One of the causes of photosynthesis inhibition in plant is thermal instability of Rubisco Activase, a chaperone protein required for proper functioning of Rubisco. Silencing of Rubisco Activase gene in tobacco plants has shown to increase plants' sensitivity to heat stress than in control plants (Sharkey et al. 2001). Thus, efforts can be made to develop heat-tolerant plant by improving the expression of Rubisco Activase.

5.5 Conclusion

Crop losses from heat stress have serious economic impacts. It has been estimated that heat stress may cause multi-billion dollar crop damage worldwide. Loss in agricultural productivity will have other negative consequences in farming sector including rise of food prices, and higher cost of livestock production. These effects are more pronounced in the developing countries. However, heat stress is inevitable and plants have already developed various molecular physiological strategies to combat heat stress. There are numerous genes and transcription factors that work in synchronized way to produce protective proteins. With the advent of climate change and global warming, research on heat stress will become more relevant. Understanding modes of heat tolerance in plants will help us to develop better thermotolerant food crops. Availability of next generation sequencing technologies will help us understand the genomic make up of heat-tolerant plants. High-throughput computing tools will help us to identify economically important thermotolerant genes. The next step would be genetic modification of plants with thermotolerant genes. Transgenic approach to produce thermotolerant plant may not be the answer to combat crop loss due to heat stress; however, it is surely one of the options for agricultural scientists.

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Chapter 6
The Omics of Cold Stress Responses in Plants

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Abstract Low temperature (LT) is a major threat that limits growth, development, and distribution leading to plant damage and crop losses. Plants respond to cold stress through a phenomenon known as cold acclimation, which is a complex process involving changes at multiple levels that include physiological and biochemical modifications, alterations in gene expression, and changes in concentrations of proteins and metabolites. Perception of cold stress by the cell membranes results in activation of cold-responsive genes and transcription factors that help in combating cold stress. Transcriptional responses to cold are guided by both ABA-dependent and -independent pathways that induce the expression of cold-regulated (*COR*) genes, thereby changing protein and metabolite homeostasis. Recent advances in the field of genomics, proteomics, and metabolomics has led to new discoveries, which has augmented our understanding of this intricate phenomenon. Here, we discuss the various aspects of cold stress responses in plants to develop a holistic understanding in the field of stress-mediated signaling.

Keywords Cold acclimation • Low temperature stress • C-repeat binding factor • Signal transduction

6.1 Introduction

Abiotic stresses adversely affect growth, productivity and survival of the plants, and hence are considered as key determinants of the crop losses worldwide. The human population is expected to touch 9.6 billion by 2050. To meet the food demand of this

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rapidly growing population, the agricultural production needs to be double by 2050. However, a continuous increase in the intensity, duration and unpredictability of abiotic stresses impose major limitation to achieve the required food production. Low temperature is one of the most intimidating abiotic stresses that affect the plant growth and development, thereby limiting the distribution of crop species. Based on its intensity, cold stress can be broadly classified into chilling and freezing stresses. Exposure to temperatures below 0 °C results in freezing stress, whereas chilling stress occurs at temperatures ranging from 0 to 20 °C. Plants such as rice, maize, and tomato that grow in tropical and subtropical regions are chilling sensitive whereas the plants from temperate region are chilling tolerant (Chinnusamy et al. 2007; Solanke and Sharma 2008). However, plants have the ability to acquire tolerance to chilling and freezing conditions if they are pre-exposed to non-freezing temperatures, a process known as cold acclimation (Levitt 1980). Cold acclimation helps plants to fine tune their metabolism and improve freezing tolerance by initiating signaling cascades that leads to several biochemical and physiological changes including modification of membrane lipid composition and changes in gene expression (Shinozaki and Yamaguchi-Shinozaki 1996; Thomashow 1998; Gilmour et al. 2000; Chinnusamy et al. 2003). The altered gene expression leads to accumulation of several protective proteins such as antifreeze proteins (AFPs) (Griffith et al. 1997), late embryogenesis abundant (LEA) proteins (Antikainen and Griffith 1997), heat shock proteins (HSP) (Wisniewski et al. 1996), cold-regulated (COR) proteins, and various metabolites such as amino acids, soluble sugars, organic acids, pigments (Krause et al. 1999), polyamines (Bouchereau et al. 1999), and antioxidants (Hausman et al. 2000). These metabolites and proteins help in protecting plant membranes and prevent cell disruption during cold stress by stabilizing the membrane lipids, proteins, maintaining the hydrophobic interactions, ion homeostasis and scavenging the reactive oxygen species (ROS) (Hare et al. 1998; Gusta et al. 2004; Chen and Murata 2008; Janska et al. 2010). In this chapter, we focus on the various aspects of cold acclimation such as physiological and biochemical effects of cold stress, cold sensing and signal transduction, cold-responsive pathway and role of various cold-responsive genes and transcription factors. We have discussed the progress in proteomic, metabolomics, and transcriptomic approaches that have been used to unravel the various biochemical pathways, cellular responses and complex gene interactions during low temperature stress in plants. These system approaches can give a deeper insight into the network of genes, proteins, and metabolites underlying the stress responses (Cramer et al. 2011).

6.2 Physiological Changes During Cold Stress

Plants respond variably to stress condition depending largely upon their phenological stages and the severity of cold they are subjected to. The various symptoms of chilling injury includes poor germination, stunted seedlings, surface lesions, water soaked appearance, desiccation, discoloration, tissue breakdown, and accelerated senescence among others (Sharma et al. 2005). Cold stress negatively influences reproductive development of plants resulting in flower sterility, as observed in rice at the time of anthesis (flower opening) (Jiang et al. 2002).

Freezing injury in most plants causes membrane damage due to severe cellular dehydration caused by ice formation in inter- and intracellular spaces. Since the chemical potential of ice is less than that of liquid water, extracellular ice formation leads to decrease in water potential outside the cell. Consequently, unfrozen water from inside the cell moves to the intercellular spaces resulting in cellular dehydration. This can potentially result in physical disruption of cells and tissues. Further, the magnitude of injury to the membrane also varies with the freezing temperature and the level of cellular dehydration (Steponkus et al. 1993). At temperatures between −2 to −4 °C, the major injury observed in non-acclimated plants is expansion-induced lysis caused by osmotic contraction and expansion. At lower temperatures of around −4 to −10 °C, the predominant form of injury in non-acclimated plants is freezing-induced lamellar-to-hexagonal-II phase transitions. Another form of injury is the fracture jump lesion that occurs at temperature below −10 °C between the plasma membrane and closely appressed cytoplasmic membrane (Webb and Steponkus 1993). Extreme cold also results in protein denaturation and solutes precipitation due to freezing-induced dehydration in plants (Uemura et al. 1995; Thomashow 1998; Salinas 2002). The lipid composition of plasma membrane is also altered significantly under cold stress (Steponkus et al. 1993; Uemura et al. 1995). Chilling stress also decreases the membrane fluidity by causing fatty acid unsaturation, altering lipid composition and ratio of lipids to proteins in cell membrane (Wang et al. 2006). An increase in the proportion of phospholipids during cold acclimation has been observed in various plant species (Uemura and Yoshida 1984; Uemura and Steponkus 1994; Ishikawa and Yoshida 1985; Uemura et al. 1995). In response to low temperature, the relative proportion of di-unsaturated phosphatidylcholine and phosphatidylethanolamine increases and that of mono-unsaturated phospholipids, cerebrosides and free sterols decreases in *Arabidopsis* (Uemura et al. 1995). Molecules of 16:0/16:0 phosphatidyl choline, phosphatidyl glycerol or cerebrosides undergo phase transition from a highly fluid liquid crystalline phase to the more rigid gel phase. The gel phase interferes with normal functioning of integral membrane protein in maintaining an efficient permeability barrier. These lead to ion leakage across the membrane and eventually cell dysfunction. Role of lipid unsaturation in cold tolerance was confirmed using transgenic tobacco plants expressing acyl-ACP:glycerol-3-phosphate acyl transferase (GPAT) from chilling-sensitive squash and chilling-tolerant *Arabidopsis*. Transgenic tobacco carrying GPAT from squash had higher levels of saturated phosphotidyl glycerol, while those with enzyme from *Arabidopsis* had decreased levels of saturated lipids (Murata et al. 1992). Transgenic tobacco expressing *Arabidopsis* ω-3 fatty acid desaturase (FAD7) resulted in higher levels of trienoic fatty acids and enhanced freezing tolerance (Kodama et al. 1994). Knockdown mutants of *fad2, fad5,* and *fad6* in *Arabidopsis* resulted in growth defect and chlorotic plants at low temperature (Hugly and Somerville 1992; Miquel et al. 1993). Mutation in acyllipid desaturase leads to increased sensitivity to chilling and freezing temperatures in *Arabidopsis* (Chen and Thelen 2013). Cold stress causes inactivation of H^+-ATPase in plasma membrane of chilling-sensitive plants (Yoshida 1991; Kasamo 1988). It is evident that an increase in membrane lipid unsaturation and bilayer fluidity are important adaptations for effective cold acclimation (Steponkus et al. 1993). The accumulation of sucrose, simple sugars, osmolytes, and LEA proteins during cold acclimation contributes to stabilization of membranes (Thomashow 1999).

6.3 Cold Sensing and Signaling

The plasma membrane appears to be the primary site for the perception of temperature change (Sangwan et al. 2002; Uemura et al. 2006; Vaultier et al. 2006; Wang et al. 2006), as low temperature can quickly induce membrane rigidification (Vaultier et al. 2006). Membrane rigidification is caused by the expression of a DMSO (dimethyl sulfoxide)-induced membrane rigidifier protein *COR*, even at normal growth temperatures, while benzyl alcohol, by negatively regulating the expression of *COR* gene at low temperatures confers membrane fluidity in alfalfa and *Brassica napus* (Orvar et al. 2000; Sangwan et al. 2001). Membrane rigidification probably leads to rearrangement of cytoskeletal microtubules and actin filaments, which then may activate mechanosensitive calcium channels to modulate Ca^{2+} signature (Nick 2000; Orvar et al. 2000; Sangwan et al. 2001).

A transient increase in cytosolic Ca^{2+} levels is observed as an early response to cold (Knight et al. 1991). Transgenics of *Arabidopsis* and tobacco expressing the calcium-sensitive luminescent protein aequorin demonstrated a rise in cytosolic Ca^{2+} concentration in response to low temperature (Knight et al. 1991). Cytosolic Ca^{2+} is an important second messenger in plant cells involved in cold signal transduction. A positive correlation was observed between cold-induced Ca^{2+} influx and accumulation of cold-induced transcripts in alfalfa (Monroy and Dhindsa 1995; Reddy and Reddy 2004) and *Arabidopsis* (Henriksson and Trewavas 2003). Use of various chelators and channel blockers also supported the role of cytosolic Ca^{2+} influx as second messenger in response to cold in alfalfa (Monroy et al. 1993; Knight et al. 1996) and *Arabidopsis* (Tahtiharju et al. 1997). The elevation in cytosolic Ca^{2+} is sensed by Ca^{2+} sensor proteins, which bind to Ca^{2+} and undergo conformational changes. The major Ca^{2+} sensors in plants are calmodulin (CaM), calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs) (Luan et al. 2002; Pandey 2008; Tuteja and Mahajan 2007). Different Ca^{2+} binding proteins distinguishes different Ca^{2+} signatures and protein kinases and decoding of these signals causes changes in gene expression leading to appropriate physiological responses (Yang and Poovaiah 2003; Sathyanarayanan and Poovaiah 2004; Pandey 2008).

CaM is one of the most conserved Ca^{2+} binding proteins in eukaryotes mediating various signaling responses against both developmental and environmental stimuli (Kim et al. 2009; Das and Pandey 2010). Studies with alfalfa cells (Monroy et al. 1993) and *Arabidopsis* (Tahtiharju et al. 1997) have shown that CaM antagonist prevents cold acclimation and reduces expression of cold-regulated genes, indicating a positive role of CaM in low temperature stress signaling. However, overexpression of CaM in *Arabidopsis* resulted in reduced expression of cold-responsive genes, implying it might also function as negative regulator of cold acclimation (Townley and Knight 2002).

Similarly, CDPKs are important sensors in abiotic stress signaling including cold stress (Cheng et al. 2002; Chinnusamy et al. 2004). Studies have shown that, in alfalfa cold induce the expression of CDPKs significantly (Monroy and Dhindsa 1995). Likewise, in rice, the expression of *OsCPK4*, *OsCPK5*, and *OsCPK13* (*OsCDPK7*) was found to be upregulated in response to cold (Ray et al. 2007). The

overexpression of *OsCDPK7* in rice confers enhanced tolerance toward cold as well as salt and drought stresses (Saijo et al. 2001). Overexpression of *OsCDPK13* and calreticulin interacting protein (CRTintP1) also conferred cold tolerance in rice (Komatsu et al. 2007).

CBLs were first identified in *Arabidopsis*. *AtCBL1* was identified as a highly induced protein under cold and drought stress conditions (Kudla et al. 1999). Overexpression of *AtCBL1* causes freezing sensitivity and alteration of expression of *CBF/DREB* transcription factor, while mutation in this gene resulted in freezing tolerance and changes in the expression of COR genes (Cheong et al. 2003; Albrecht et al. 2003). CIPKs act downstream of Ca^{2+} signal but upstream of transcription factors regulating cold stress (Kim et al. 2003; Pandey 2008). CBLs relay the Ca^{2+} signal by interacting with and regulating the family of CIPKs. The role of *CIPK3* in cold signaling was shown via changes in expression pattern of *RD29A* (*Responsive to desiccation 29A*), *KIN1* (*cold-inducible1*), and *KIN2* (*cold-inducible2*) genes in *Arabidopsis* (Kim et al. 2003). In rice, overexpression of *OsCIPK03*, *OsCIPK12*, and *OsCIPK15 cause* increased tolerance toward cold, drought, and salt stress (Xiang et al. 2007). Another study suggested that chilling hypersensitivity of *cbl1* mutant (Cheong et al. 2003) indicates its role in the regulation of cold response by interacting with CIPK7 (Huang et al. 2011).

Protein phosphatases also act as Ca^{2+} sensors. The *Arabidopsis* protein phosphatase 2C, *AtPP2CA*, is cold inducible, and attains maximum expression within 12 h and thereafter remain high (Tähtiharju and Palva 2001). Monroy et al. (1998) showed that cold-induced inactivation of protein phosphatase 2A (PP2A) was mediated by Ca^{2+} influx in alfalfa cells. This suggests a possible role of protein phosphatases in Ca^{2+}-mediated cold stress signaling pathway.

6.4 Reactive Oxygen Species

Both low and freezing temperatures result in the oxidative stress due to generation of ROS, which includes hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (HO·) (Halliwell 2007). ROS accumulates in cells exposed to abiotic stresses and mediate signaling and gene expression even at moderate level (Chinnusamy et al. 2007; Renaut et al. 2008). ROS signals can induce the activation of redox-responsive proteins, such as transcription factors and protein kinases (Hung et al. 2005; Chinnusamy et al. 2007). ROS is also known to be involved in the regulation of Ca^{2+} channels (McAinsh and Pittman 2009), mitogen-activated protein kinase (MAPK) cascade (Knight and Knight 2001; Mittler et al. 2004; Hung et al. 2005; Colcombet and Hirt 2008), and activation of ZAT12 TF (Dat et al. 2000; Mittler et al. 2004; Davletova et al. 2005). The *Arabidopsis* MPK1, MPK2, MPK3, MPK4, MPK6, and MPK7 are induced by H_2O_2 (Kovtun et al. 2000; Nakagami et al. 2006; Doczi et al. 2007; Ortiz-Masia et al. 2007). MAP kinase activity has been shown to be enhanced by cold in alfalfa (Jonak et al. 1996; Sangwan et al. 2002) and *Arabidopsis* (Ichimura et al. 2000). *Arabidopsis* MPK4 and MPK6 were found to be phosphorylated by MKK2 (MAP kinase kinase2)

when exposed to cold stress. Overexpression of MKK2 exhibited upregulation of *CBF/DREB1s* and cold tolerance (Teige et al. 2004). The cold activation of SAMK, an alfalfa MPK, was inhibited by blocking the influx of extracellular Ca^{2+} and an antagonist of CDPKs, suggesting that Ca^{2+} fluxes and CDPKs are essential for the activation of MPK cascades in alfalfa (Sangwan et al. 2002).

Excessive accumulation of ROS leads to cellular injury, which ultimately leads to death of the plant due to damage of photosystem II reaction center and membrane lipids (Prasad et al. 1994; Suzuki and Mittler 2006). The *Arabidopsis fro1* (frostbite1) mutant, which constitutively accumulates high levels of ROS, exhibited impaired expression of *COR* genes and hypersensitivity to chilling and freezing. *FRO1* encodes the Fe–S subunit of complex I (NADH dehydrogenase) of the respiratory electron transfer chain in mitochondria, and its disruption leads to high levels of ROS generation (Lee et al. 2002a, b; Chinnusamy et al. 2007). Another *Arabidopsis* mutant, *chy1*, which is defective in a peroxisomal β-hydroxyisobutyryl-CoA hydrolase involved in fatty acid β-oxidation and valine catabolism, also accumulates high levels of ROS. The *chy1* mutant showed a reduced cold induction of *CBF* genes, and is defective in chilling and freezing tolerance (Zhu et al. 2007). *Arabidopsis* AtHAP5A (heme-associated protein HAPs, also known as NUCLEAR FACTOR Y subunit A/B/C) binds to AtXTH21, and these proteins inhibit cold stress-induced ROS accumulation and modulate freezing stress resistance via CBF-independent pathway (Shi et al. 2014).

6.5 Regulation of Gene Expression During Cold Stress

6.5.1 ABA-Independent Cold Signaling Pathway

6.5.1.1 CBF/DREB-Responsive Pathway

Several COR genes such as *Arabidopsis COR15A* (Baker et al. 1994), COR78/*RD29A* (Yamaguchi-Shinozaki and Shinozaki 1994), the *Brassica napus* gene *BN115* (Jiang et al. 1996), and the wheat gene *WCS120* (Ouellet et al. 1998) are involved in cold acclimation and enhance freezing tolerance of plants in response to low temperature. Yamaguchi-Shinozaki and Shinozaki (1994) identified two 9-bp *cis*-acting DNA regulatory elements (TACCGACAT), in the promoter region of *RD29A* gene, which contained the conserved low-temperature DRE core sequence (CCGAC). DRE was named as C-repeat by Thomashow and colleagues (Baker et al. 1994). This *cis*-element, also referred to as LTRE (Low temperature responsive element), was present in the promoter of other COR genes and was found to be crucial for low temperature response. While many of the changes in gene expression that occur during cold acclimation in response to low temperature are mediated by ABA (Bray 1993; Welin et al. 1994), the C-repeat/DRE appears to be mainly regulated by ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 1994). The *CBF/DREB1* pathway that regulate ABA-independent expression of *COR* genes was first

identified in *Arabidopsis* (Stockinger et al. 1997; Liu et al. 1998), and now it is the most characterized pathway involved in cold acclimation and freezing tolerance across evolutionarily diverse plant species.

C-repeat binding factors (CBFs) or dehydration-responsive element binding factor 1 (DREB1) are the transcription factors belonging to ethylene-responsive element binding protein/APETALA2 (EREBP/AP2) family (Stockinger et al. 1997; Liu et al. 1998) and DREB subfamily (Chen et al. 2009). DREB subfamily is further subdivided into six groups A-1 to A-6. *DREB1/CBF*-like genes belong to the A-1 subgroup and are responsive to low temperature. The *CBF/DREB1* were first isolated and characterized in *Arabidopsis* (Stockinger et al. 1997; Liu et al 1998). In *Arabidopsis*, three cold inducible *CBF/DREB1* genes are known, which are present in tandem on chromosome 4 in the order: *DREB1B/CBF1, DREB1A/CBF3*, and *DREB1C/CBF2* (Medina et al. 1999). The CBFs have two signature sequences, PKK/RPAGRxKFxETRHP and DSAWR, located immediately upstream and downstream of AP2 domain, respectively (Canella et al. 2010). These sequences are highly conserved in the CBF proteins from different plant species. They have recognition sites for protein kinase C and casein kinase II. All the three *CBF* genes are induced within 15 min of plants exposure to cold, and they induce transcription of *CBF/DRE*-regulated *COR* genes accumulated within 2 h of exposure to cold stress (Gilmour et al. 1998). The absence of CRT sequence (CCGAC) in the promoters of *CBF* genes and negligible effect of overexpression of *CBF1* on *CBF3* transcript levels suggested that the *CBF* gene family does not involve auto-regulation (Gilmour et al. 1998), rather it is controlled by a set of interacting transcription factors (Vogel et al. 2005; Chinnusamy et al. 2003, 2010; Agarwal et al. 2006; Doherty et al. 2009). Ectopic expression of *CBFs* in transgenic *Arabidopsis* plants activated the expression of *COR* genes and enhanced freezing tolerance without a prerequisite low temperature acclimation (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). Microarray analysis of *CBF*-overexpressing transgenic plants revealed several CBF target genes involved in various pathways such as signaling, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism, and stress response (Fowler and Thomashow 2002; Maruyama et al. 2004). Overexpression of *AtCBF*1/3 has increased cold, drought, and salt stress tolerance in *Brassica* species (Jaglo et al. 2001), wheat (Pellegrineschi et al. 2004), tomato (Hsieh et al. 2002), tobacco (Kasuga et al. 2004), and rice (Oh et al. 2005). However, the constitutive overexpression of *CBFs* under the transcriptional control of the 35S cauliflower mosaic virus promoter in transgenic plants resulted in severe growth retardation under normal growth conditions in diverse plant species such as *Arabidopsis*, *B. napus*, tomato, potato, and rice. This negative impact on the plant growth was reduced by expressing the *CBF* genes under the control of stress inducible *RD29A* promoter instead of the constitutive CaMV 35S promoter (Kasuga et al. 1999).

Molecular analysis with a *cbf2* null mutant of *Arabidopsis* showed that *cbf2* null mutation conferred enhanced expression of *DREB1B/CBF1* and *DREB1A/CBF3* and leads to freezing tolerance. This suggested that DREB1C/CBF2 is a negative regulator of *DREB1B/CBF1* and *DREB1A/CBF3* (Novillo et al. 2004). Studies on *CBF1-RNAi* and *CBF3*-RNAi lines demonstrated that these are involved in the regulation

of other *CBF/DREB1s* genes and they induce cold acclimation by activating same set of *CBF/DREB1* regulon genes. The functions of CBF1 and CBF3 appear to be different from that of CBF2 (Novillo et al. 2007). CBF3 appears to be a negative regulator of CBF2 (Chinnusamy et al. 2003, 2006). Whole transcriptome analysis of transgenic *Arabidopsis* plant overexpressing *CBF* have shown that only 12 % of the COR genes are controlled by the *CBF* regulon, indicating that other regulatory pathways are also activated in response to low temperature. Transcription factors such as AP2/ERF factors RAP2.1 and RAP2.6, and the C2H2-type zinc finger STZ/ZAT10 belong to the CBF regulon (Fowler and Thomashow 2002; Vogel et al. 2005).

The homologs of *Arabidopsis* CBF have cloned and characterized from several species such as *Brassica napus* (Jaglo et al. 2001; Gao et al. 2002), barley (Xue 2003), rice (Dubouzet et al. 2003), wheat (Shen et al. 2003), corn (Qin et al. 2004), tomato (Zhang et al. 2004), sweet cherry (Kitashiba et al. 2004), alfalfa (Pennycooke et al. 2008a, b), potato (Pennycooke et al. 2008a, b), etc.. Overexpression of these genes in *Arabidopsis* and other plants conferred enhanced tolerance to multiple abiotic stresses. Overexpression of *OsDREB1A* (Dubouzet et al. 2003), maize *DREB1* (Qin et al. 2004), a modified AP2/ERF transcription factor from *Brassica napus* (Xiong et al. 2013), *Vaccinium myrtillus CBF/DREB1* (Oakenfull et al. 2013) in transgenic *Arabidopsis* plants resulted in enhanced freezing tolerance. Transgenic barley lines overexpressing *TaCBF14* and *TaCBF15* exhibited enhanced freezing tolerance (Soltesz et al. 2013).

6.5.2 Regulation of CBF/DREB1 Transcription Factors

6.5.2.1 Inducer of CBF Expression (ICE1)

A systematic genetic analysis of transgenic *Arabidopsis* plant expressing the firefly luciferase gene under the control of *CBF3* promoter led to the identification of a constitutively expressed and nuclear-localized transcription factor, *Inducer of CBF expression 1* (*ICE1*). It encodes an MYC-type basic helix-loop-helix transcription factor that binds to the MYC recognition elements (CANNTG), known as ICE box, present in the promoter of *CBF3* gene, which in turn regulates the expression of downstream genes during cold acclimation leading to freezing tolerance (Chinnusamy et al. 2003; Chen et al. 2009). The *ice1* mutant is highly sensitive to both chilling and freezing temperature and induction of *CBF3* gene was impaired in the mutant. Constitutive overexpression of *ICE1* in transgenic *Arabidopsis* conferred enhanced expression of *CBF3*, *CBF2*, and downstream *COR* genes resulting in freezing tolerance Figure 6.1 (Chinnusamy et al. 2003).

Transcriptome analysis of *Arabidopsis* genome in response to cold revealed that about 40 % of cold-regulated genes, in particular 46 % of cold-regulated transcription factor genes are impaired in dominant *ice1* mutant (Lee et al. 2005a, b). Therefore, ICE1 is considered as a master regulator of CBFs and many other cold-responsive regulons. *ICE1* is functionally conserved in higher plants. During ambient

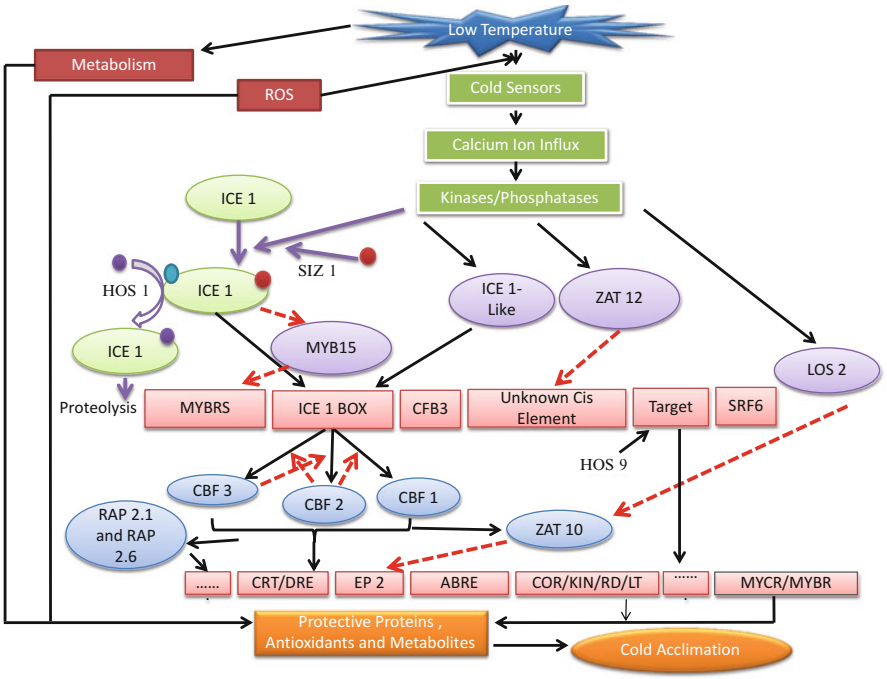

Fig. 6.1 Schematic diagram of regulatory networks involved in low temperature responses. Plants sense low temperature through membrane rigidification and cellular changes that induce calcium signaling and activate protein kinases. Constitutively expressed ICE1 is activated by cold stress via sumoylation and phosphorylation. SIZ1, a SUMO E3 ligase causes sumoylation of ICE1 at K393, which is critical for ICE1 activation of transcription of CBFs and repression of MYB15. HOS1, a RING finger ubiquitin E3 ligase, mediates the ubiquitination and proteosomal degradation of ICE1 and, thus, negatively regulates CBF regulons. CBFs regulate the expression of COR genes that confer freezing tolerance. The expression of CBFs is negatively regulated by MYB15 and ZAT12. CBFs induce the expression of ZAT10, which downregulate the expression of COR genes. Cold-upregulated LOS2 represses the transcription of ZAT10. ZAT10 and ZAT12 are two C2H2 zinc finger transcription factors. Broken arrows indicate negative regulation; purple arrows indicate post-translational regulation; activation solid arrows indicate activation, the (⋯) indicate unknown *cis*-elements. Abbreviations: CBF, C-repeat binding factor (an AP2-type transcription factor); CRT, C-repeat elements; DRE, dehydration-responsive elements; HOS1, high expression of osmotically responsive genes1; ICE1, inducer of CBF expression 1 (an MYC-type bHLH transcription factor); LOS2, low expression of osmotically responsive genes 2; MYB, myeloblastosis; MYBR/MYCR, MYB/MYC transcription factor recognition sequence; SIZ1, SAP and MiZ1 (a SUMO E3 ligase); ROS, Reactive oxygen species; Phosphorylation SUMO protein Ubiquitin

temperature ICE1 remains in an inactive state at warm temperatures and is activated upon exposure to cold, inducing CBF gene expression. *ICE1* mainly affects the expression of CBF3*/DREB1A* whereas overexpression analysis showed that *ICE2* (At1g12860, a homolog of ICE1) induces the expression of *CBF1/DREB1B* and confers increased freezing tolerance in *Arabidopsis* after cold acclimation (Fursova et al. 2009).

ICE1 homologs have been identified in barley (Skinner et al. 2006; Tondelli et al. 2006), wheat (Badawi et al. 2008), maize (Hu et al. 2011), rice (Nakamura et al. 2011), tomato (Miura et al. 2012; Feng et al. 2013), tea (Wang et al. 2012), banana (Zhao et al. 2013), wild grapes (Dong et al. 2013; Xu et al. 2014), orchid (Peng et al. 2014), etc. Overexpression of *Arabidopsis ICE1* enhanced proline accumulation and improved chilling tolerance in rice (Xiang et al. 2008) and cucumber (Liu et al. 2010). As in case of *Arabidopsis*, the ICE1 homologs of wheat *TaICE141* and *TaICE187* are constitutively expressed and induce the expression of the wheat group IV CBFs, which are associated with freezing tolerance. Overexpression of *TaICE141* and *TaICE187* increased the *CBF/DREB1*-dependent *COR* gene expression and freezing tolerance in *Arabidopsis* (Badawi et al. 2008). Overexpression of *SlICE1* enhances chilling tolerance in tomato through increased accumulation of antioxidants, free amino acids, amines and sugars in red tomato fruits of *SlICE1-overexpressing* plants (Miura et al. 2012). Cold stress upregulates *OsICE1* and *OsICE2* and *OsDREB1B*, *OsHsfA3* (rice heat shock factor A3), and *OsTPP1* (rice trehalose 6-phosphate phosphatase) in rice (Nakamura et al. 2011). Overexpression of *ICE1* or *CBFs* enhanced cold and other abiotic stress tolerance in different species suggesting evolutionarily conserved role of ICE1–CBF pathway in cold tolerance (Table 6.1).

Transcriptome analyses revealed that ICE1 regulates several families of TFs in addition to CBF family (Chinnusamy et al. 2003; Lee et al. 2005a, b). The results from transgenic overexpression in *Arabidopsis* and other plants support this, as ICE1 overexpression conferred tolerance to cold and other abiotic stresses in these transgenic plants through diverse mechanisms (Table 6.1). In addition to the vegetative tissues, ICE1–CBF pathway also regulates cold tolerance of fruits. Banana ripening-induced MaNAC1, an NAC (NAM, ATAF1/2, and CUC2) transcription factor (TF) gene is involved in cold tolerance of banana fruits. MaICE1 binds to the promoter of MaNAC1 and induces the expression of MaNAC1. Interestingly, MaNAC1 interacted with MaCBF1 in yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) analyses. These results suggest that the cold induction of MaNAC1 in banana fruits is mediated by ICE1–CBF cold signaling pathway (Shan et al. 2014).

In addition to chilling temperature (<10 °C)-mediated acclimation, responses to moderate temperature decrease at the non-extreme stress range are also regulated by ICE1 in *Arabidopsis*. Transgenic *Arabidopsis* plants expressing CdICE1 when acclimated at 16 °C showed ICE1-mediated induction of miR398–CSD pathway (Chen and Thelen 2013). Shifting of *Arabidopsis* plants from 28 °C to 22 °C resulted in induction of several genes including the *BON1-ASSOCIATED PROTEIN1* (*BAP1*) gene. BAP1 is under the transcriptional control of ICE1. The *ice1* mutant showed low induction of BAP1 and enhanced resistance to *Pseudomonas syringae* pv tomato (Zhu et al. 2011). Mutational analysis of 125 bp sequence of *CBF2* promoter identified two *cis*-acting elements, *ICEr1* and *ICEr2* that function together and induce gene expression in response to cold. *ICEr1* contains CACATG sequence, which has recognition site for bHLH proteins, CANNTG. So, *ICEr1* is the potential binding site for ICE1 (Zarka et al. 2003). In the promoter of maize, *ZmDREB1*, after cold treatment histones H3 and H4 gets hyperacetylated and DNA demethylation, occur in the ICE1-binding region, also followed by chromatin decondensation, indicating the role of chromatin in the regulation of *CBF/DREB1* by ICE1 (Hu et al. 2011).

Table 6.1 ICE1–CBF/DREB1 pathway confers tolerance to cold and other stresses in various plants

Gene	Source plant	Transgenic plant	Phenotype and effects	References
AtICE1	*Arabidopsis thaliana*	Constitutive overexpression (Super promoter) in *Arabidopsis thaliana*	Freezing tolerance; activation of *CBF3/DREB1A*	Chinnusamy et al. (2003)
AtICE1	*Arabidopsis thaliana*	*Oryza sativa*	Chilling tolerance	Xiang et al. (2008)
AtICE1	*Arabidopsis thaliana*	*Cucumis sativus*	Chilling tolerance; dwarf	Liu et al. (2010)
AtICE2	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Freezing tolerance; activation of *CBF1/DREB1B*	Fursova et al. (2009)
SlICE1	*Solanum lycopersicum*	*Solanum lycopersicum*	Chilling tolerance; accumulation of antioxidants	Miura et al. (2012)
SlICE1	*Solanum lycopersicum*	Nicotiana tabacum	Confers cold, osmotic and salt tolerance	Feng et al. (2013)
TaICE141, TaICE187	*Triticum aestivum*	*Arabidopsis thaliana*	Freezing tolerance	Badawi et al. (2008)
EcaICE1	*Eucalyptus camaldulensis*	Nicotiana tabacum	Enhanced cold tolerance through enhanced expression of CBF1, CBF3, and group 2 LEA protein NtERD10C	Lin et al. (2014)
ItICE1	*Isatis tinctoria*	*Oryza sativa*	Enhanced chilling tolerance by enhancing CBF pathway, antioxidants, and proline accumulation	Xiang et al. (2013)
CdICE1	*Chrysanthemum dichrum*	*Chrysanthemum grandiflorum*	Improves cold, salinity, and drought tolerance	Chen et al. (2012a, b)
CdICE1	*Chrysanthemum dichrum*	*Arabidopsis thaliana*	CdICE1 conferred cold tolerance via CBF pathway and miR398–CSD when acclimated at 4 and 16 °C, respectively	Chen and Thelen (2013)
VaICE1	*Vitis amurensis*	*Nicotiana tabacum*	Confers chilling tolerance, in part, due to enhanced level of antioxidant enzymes	Dong et al. (2013)

(continued)

Table 6.1 (continued)

Gene	Source plant	Transgenic plant	Phenotype and effects	References
VaICE1, VaICE2	*Vitis amurensis*	*Arabidopsis thaliana*	Increase freezing tolerance in nonacclimated plants due to high levels of proline, reduced contents of malondialdehyde (MDA) and decreased levels of electrolyte leakage	Xu et al. (2014)
VvICE1a, VvICE1b	*Vitis vinifera*	*Arabidopsis thaliana*	Improved the tolerance to cold, drought and salinity stresses	Li et al. (2014)
MdCIbHLH1 (=*MdICE1*)	*Malus*×*domestica*	*Nicotiana tabacum*	Conferred enhanced chilling tolerance	Feng et al. (2012)
PtrbHLH (=*ICE2*)	*Poncirus trifoliata*	*Nicotiana tabacum, Citrus limon*	Overexpression conferred enhanced tolerance to chilling and freezing temperatures, while RNAi orange plants exhibited hypersensitivity to cold stress	Huang et al. (2013)
AtCBF1, AtCBF2, AtCBF3	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Freezing, salt and drought tolerance; constitutive expression of *COR*	Gilmour et al. (2004), Jaglo-Ottosen et al. (1998), Liu et al. (1998)
AtCBF1, AtCBF2, AtCBF3	*Arabidopsis thaliana*	*Brassica napus*	Freezing tolerance; constitutive expression of *COR*	Jaglo et al. (2001)
AtCBF1	*Arabidopsis thaliana*	*Fragaria ananassa*	Freezing tolerance	Owens et al. (2002)
AtCBF1	*Arabidopsis thaliana*	*Populus tremula* × *alba*	Freezing tolerance	Benedict et al. (2006)
AtCBF3	*Arabidopsis thaliana*	*Solanum tuberosum*	Freezing tolerance	Behnam et al. (2007)
AtCBF3	*Arabidopsis thaliana*	*Triticum aestivum*	Freezing tolerance	Pellegrineschi et al. (2004)
AtCBF3	*Arabidopsis thaliana*	*Nicotiana tabacum*	Freezing tolerance	Kasuga et al. (2004)
OsDREB1A, OsDREB1B, OsDREB1C	*Oryza sativa*	*Oryza sativa*	Chilling, salt and drought tolerance; dwarf	Ito et al. (2006)

HvCBF4	*Hordium vulgare*	*Oryza sativa*	Chilling, drought and salt tolerance	Oh et al. (2007)
TaDREB2TaDREB3	*Triticum aestivum*	*Triticum aestivum*	Freezing and drought tolerance; dwarf	Morran et al. (2011)
TaDREB1	*Triticum aestivum*	*Arabidopsis thaliana*	Cold, dehydration stress tolerance	Shen et al. (2003)
SlCBF1	*Solanum lycopersicum*	*Arabidopsis thaliana*	Chilling and oxidative tolerance	Hsieh et al. (2002)
SlCBF1	*Solanum lycopersicum*	*Arabidopsis thaliana*	Freezing tolerance	Zhang et al. (2004)
OsDREB1A	*Oryza sativa*	*Arabidopsis thaliana*	Freezing, drought and salt tolerance	Dubouzet et al. (2003)
ZmDREB1A	*Zea mays*	*Arabidopsis thaliana*	Freezing and drought tolerance; dwarf	Qin et al. (2004)
VrCBF1, VrCBF4	*Vitis riparia*	*Arabidopsis thaliana*	Freezing and drought tolerance; dwarf	Siddiqua and Nassuth (2011)
HvCBF3	*Hordeum vulgare*	*Arabidopsis thaliana*	Freezing tolerance	Skinner et al. (2005)
LpCBF3	*Lolium perenne*	*Arabidopsis thaliana*	Freezing tolerance; dwarf	Xiong and Fei (2006), Zhao and Bughrara (2008)
SlCBF1	*Solanum lycopersicum*	*Arabidopsis thaliana*	Chilling and oxidative tolerance	Hsieh et al. (2002)
MbDREB1	*Malus baccata*	*Arabidopsis thaliana*	Chilling, drought and salt tolerance	Yang et al. (2011)
GmDREB3	*Glycine max*	*Arabidopsis thaliana*	Freezing, drought and salt tolerance	Chen et al. (2009)
BpCBF1, BpCBF1	*Betula pendula*	*Arabidopsis thaliana*	Freezing tolerance; dwarf	Welling and Palva (2008)
OsDREB1B	*Oryza sativa*	*Nicotiana plumbaginifolia*	Freezing, oxidative and drought tolerance; disease resistance	Gutha and Reddy (2008)
Wcor410	*Triticum aestivum*	*Fragaria ananassa*	Freezing tolerance	Houde et al. (2004)
Wcor15	*Triticum aestivum*	*Nicotiana tabacum*	Freezing tolerance	Shimamura et al. (2006)
WCBF2	*Triticum aestivum*	*Nicotiana tabacum*	Freezing tolerance	Takumi et al. (2008)
BpCBF1, BpCBF2	*Betula pendula*	*Arabidopsis thaliana*	Freezing tolerance	Welling and Palva (2008)
CbCOR15	*Chorispora bungeana*	*Nicotiana tabacum*	Freezing tolerance	Si et al. (2009)
PaCBF1	*Phalaenopsis aphrodite*	*Arabidopsis thaliana*	Enhanced cold tolerance	Peng et al. (2014)

6.5.3 *Regulation of* ICE1

Ubiquitination of ICE1 is mediated by a negative regulator of CBF regulon, HOS1, which is a functional RING-finger protein having E3 ubiquitin ligase activity for proteasomal protein degradation (Dong et al. 2006). The COR genes, such as *RD29A*, *COR15A*, *KIN1*, and *ADH*, showed enhanced expression in loss-of-function *hos1* mutant plants (Ishitani et al. 1998). It encodes a protein of 915 amino acids and contains a short motif near N-terminus, which is similar to RING-finger protein found in the inhibitor of apoptosis (IAP) group of animal protein (Lee et al. 2001). At normal growth temperatures, HOS1 protein resides in the cytoplasm but moves into nucleus upon exposure to cold (Lee et al. 2001). Yeast two-hybrid assays showed that HOS1 physically interacts with ICE1 and mediates its degradation by polyubiquitination both in vitro and in vivo. Once the CBF regulon is activated by ICE1, ICE1 protein is degraded during prolonged duration of cold stress and cold-induced proteolysis of ICE1 is impaired in *hos1* mutant. Overexpression of *HOS1* in *Arabidopsis* transgenic plants lowers freezing tolerance and decreases the expression of *CBFs* and their downstream genes (Dong et al. 2006). The *hos1-1* mutant plant showed early flowering under short day conditions, and the expression level of *Flowering Locus C* (*FLC*) was downregulated in the mutant plant, indicating its role in vernalization (Ishitani et al. 1998). HOS1 has also been demonstrated to cause the degradation of CO (CONSTANS) under cold stress and regulate *Arabidopsis* flowering (Jung et al. 2012; Lazaro et al. 2012). The involvement of HOS1 in modulating flowering time in *Arabidopsis* in response to low ambient temperature was later shown by Lee et al. (2012a, b, c). Under cold stress, HOS1 binds to *FLC* chromatin, preventing its association with histone deacetylase 6 (HDA6) resulting in *FLC* induction and delayed flowering (Jung et al. 2013). In wheat, *Triticum durum*, the RING-finger E3 ligase TdRF1 (RING-finger protein1) interacts with another RING-finger E3 ligase, WVIP2 (wheat viviparus1 interacting protein2), and both of these genes are upregulated by cold treatment (Guerra et al. 2012). *HOS1* has been suggested to affect cold signaling partially through altered mRNA export and disruption of circadian clock (MacGregor et al. 2013). A novel *HOS1* gene, *VvHOS1* from "Muscat Hamburg" grapevine (*Vitis vinifera*) has been isolated and characterized. Overexpression of *VvHOS1* in *Arabidopsis* reduced the tolerance of plant to cold, drought, and salt stress. It was also shown to negatively regulate the expression level of two stress-responsive genes, *AtRD29A* and *AtCOR47* (Li et al. 2014).

Polyubiquitination of ICE1 is blocked by SUMO (Small Ubiquitin Modifier) E3 ligase SIZ1 (SAP and Miz1)-mediated sumoylation of ICE1 at K393 position, thereby increasing ICE1 activity (Miura et al. 2007). The *siz1* null mutant is impaired in the accumulation of SUMO conjugates and result in reduced expression of *CBFs* and COR genes, thus leads to hypersensitivity to chilling and freezing stresses. However, there is enhanced expression of *AtMYB15*, a negative regulator of CBF expression, in *siz1* mutant (Miura et al. 2007). A K393R substitution in ICE1 blocks SIZ1-mediated sumoylation. It was studied that the serine 403 residue of ICE1 protein regulates the transactivation and stability of ICE1 during cold stress. Substitution of serine 403 by alanine in ICE1 increased ICE1 transactivation activity in the

protoplast of *Arabidopsis*. Transgenic plants overexpressing *ICE1* (*S403A*) showed higher level of freezing tolerance and expression of COR genes as compared to *ICE1* (*WT*) in *Arabidopsis*. Cold treatment did not alter the protein level of ICE1(S403A) whereas the level of WT ICE1 protein was significantly reduced (Miura et al. 2011).

6.5.4 CAMTA Transcription Factor

The CaM binding transcriptional activator (CAMTA) family was found to regulate the expression of *CBF2* gene during cold stress by binding to the CCGCGT sequence (CM2 motif) present in the promoter of *CBF2* gene. Mutation of c*amta3* resulted in 50 % reduction in cold-induced transcripts of *CBF2*. However, this mutation has no effect upon cold acclimation of the plant but the double mutant c*amta1/camta3* altered the freezing tolerance in the plant (Doherty et al. 2009).

6.5.5 Dead Box RNA Helicase

6.5.5.1 LOS4 (Low Expression of Osmotically Responsive Gene 4)

LOS4 encodes a DEAD box RNA helicase, AtNUP160, and is localized in nucleus. It is involved in cold-responsive gene expression and freezing tolerance (Gong et al. 2005). It is essential for the mRNA export in the plant cell. Induction of CBFs and the downstream target genes is impaired in *los4-1* mutants and plant is hypersensitive to cold. Overexpression of *CBF3* gene in *los4-1* mutants reverses the sensitivity effect (Gong et al. 2002). Another mutant *cryophyte* (*los4-2*) was reported to be more freezing tolerant than the wild type *Arabidopsis* plant due to enhanced induction of *CBF2* in the mutant (Gong et al. 2005).

6.5.5.2 Regulator of CBF Gene Expression1 (rcf1-1)

A cold inducible DEAD box RNA helicase, RCF1, was identified through a forward genetic approach where *CBF2* promoter was fused to a firefly *luciferase* reporter gene (*CBF2:LUC*). It functions in maintaining proper splicing of pre-mRNAs of nuclear encoded genes and is important for cold-responsive gene regulation. The *rcf1-1* mutant was found to be hypersensitive to chilling and freezing stress, while overexpression of *RCF1* in *Arabidopsis* increased freezing tolerance (Guan et al. 2013).

6.5.6 Negative Regulators of CBF Regulon

MYB transcription factor: An R2R3 type MYB transcription factor binds to the MYB recognition elements (MYBRS) in the promoter region of *CBF/DREB1* and negatively regulates its expression and thereby freezing tolerance. Increased

expression of *MYB15* in *ice1* mutant during cold stress revealed that ICE1 represses the activity of MYB15 either directly by binding to its promoter or indirectly through its downstream genes (Chinnusamy et al. 2003; Badawi et al. 2008). *AtMYB15* physically interacts with ICE1 in yeast-two hybrid and in vitro pull down assay (Agarwal et al. 2006). It binds to the promoter region of *CBF1*, *CBF2*, and *CBF3* genes. Transgenic plants overexpressing *MYB15* were sensitive to freezing stress and there was decrease in the transcript levels of *CBF* genes. Knockout mutants of *MYB15* displayed increased freezing tolerance and expression of *CBF* genes. The downstream cold-responsive genes remain unaffected by overexpression or mutation of *MYB15* in plants. *MYB15* is localized in nucleus and is present in low levels in all tissues. Upon exposure to cold, its expression is upregulated (Agarwal et al. 2006). In the *siz1* mutant, enhanced induction of *AtMYB15* was reported (Miura et al. 2007). Another MYB transcription factor, *AtMYB14*, was found to positively regulate cold tolerance in *Arabidopsis*. Knockdown mutants of *AtMYB14* led to increased cold tolerance and enhanced expression of *CBF* and downstream genes (Chen and Thelen 2013). Similarly overexpression of a single repeat R3-MYB transcription factor, *MYBC1*, increased cold tolerance whereas the *mybc1* mutants were sensitive to freezing stress (Zhai et al. 2010).

ESK1: The constitutively freezing tolerant *eskimo1* (*esk1*) mutant of *Arabidopsis* was identified through a freezing tolerant genetic screening. The *esk1* plants accumulated 30-fold higher levels of free proline than that in the wild type plants. Additionally, the expression level of *RAB18*, a cold-responsive *LEA II* gene was threefold higher. This suggested that *ESK1* acts as a negative regulator of cold (Xin et al. 1998). *ESK1* encodes a 56.7-kDa protein that contains plant-specific DUF231 (domain of unknown function 231) domain. Transcriptome analysis of the *esk1* mutant revealed 312 genes with altered expression, 173 genes were upregulated and 139 were downregulated. The expression of only 12 % of the genes showed overlap with genes regulated by cold acclimation pathway. Other genes were found to be regulated by salt, osmotic and ABA treatment, suggesting that *esk1*-induced freezing tolerance pathway is distinct from CBF-dependent pathway (Xin et al. 2007).

ZAT12 is a C_2H_2 zinc finger protein that contains an EAR motif-like sequence, which functions as a repressor domain. It plays a role in cold acclimation and oxidative stress (Vogel et al. 2005). Transcriptome analysis of *ZAT12* overexpressing *Arabidopsis* plant, ZAT12 regulon consists of 24 COS (cold standard set) genes, nine of which are cold-induced and rest are cold-repressed genes (Vogel et al. 2005; Chinnusamy et al. 2007). Constitutive expression of *ZAT12* reduced the expression of *CBF* genes in response to low temperature, indicating that ZAT12 negatively regulates CBF cold-responsive pathway (Vogel et al. 2005; Chinnusamy et al. 2007).

ZAT10/STZ: Molecular analysis of *los2* mutant of *Arabidopsis* led to the identification of another C2H2 zinc finger protein, ZAT10/STZ, as a negative regulator of COR genes. The expression of *ZAT10/STZ* is induced rapidly and transiently upon cold exposure in the wild type plant and there is increased induction of *ZAT10* in *los2* mutant. Transient expression studies of *ZAT10* in *Arabidopsis* have shown that ZAT10 represses the expression of *RD29A* gene (Lee et al. 2002a, b). *ZAT10/STZ*

binds to the STZ recognition site at −554 to −522 in the promoter region of *RD29A* gene. It binds specifically to A(G/C)T *cis*-element within the EP2 sequence of *RD29A* gene (Lee et al. 2002a, b).

LOS2 is a bifunctional enolase, which negatively regulates the expression of *ZAT10* by binding to the MYC recognition elements present in the promoter of *ZAT10*. The expression of COR genes *COR/KIN/RD/LTI* is impaired in *los2* mutant, while there is no difference in the expression levels of *CBF* genes, indicating that *LOS2* has a functional role downstream of the CBF transcription factors and act as a positive regulator of *COR* genes (Lee et al. 2002a, b). This effect was similar to that of *SFR6* (sensitive to freezing) gene. Mutation in *SFR6* leads to reduced expression of *COR* genes having CRT/DRE elements, while the expression of *CBF* genes remains unaltered, suggesting that SFR6 may be the transcriptional activator of *COR* genes (Knight et al. 1999).

FIERY2 (*FRY2*): The *FRY2* locus was identified in *Arabidopsis* by *PRD29A::LUC* reporter gene-based genetic screening. The *fry2* mutant exhibited an increased expression of COR genes such as *RD29A, COR47, COR15A* and *KIN1* as compared to wild type when treated with cold, salt or ABA. The transcript levels of *CBF* genes were also higher in *fry2* mutant suggesting that *FRY2* is a negative regulator of *CBF/DREB1* transcription factor (Xiong et al. 2002). Genetic analyses of *fry2* mutant in *Arabidopsis* have shown that *FRY2/CPL1* encodes a novel transcriptional repressor having two double-stranded RNA binding domains and a region homologous to the catalytic domain of RNA polymerase II C-terminal domain phosphatases found in yeast and in animals. This region controls transcription and mRNA processing by dephosphorylation of RNA polymerase II (Koiwa et al. 2004; Xiong et al. 2002).

6.5.7 CBF-Independent Pathways

Cold-responsive pathways are mediated both in ABA-independent and -dependent manner and ABA acts synergistically with cold signaling (Xiong et al. 1999). The ABA-dependent pathway is regulated by transcription factors belonging to bZIP such as ABRE binding factors (AREBs), MYC and MYB families. *SCOF1* is a cold-inducible C2H2 zinc finger protein isolated from soybean. Overexpression of *SCOF1* in *Arabidopsis* resulted in increased *COR* gene expression and enhanced cold tolerance in non-acclimated plants. SCOF1 does not bind directly to the *DRE* or *ABRE* sequences, rather facilitates the DNA binding of a cold inducible bZIP transcription factor, soybean G-box binding factor 1 (SGBF1) to *ABRE* and induces ABA-dependent cold acclimation pathway (Kim et al. 2001).

High expression of osmotically responsive genes, *HOS9* was identified by RD29A::LUC genetic screen. *HOS9* encodes a homeodomain MYB transcription factor that is localized to the nucleus and is required for basal freezing tolerance (Zhu et al. 2004). Microarray analysis revealed that HOS9 controls expression of about 175 genes that does not belong to the *CBF* regulon. Also, 41 of these genes targeted by *HOS9* were reported to be cold-induced. Knockout mutants of *HOS9*

gene resulted in reduced basal and acquired cold tolerance. The *hos9* mutation also resulted in the alteration of other characteristics including trichome development, growth rate, and flowering time. These results suggested that *HOS9* is involved in plant growth and development and affects freezing tolerance in a CBF-independent manner (Zhu et al. 2004).

Overexpression of *OsMYB4* in *Arabidopsis* plants enhanced *COR* gene expression, increased proline content and exhibited chilling and freezing tolerance (Vannini et al. 2004; Pasquali et al. 2008). *OsMYBS3* and *OsMYB2* confer increased tolerance in response to cold in rice (Su et al. 2010; Yang et al. 2012).

6.6 Transcriptomics Approach

Transcriptomics or transcript profiling is a widely used technique to study spatial and temporal gene expression and their regulation by various internal or external stimuli. Several techniques, such as DNA microarrays, Expressed Sequence Tags (ESTs), Serial Analysis of Gene Expression (SAGE), Digital Gene Expression (DGE) profiling, and Next-Generation Sequencing (NGS)-based tools such as RNA sequencing (RNA-seq) are being exploited to capture and identify the differentially regulated genes during stress conditions in plants (Cramer et al. 2011; Lister et al. 2009; Wang et al. 2013) (Table 6.2). It provides an in-depth knowledge of the global gene expression pattern of plant response to stress stimuli (Jung et al. 2003).

Using cDNA microarrays or whole genome arrays, the expression pattern of genes in response to chilling stress has been analyzed in *Arabidopsis*, rice, sunflower and several other plants (Seki et al. 2002; Rabbani et al. 2003; Fernandez et al. 2008). Seki and coworkers (2001) used a full-length cDNA microarray for 1,300 *Arabidopsis* genes, and identified 19 *COR* genes, among which the newly identified genes were ferritin, a nodulin-like protein, LEA protein and glyoxalase. In a different study, Fowler and Thomashow (2002) reported 306 *COR* genes using microarray for 8,000 genes. Of these 306 genes, 218 were upregulated and 88 were downregulated and 45 of these *COR* genes were found to be expressing under the control of CBF1. Differentially regulated genes, between two cultivars of wheat, during cold acclimation were identified by microarray and these genes encode protein kinases, transcription factors, calcium binding proteins and proteins involved in photosynthesis (Gulick et al. 2005). A transcriptome profiling of cassava apical shoots subjected to a progressive cold stress was performed using a 60-mer oligonucleotide microarray representing 20,840 cassava genes, which led to the identification of 508 transcripts as early cold-responsive genes including genes for signal transduction components (*MAPK4*), transcription factors (*RAP2.11* and *AP2-EREBP*), and active oxygen species scavenging enzymes (catalase 2), as well as photosynthesis-related genes (*PsaL*). An increase in transcripts of genes encoding ROS scavenging enzymes and the genes for accumulation of total soluble sugars (including sucrose and glucose) were also detected (An et al. 2012). Genome-wide transcriptome analysis uncovered various molecular components involved in

Table 6.2 Transcriptome analysis in response to low temperature

System	Treatment	Method	Major result	Reference
Poncirus trifoliata	Progressive treatment at 4 °C	Suppression subtractive hybridization	Cell wall structural genes, genes involved in synthesis of osmolytes, β-amylase, gene encoding antioxidants	Peng et al. (2012)
Ammopiptanthus mongolicus	Progressive treatment at 4 °C	ESTs from cDNA library	Genes involved in photosynthesis, carbohydrate metabolism, protein metabolism, ROS scavenging, signal transduction, TFs, cellular transport, defense response	Liu et al. (2013)
Populus simonii	6 h stress at 4 °C	Microarray hybridization	Genes involved in photosynthesis, calcium/calmodulin-mediated signaling, TFs, hormone biosynthesis, antioxidants	Song et al. (2013)
Triticum aestivum	Cold acclimated at 6 °C	Microarray hybridization	Genes involved in transport, cellular carbohydrate metabolism, transcription regulation, lipid metabolism, oxidation–reduction, DNA metabolism, chromatin organization	Laudencia-Chingcuanco et al. (2011)
Oryza sativa ssp. *indica*	1 h and 5 h stress at 5 ± 1 °C	Microarray hybridization	Protein kinases, TFs, ubiquitin protein ligase, auxin responsive, metabolism related	Mittal et al. (2012)
Vitis amurensis	8 h cold treatment (first 4 h with temperature dropped at 5 °C per hour and then held for 4 h at 4 °C)	Next generation sequencing using IlluminaHiSeq 2000 system	Genes involved in metabolism, signal transduction, transcription, transport, stress-related genes	Xin et al. (2013)
Ammopiptanthus mongolicus	14 days at 4 °C	Next generation sequencing using IlluminaHiSeq 2000 system	Genes involved in lipid transport and metabolism, intercellular osmoprotectant, antioxidant enzyme systems, calcium and ABA, TFs (*COR*, *LEA*, *CBF*, and *DREB*)	Pang et al. (2013)

(continued)

Table 6.2 (continued)

System	Treatment	Method	Major result	Reference
Chorispora bungeana	24 h chilling stress	Next generation sequencing using IlluminaHiSeq 2000 system	COR15A, ABR1, cytochrome P450, plant invertase, mitogen-activated protein kinase, *HSFA1E*, *GA2OX6*, LEA proteins, glucosyltransferase, low temperature induced 65, ubiquitin ligase, zinc finger family protein,DUF21/295	Zhao et al. (2012)
Camellia sinensis	Sample harvested from field conditions at temperature higher than 15 °C	Next generation sequencing using IlluminaHiSeq 2000 system	Cold sensor or signal transduction genes, cold-responsive TFs, AFPs, PR proteins	Wang et al. (2013)
Oryza sativa ssp. *japonica*	24 h stress at 4 °C	Massive parallel signature sequencing (MPSS) and sequencing by synthesis (SBS)	Glycosyl hydrolases, ABC transporter ATP-binding protein, early responsive to dehydration, bZIP transcription factor, fatty acid hydroxylase, ethylene responsive transcription factor, calmodulin-binding motif domain containing protein	Venu et al. (2013)

chilling tolerance and susceptibility in grapefruit. Out of 30,171 transcripts, 1,345 were found to play a role in low temperature response and were termed as chilling response regulons. Transcripts related to cell wall, defense, photosynthesis, respiration and secondary metabolism were observed to be downregulated, while transcripts encoding membrane proteins, lipid and carbohydrate metabolism, hormone biosynthesis and transcription factors were upregulated under cold stress (Maul et al. 2008). Cold-acclimated wheat plants showed the upregulation of *CBF* genes, WRKY, kinases, phosphatases and genes involved in signal transduction during freezing stress (Skinner 2009).

Based upon ESTs, high expression of dehydrin, *COR* genes, antifree proteins (AFPs), and pathogenesis-related (PR) proteins was observed in seabuckthorn (Ghangal et al. 2012). Again, the analysis of ESTs in *Vitis amurensis* showed the upregulation of *ERD* genes, chitinases, β-glucanase, ubiquitin ligase, and calcium signaling-related genes in response to low temperature (Zhang et al. 2013). Global

transcriptome analysis of chickpea during cold acclimation by cDNA-AFLP approach lead to the identification of several transcripts having functions associated with metabolism, transport, signal transduction, and transcription (Dinari et al. 2013).

SAGE was applied to capture the genes involved in freezing tolerance in *Arabidopsis*, where around 272 differentially expressed genes were identified from cold-treated leaves. The genes induced by cold mostly included *COR* genes, alcohol dehydrogenase genes involved in glucose metabolism pathways, and lipid transfer protein genes. *COR15a* was induced over 300-fold, other upregulated genes were *COR47*, dehydrin *Xero 2*, and *Rab18* (Jung et al. 2003). SAGE analysis revealed that poor accumulation of *COR*, lipid transfer protein and *β*-amylase in *Arabidopsis* pollen are the cause of cold sensitivity (Lee and Lee 2003). Analysis of five different SAGE libraries constructed from *Arabidopsis* leaf tissues collected at various durations of exposure to cold identified 920 low-temperature responsive genes. Consequently, transcripts of around 63 genes showing *COR* gene like expression accumulated after 2 days of cold stress treatment and 47, 19, 13 genes exhibiting pattern of *CBF1*, *CBF2,* and *CBF3*, respectively, were identified (Robinson and Parkin 2008). Long SAGE was also performed in *Arabidopsis* leaf to study the gene expression during early response to cold (Byun et al. 2009).

Global transcriptome analysis and gene expression profile of *Jatropha curcas* in response to cold were determined by high-throughput sequencing using Illumina Hi-Seq 2000 followed by DGE analysis. Plants were subjected to 12, 24 and 48 h stress at 12 °C. Several genes such as starch catabolism-related genes, phospholipase Dδ (*PLDδ*), calcium-dependent protein kinase (*CDPK*), mitogen-activated kinase (*MAPK*), protein kinase C (*PKC*), omega-3-fatty acid desaturase, peroxidase, superoxide dismutase, glutathione reductase, osmoprotectants, LEA proteins, *CBFs*, *HOS1*, *LOS2*, *LOS4*, *ZAT10*, and *ZAT12* were observed to be upregulated. *MYB15* was found to be upregulated after 12 and 24 h of stress but after 48 h it gets downregulated (Wang et al. 2013).

6.7 Proteomics Approach

Although transcriptome studies have enhanced our understanding of stress-responsive gene expression enormously, it is a poor indicator of predicting the expression level of the proteins. Proteins can be identified by differential display pattern following their separation by two-dimensional electrophoresis (2-DE) (Wittmann-Liebold et al. 2006) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Fournier et al. 2007) and the structures can be predicted by MALDI-TOF/TOF MS. Abundance in protein levels during cold has been studied in several plants such as *Arabidopsis thaliana* (Bae et al. 2003; Kawamura and Uemura 2003; Amme et al. 2006), rice (Imin et al. 2004; Cui et al. 2005; Yan et al. 2006), peach (Renaut et al. 2008), chicory (Degand et al. 2009), soybean (Cheng et al. 2010), pea (Dumont et al. 2011), and wheat (Rinalducci et al. 2011).

Analysis of *Arabidopsis* leaves and nuclear proteome during cold stress have revealed 38 plasma membrane proteins and 54 nuclear proteins (Bae et al 2003; Kawamura and Uemura 2003). The nuclear proteome showed abundant change in expression for DEAD box RNA helicase, serine acetyl-transferase, heat-shock proteins, myrosinase-binding proteins, CaMs, and several other families of transcription factors. Cold induced 60S acidic ribosomal protein P2-A, which is involved in elongation step of protein synthesis (Wahl and Moller 2002). A group of putative proteins containing sequences or motifs relevant to RNA-associated functions such as putative U2 snRNP-A, serine-rich protein with a sequence similar to ribosomal protein S1, and putative DEAD box RNA helicase were revealed. Yan et al. (2006) studied total proteins in rice leaves during cold stress using 2D electrophoresis and identified around 85 differentially expressed proteins by mass spectrometry, which included both novel and known cold-responsive proteins. It also showed the involvement of proteins in regulatory and functional network during chilling stress and supported the idea of protein degradation during low temperature. The photosynthetic proteins were found to be highly susceptible to degradation, 19 fragments of Rubisco large subunit were identified during this study. Disassembly of Rubisco under chilling conditions has been suggested to be the cause for reduction in rate of photosynthesis in *Arabidopsis* (Yi et al. 2011). In addition, glycine dehydrogenase and RcbA, involved in photorespiration, also get degraded during cold stress. It has been shown that ROS may induce degradation of Rubisco by proteases (Desimone et al. 1996). Proteins such as WAK1, armadillo repeat containing protein, HSP, putative acidic ribosomal protein P3a were found to be upregulated in rice during cold. Hashimoto and Komatsu (2007) also reported several differentially expressed proteins in rice seedlings during low temperature. This study also revealed for the first time the downregulation of phosphoglucomutase in response to cold. Proteins involved in cellulose synthesis, such as UDP-glucose pyrophosphorylase were found to be in abundance inside the cells.

Rice roots subjected to 24–72 h of cold stress initiate the activation of metabolic processes as indicated by the presence of several proteins related to metabolism such as phosphogluconate dehydrogenase, NADP-specific isocitrate dehydrogenase, fructokinase, and cytoplasmic malate dehydrogenase. In addition, higher abundance of pyruvate orthophosphate dikinase precursors (PPDK), aconitate hydratase, glycine dehydrogenase, and enolase were also identified in chilling stress (Lee et al. 2009).

Cold stress generates ROS and their precursors that cause oxidative damage to plants. Plants produce antioxidants and detoxifying proteins to combat these damages. Three peroxidases were found in rice proteome, which can scavenge and control ROS levels in plants (Yan et al. 2006). Glyoxalase I and oxalyl-CoA decarboxylase, which are involved in detoxification and prevention of cell damage, were shown to be upregulated in rice roots during cold stress (Lee et al. 2009). Glyoxalase I functions to detoxify methylglyoxal produced during the stress condition whereas oxalyl-CoA decarboxylase causes decarboxylation of activated oxalate molecule that generates ROS. It also causes generation of hydroxyl or carbonate radicals through its interaction with hydrogen peroxide (Espartero et al. 1995;

Urzua et al. 1998). The ROS scavengers such as superoxide dismutase, catalase, and ascorbate peroxidase were found in abundance in chicory roots and chickpea during cold stress (Lee et al. 2009; Heidarvand and Maali-Amiri 2013).

Cold stress leads to pollen sterility in rice. Proteome analysis suggest that breakdown of β-expansin and glycogen phosphorylase may possibly lead to incomplete pollen tube growth and starch formation and, thus causing pollen sterility in plants (Imin et al. 2004). Various proteins associated with metabolism, biosynthesis of cell wall components, antioxidative/detoxifying reactions, and energy production were found to be induced in response to cold in rice (Cui et al. 2005). The comparative proteome study of non-transgenic and *rd29A:RdreB1B1* transgenics of strawberry under cold stress revealed that the *rd29A:RdreB1B1* transgenics showed higher expression level of Cu/Zn superoxide, LEA14-A, eukaryotic translation initiation factor 5A (eIF5A) and photosynthetic proteins which confers higher cold stress tolerance as compared to non-transgenic plants. It was also inferred that the DREB transcription factor regulates the accumulation of eIF5A (Gu et al. 2013). Proteome analysis of spring wheat cultivar revealed increased accumulation of COR proteins, glycine-rich RNA binding protein, proteins involved in amino acid and prosthetic group metabolism, detoxification of ROS and chaperonins (Rinalducci et al. 2011).

Increased level of RNA binding protein, cp29, has been reported during cold (Amme et al. 2006; Gao et al. 2009). It is localized in chloroplast stroma and is involved in photoprotection mechanism that in turn is regulated by phosphorylation. The sensitive genotype of maize lacks phosphorylation of cp29 resulting in photoinhibitory damage (Mauro et al. 1997). Cold stress leads to degradation of glycine dehydrogenase and RcbA, which are important components in sustaining electron flow (Yan et al. 2006). Goulas et al. (2006) identified 43 (35 in stroma and 8 in lumen of the chloroplast) differentially expressed proteins that participate in photosynthesis, other plastid metabolic functions, hormone biosynthesis and stress sensing and signal transduction during cold acclimation. Proteomic studies during freeze–thaw injury in onion scales revealed the absence of several dehydrins, proteins associated with energy and secondary metabolism, chaperones and antioxidants (Chen and Thelen 2013). Proteins related to recovery after freeze–thaw were also examined. As a result, numerous proteins such as those involved in ion homeostasis, ethylene production, cell wall remodeling, molecular chaperones, detoxifying enzymes, sucrose and fructokinase were identified (Chen and Thelen 2013). Furthermore, reports suggest the activation of defense-related proteins such as protein disulfide isomerase and disease resistance response proteins in pea roots under low temperature stress (Dumont et al. 2011).

The several types of proteins that are expressed under cold stress include AFPs, dehydrins and LEA proteins, HSPs, cold shock domain proteins (CSDPs), chaperones, pathogenesis-related (PR) proteins, and proteins involved in signal transductions (Heidarvand and Maali Amiri 2010).

1. COR/LEA proteins and dehydrins: LEA proteins are low-molecular-weight proteins (10–30 kDa) and provide protection to plants during stresses (Debnath et al. 2011). They are highly hydrophilic and functions as high molecular osmoprotectants (Kosova et al. 2011).

Dehydrins are the subgroup members of LEA proteins. These are heat stable and rich in glycine. During cellular dehydration caused by abiotic stresses, dehydrins play a role in membrane stabilization and protects other proteins from denaturation (Allagulova et al. 2003). DHNs (dehydrins) are characterized by three highly conserved domains known as the K, Y, and S segments. The various DHN subclasses are defined by the number and order of the Y, S, and K: Y_nSK_n, Y_nK_n, SK_n, K_n, and K_nS. The SK_n- and K-type of DHNs are suggested to be involved in CA processes (Rorat 2006). Induction of dehydrins by cold stress has been reported in *Arabidopsis* (Nylander et al. 2001; Kawamura and Uemura 2003), wheat (Ohno et al. 2003), rice (Lee et al. 2005a, b), and Citrus fruits (Porat et al. 2004). Wisniewski et al. (2006) suggested freeze-responsive role of dehydrins in both herbs and woody plants. They also found intracellular dehydrin in peach had AFPs-like activity. Renaut et al. (2004) reported the expression of single 100 kDa LEA protein in poplar. Similarly, a dehydrin from *Solanum sogarandinum* increased chilling tolerance in cucumber (Yin et al. 2006). Dehydrins include COR (cold responsive), LTI (Low temperature induced), RAB (responsive to abscisic acid), KIN (cold-induced), or ERD (early responsive to dehydration) (Theocharis et al. 2012). All of the ERD10, ERD14, COR47, and LTI30 accumulate in response to cold stress in plants (Nylander et al. 2001). ERD10 (SK3) and ERD14 (SK2) proteins in *Arabidopsis* were known to function as chaperones (Kovacs et al. 2008), whereas COR15a prevents protein aggregation (Nakayama et al. 2008) and thus act as a protectant. Thirteen dehydrin genes *DHN1* to *DHN13* are known in barley. Two of these, *DHN5* and *DHN8*, were reported to express manifold during cold stress (Heidarvand and Maali Amiri 2010) in barley. Three *COR* genes that are well-identified dehydrins, *Wcs*120(K6), *Wcor*410(SK3), and *Wcor*14 have been shown to accumulate during the initial response to low temperature in wheat (Ganeshan et al. 2008). *Wcs120* and *Wcor14* have CBF recognition sequences in their promoter region and they are activated by the CBF transcription factors (Heidarvand and Maali Amiri 2010). A COR protein, WCOR18 showed upregulation in wheat leaves subjected to cold stress (Rinalducci et al. 2011). COR WCOR719 is highly induced by low temperature and protects plants from freezing by preserving the structural integrity of plant cells (Danyluk et al. 1996). Cold acclimation-specific protein 15 (CAS15) was identified in chicory roots under chilling stress (Degand et al. 2009). CAS15 protein contains dehydrin K and S segments and contributes to cold tolerance like dehydrins (Pennycooke et al. 2008a, b).

2. Antifreeze proteins: These proteins accumulate in the apoplastic fluid during cold acclimation and inhibit growth of ice crystals (Griffith and Yaish 2004; Griffith et al. 2005; Yaish et al. 2006). These proteins bind to the surface of ice crystals and affect their shape. They also function to depress the freezing point in cold-acclimated leaves and once the leaves are frozen, these prevent ice recrystallization and formation of larger ice crystals. Thus, protecting the plants from mechanical injury. PR proteins like β-1,3-glucanases, chitinases, or thaumatin-like proteins exhibit antifreeze activities (Griffith and Yaish 2004). Wheat TaIRI (ice recrystallization inhibition) proteins have a peculiar bipartite

structure. They have a leucine-rich repeat receptor domain at their N terminus and ice recrystallization inhibition domains at their C terminus (Ouellet 2007). It was reported that the hormone ethylene, salicylic acid, ABA, and drought are involved in regulating antifreeze activity in response to cold in plants (Atici and Nalbantoglu 2003).

3. Heat shock proteins (HSPs): Though mainly known to be heat stress inducible, these are also reported to be accumulated in response to other abiotic stresses (Sabehat et al. 1998). HSP90, HSP70, small HSPs, chaperonins 60 and 20 are highly induced by low temperature (Lopez-Matas et al. 2004). Two Hsp70-like proteins were found to be upregulated in response to low temperature in peach (Renaut et al. 2008). Four cytoplasmic and two mitochondrial proteins of *Arabidopsis* HSP70 family were also revealed to be upregulated in response to low temperature (Heidarvand and Maali Amiri 2010). During analysis of *Arabidopsis* nuclear proteome, HSP70-1 was identified (Bae et al. 2003) as a dnaK-type molecular chaperone (Sung et al. 2001). These proteins participate in membrane protection, translation, and translocation into organelles, refolding of denatured proteins and prevent aggregation of denatured proteins (Tsvetkova et al. 2002; Renaut et al. 2006; Timperio et al. 2008).
4. Pathogenesis-related (PR) proteins: Apart from being expressed during pathogen attack, these proteins are also produced during various abiotic stresses. These include β-1,3-glucanases, chitinases, thaumatin-like proteins and lipid transfer proteins (Liu et al. 2003). These are involved in signal transduction during cold stress (Hoffmann-Sommergruber 2000). Four PR proteins, two isoforms of β-1,3-glucanases, one chitinase-3-like protein precursor and one glucan endo-1,3-β-glucosidase, were found in onion scales recovered after freeze–thaw injury (Chen and Thelen 2013).
5. Histones: The exploration of the proteomic response to cold stress in rice seedlings for various durations showed the involvement of histone regulation. Histones H4, H2B.9, H3.2, linker histones H1 and H5 were downregulated after cold stress while H2A.3 was found to be upregulated. Additionally, WD-40 repeat protein that is known to modify histones was upregulated after 96 h of stress (Neilson et al. 2011).
6. Vitamin B biosynthetic proteins: Proteins responsible for folate and riboflavin synthesis (vitamin B9 and B2, respectively) were downregulated while thiamine biosynthetic enzyme (vitamin B1) was expressive at all time points of cold stress in rice (Neilson et al. 2011).

6.7.1 Plasma Membrane Proteome During Cold Stress

Plasma membrane plays a pivotal role in signal perception, cellular homeostasis, and stress tolerance in plants (Takahashi et al. 2013). Plasma membrane proteomics is being exploited to enhance freezing tolerance in plants. An enhanced accumulation of phospholipase Dδ (PLDδ), a plasma membrane-associated protein, which

hydrolyses phospholipids and generates phosphotidic acid (PA), was reported to express in *Arabidopsis* during cold acclimation (Kawamura and Uemura 2003). Li et al. (2004) showed that knockdown of PLDδ gene results in freezing-sensitive plants, whereas overexpression leads to improve freezing tolerance in *Arabidopsis*. The PLDδ alterations were not associated with change in expression of COR47 or COR78 and also did not affect the soluble sugars or proline. Lipocalin-like protein (temperature-induced lipocalin, TIL) also exhibits enhanced cold tolerance (Kawamura and Uemura 2003; Uemura et al. 2006).

Cold acclimation leads to an increase in P-type ATPase activity, disassembly of microtubules, and accumulation of different types of dehydrin family proteins on the plasma membrane (Ishikawa and Yoshida 1985; Abdrakhamanova et al. 2003; Kosová et al. 2007). Dehydrins, EDR10 and EDR14, protect proteins and membranes against freeze-induced injury and dehydration (Uemura et al. 2006; Kosová et al. 2007). A novel protein expressed during cold acclimation synaptotagmin 1 (SYT1) was identified in plants, an isoform of which is supposed to be involved in membrane resealing in animals. It was later reported that SYT1 is involved in resealing and repairing of membrane and thus conferring freezing tolerance (Yamazaki et al. 2008).

Analysis of plasma membrane proteome in rice roots during low temperature has revealed the role of proteins involved in membrane permeability and signal transduction in cold response (Hashimoto et al. 2009). Members of annexin and hypersensitive-induced response (HIR) protein families fall under this category. Annexins are Ca^{2+}-dependent membrane binding proteins and help in membrane trafficking and organization (Gerke and Moss 2002), while HIR proteins are involved in ion channel activity (Nadimpalli et al. 2000). In *Arabidopsis*, two cold-responsive plasma membrane proteins have been suggested to have a positive role in freezing tolerance (Tominaga et al. 2006). Outer membrane lipoprotein like protein (lipocalin-like protein, *AtLCN*) and ERD14 were found to increase during cold acclimation and affect the cryobehavior of plasma membrane (Kawamura and Uemura 2003). Transgenic plants overexpressing *AtLCN* showed greater freezing tolerance than wild type plants without cold acclimation (Uemura et al. 2006). *ERD14* transgenic plants displayed decrease in intracellular freezing in the protoplast (Uemura et al. 2006).

6.8 Metabolomics Approach

To survive in cold stress environmental conditions, plant needs to adjust its physiological pathways to restore its metabolic homeostasis in order to accomplish its basic requirements of carbon source, energy, membrane stability, osmolarity, and resistance against ROS produced during stress. Various metabolic pathways are altered in response to cold. Some of the important findings of metabolic adaptations revealed from recent metabolomics studies are summarized in Table 6.3.

Table 6.3 A summary of metabolomics studies on plants subjected to cold stress

Metabolite/Enzymes	Plant species	Conclusion	Reference
RFOs	*Ajuga reptans*	RFO generation helps to stabilize plants facing cold stress	Bachmann et al. (1994)
Antioxidant molecules	*Arabidopsis thaliana*	Activity of catalase, ascorbate peroxidase, and glutathione increased during cold stress	O'Kane et al. (1996)
Carbohydrates and Pi	*Hedera helix* L.	Photosynthetic activity reduces and soluble sugar levels increases in cold-acclimated plants	Bauer et al. (1996)
Photosynthetic activity	*Arabidopsis thaliana*	Enzymes involved in sucrose synthesis increases more drastically then the enzymes of starch synthesis	Strand et al. (1997)
Sucrose-phosphate synthase activity	*Solanum*	Overexpression of SPS gene provides cold tolerance to cold-sensitive species	Murchie et al. (1999)
Carbohydrates	*Arabidopsis thaliana*	Plants overexpressing SPS gene neither accumulates carbohydrates nor reduces photosynthesis activity	Signora et al. (1998)
Calvin cycle and sucrose synthesis enzymes	*Arabidopsis thaliana*	Starch synthesis declines while sucrose synthesis increases	Strand et al. (1999)
FBPase and SPS	*Arabidopsis thaliana*	SPS activity directly affects sucrose synthesis	Strand et al. (2000)
RFOs	*Populus tremuloides*	RFO's level increases in winter	Cox and Stushnoff (2001)
NO and H_2O_2	*Arabidopsis thaliana*	NO and H_2O_2 act as signaling molecules and activates genes responsible to attain cold tolerance	Neill et al. (2002)
Photosynthetic activity	*Cistus albidus* L. and *Quercus ilex* L.	Low energy assimilation rate helps plant to survive cold stress	Oliveira and Peñuelas (2004)
Sucrose	*Arabidopsis thaliana*	Accumulation of soluble sugars enhances cold tolerance	Strand et al. (2003)
Sucrose	*Arabidopsis thaliana*	Exogenous application of sucrose reduces loss of osmolarity caused due to cold stress	Uemura et al. (2003)
Metabolites	*Arabidopsis thaliana*	Differential expression of metabolites in cold stress and heat-stressed plants	Kaplan et al. (2004)
Carbohydrates, proline and polyamines	*Populus tremula* L.	Carbohydrates also provide membrane stability	Jouve et al. (2004)

(continued)

Table 6.3 (continued)

Metabolite/Enzymes	Plant species	Conclusion	Reference
Trehalose 6-phosphate	*Arabidopsis thaliana*	Trehalose 6-phosphate promotes sugar synthesis	Kolbe et al. (2005)
Proline	*Phaseolus vulgaris*	Proline accumulation helps in cold acclimation	Ruiz et al. (2002)
Rubisco protein	*Arabidopsis thaliana*	Rubisco protein levels rise, stabilizing photosynthesis in cold-stressed plants	Goulas et al. (2006)
Sucrose biosynthesis enzymes	*Arabidopsis thaliana*	Sucrose biosynthesis enzymes are present in abundance in cold stress	Kaplan et al. (2007)
Carbohydrates	*Oryza sativa* L.	Cold-tolerant species accumulate sugar and raffinose during old stress	Morsy et al. (2007)
Trehalose 6-phosphate	*Arabidopsis thaliana*	Trehalose induces accumulation of sucrose under LT	Paul (2008)
Carbohydrates	*Arabidopsis thaliana*	LT triggers starch degradation and sucrose synthesis	Maruyama et al. (2009)
Polyamine	*Cucumis sativus* L.	Polyamine acts as ROS scavenger	Zhang et al. (2009)
Carbohydrates	*Arabidopsis thaliana*	Soluble sugars and RFO's help in stabilization of plants in LT	Korn et al. (2010)
Proline	*Cicer arietinum* L.	Proline accumulate in cold-tolerant plants under cold stress	Kaur et al. (2011)
Soluble sugars, RFOs, proline and polyamines	*Thellungiella* and *Arabidopsis*	Cold-induced metabolic changes differ from plant to plant	Lee et al. (2012a, b, c)

6.8.1 Carbohydrates and Soluble Sugar Levels

Starch is the major energy storage molecule found in higher plants. As abiotic stresses inhibit existing photosynthesis while increasing the maintenance respiration for protection and repair of stress-induced damage, stored starch, and other carbohydrates are converted into soluble sugars and additional metabolic pathways are induced to convert the simple soluble sugars such as glucose and fructose into different other sugars and sugar alcohols. Abundance of soluble sugar confers resistance to cold, as it can be transported to the sink tissues for various metabolic and protective requirements. Comparison of metabolome profile of cold acclimating and non-acclimating plants revealed that more than 50 metabolites including trehalose, putrescine, and ascorbate are upregulated in cold-acclimated plants (Cook et al. 2004). Trehalose is a non-reducing disaccharide of glucose. It can reversibly absorb water and thus, protects plant from desiccation-induced damage. In vivo studies also support that trehalose protects membrane and proteins from deleterious alterations that are caused due to oxidative stress (Cook et al. 2004; Janmohammadi 2012).

6.8.1.1 Raffinose Family Oligosaccharides

It has been widely reported that starch levels fall drastically in LT conditions (Strand et al. 1997, 1999; Goulas et al. 2006) but at the same time levels of soluble sugar and Raffinose Family Oligosaccharides (RFOs) rise (Bachmann et al. 1994). RFOs are alpha-galactosyl derivatives of sucrose. These are soluble sugars such as trisaccharide (Raffinose), tetrasaccharide (Stachyose), and pentasaccharide (Verbascose) (Bachmann et al. 1994). UDP-galactose is the precursor for RFOs. Their synthesis utilizes a series of alpha galactosyl-transferase in a sequential manner (Bachmann et al. 1994). Enzymes involved in RFOs synthesis include Galactinol Synthase (GS), Raffinose Synthase (RS), and Stachyose Synthase (STS). Comparison of plants capable of cold acclimation and cold-sensitive plants revealed that STS in cold-tolerant plants but not in cold-sensitive plants is detectable at temperature below 15 °C. In Ajuga, stachyose was main carbohydrate found in phloem under cold stress. In populus, RFO levels rise in mid-winter and declines post-winter (Cox and Stushnoff 2001). RFOs have been shown to play cryoprotective role in many plants (Stushnoff et al. 1993; dos Santos et al. 2012; Egert et al. 2013). Subsequently, Raffinose was proposed to function in membrane stabilization. It also seems to appear to interact with phospholipid head groups to increase cold tolerance (Morsy et al. 2007). Raffinose was found to be having major role in membrane stabilization than other monosaccharides and disaccharides (Rontein et al. 2002).

6.8.1.2 Soluble Sugars

In cold hardy plants, synthesis of sucrose and the activity of enzymes involved in sucrose synthesis significantly rise. Mutations in the enzymes of sucrose synthesis make the plant sensitive to cold. On the other hand, mutants overexpressing enzymes of sucrose synthesis were found to be freezing tolerant than wild type plants (Strand et al. 2003).

Transgenic *Arabidopsis* plants overexpressing Sucrose Phosphate synthase (SPS) exhibited better photosynthesis, mobilization of sucrose and enhanced freezing tolerance as compared with wild type plants when shifted to 5 °C, while antisense plants were impaired in sucrose mobilization and freezing tolerance (Strand et al. 2003). Exogenous application of sucrose conferred cold tolerance to freeze-sensitive plants. Sucrose supplementation to freezing-sensitive plants also exhibited reduced loss of osmotic responsiveness to a level comparable to cold acclimation of wild type (Uemura et al. 2003).

It has been observed that during initial phase of cold acclimation, transcripts of hexose kinase and fructose kinase are abundantly expressed. These enzymes are involved in synthesis of hexose 6-phosphate and fructose 6-phosphate, which are precursors of sucrose biosynthesis. This leads to accumulation of sucrose in plants during cold acclimation (Kaplan et al. 2007). Sucrose and glucose protects a plant by directly acting as a substrate for cellular respiration or as an osmolyte to maintain

cell homeostasis (Janmohammadi 2012). Fructan, a polymer of sucrose, synthesized by fructosyl transferase, helps in membrane stabilization by binding to phosphate and choline head groups in lipid membrane (French and Waterhouse 1993). Fructan accumulation in cold-acclimating plants suggests their role in enhancing cold tolerance (Livingston et al. 2009).

Soluble sugars are also speculated to have regulatory effect on genes under cold stress conditions. COR78 gene expression levels increase when plants under LT are supplemented with sucrose. It appears that sucrose imparts control over COR78 gene and upregulates its expression so as to acclimate plant to cold conditions (Rekarte-Cowie et al. 2008). Likewise, β-amylase also regulates some COR genes (Heidarvand and Maali Amiri 2010).

6.8.2 Photosynthetic Activity

Many studies document the reduction in photosynthetic carbon dioxide uptake in chilling stress conditions as compared to normal conditions. In vitro assay has shown that at LT neither ribulose-1,5-bisphosphate carboxylase nor electron transport from water to Dichlorophenol Indophenol in isolated thylakoid is inhibited. Reduction in photosynthetic activity was not attributed either to low phosphate concentration or to low carbon dioxide supply or to low sink to source ratio under cold stress (Bauer et al. 1996). Study done on conifers revealed that the rate of photosynthesis decreases in winters due to reduction in chlorophyll content (Hansen et al. 1996).

New leaves of plants subjected to 5 °C expressed high levels of Calvin cycle enzymes along with an increase in total protein content. On the other hand, a very small increase of these enzymes is observed in mature leaves of plants that were first grown at normal temperature and later exposed to 5 °C. This suggested that leaves initiated under cold conditions are metabolically more tolerant to cold stress. Moreover, the levels of enzymes involved in sucrose synthesis in cytosol were found to increase more rapidly than enzymes of starch synthesis in chloroplast (Strand et al. 1997). An increase in Calvin cycle's enzymes was also observed. This increase might be due to overall increase in proteins during cold stress. However, the increase in Calvin cycle enzymes was still less than that of the enzymes of cytosol involved in sucrose synthesis (Goulas et al. 2006).

DIGE analysis indicated that Rubisco small and large subunit proteins increased in cold stress (Goulas et al. 2006). Heidarvand and group (2013) found that Rubisco levels increase when ATP synthesis and Electron Transport activity declines. Warm grown leaves when exposed to cold stress showed high levels of ATP synthase proteins in stromal compartment. Their levels come back to normal when new leaves develop at 5 °C but are high when plant are suddenly exposed to cold stress. The ATP/ADP ratio in plants also increases under cold stress (Hurry et al. 2000).

6.8.2.1 Xanthophylls

Xanthophylls are yellow pigments found in leaves, synthesized in plastids. These are oxygenated carotenoids and do not require light for synthesis. Their accumulation is known to surge under cold stress. They protect photosystems from oxidative damage by quenching triplet Chl and singlet oxygen during cold stress (Janmohammadi 2012). Xanthophyll acts as natural antioxidants. Free Zeaxanthin and carotienoids also seem to protect thylakoid membrane from any kind of oxidative damage caused due to change in temperature conditions (Theocharis et al. 2012).

6.8.2.2 Flavonoids

Flavonoids are secondary metabolites, synthesized by polypropanoid pathway from phenylalanine. These molecules have antioxidant activity and their concentrations increases in cold stress. Thus, stabilizing plants by scavenging oxidative species produced in cold stress (Janmohammadi 2012). Transcriptome analysis has revealed that under cold stress transcripts of flavonoid synthesis increase thereby increasing flavonoid concentration and protecting plant from oxidative damage (Theocharis et al. 2012). Flavonoids also appear to be responsible for cell membrane stabilization as they accumulate in lipid phase of cell membrane at sub-zero temperatures (Korn et al. 2008).

6.8.3 Membrane Lipids and Fluidity

Changes in temperature have a very prominent effect on permeability and composition of membrane lipids in plants. Alteration in membrane lipid composition allows plant to survive not only in cold but also during intense heat conditions (Campos et al. 2003; Sage and Kubien 2007; Heidarvand and Maali-Amiri 2013). Fatty acid composition in membrane is the key factor involved in determining fluidity of the membrane. Saturated fatty acids ensure more rigidness as they favor hydrophobic interactions in membrane whereas unsaturated FAs increase fluidity of membrane (Campos et al. 2003). It has been observed that in cold-tolerant species saturated to unsaturated fatty acid ratio decreases under cold stress. An alteration in unsaturated fatty acid (UFA) levels has also been reported in cold-stressed plants. In Chickpea, fatty acids with longer carbon chain are more abundant than shorter fatty acids. Along with this a high abundance of 18:3 UFA is seen as compared to 18:1 and 18:2. Thus, indicating importance of FAs elongation and desaturation in tolerance against stress (Heidarvand and Maali-Amiri 2013). In *C. apoata*, C16:1 contributes toward membrane stability (Campos et al. 2003). High C16:1 in thylakoid membrane promotes LHCII oligomerization which enhances photosynthesis in cold stress conditions. However in cereals, C16:1 decreases in cold stress and so does

LHCII oligomerization. This was postulated to regulate energy distribution to avoid effects of photoinhibition caused by cold stress in plants. In cold-stressed plants, lipid peroxidation increases as is evident from high MDA levels in plants experiencing cold conditions.

During recovery from cold stress, cold-tolerant plants ensure membrane stability by accumulating more of lipids that are able to withstand lower temperatures. Phosphatidylcholine (PC) and phosphatidyl ethanolamine (PE) are two phospholipids that are highly abundant in plasmalemma and mitochondrial membrane. PC has lower phase transition temperature than PE. So, in recovery phase plants under cold stress have more PC to PE ratio (Campos et al. 2003).

6.8.3.1 Electrolyte Leakage Index and Double Bond Index

Abundance of UFA in membrane increases permeability of membrane for solutes and ions. Integrity of membrane is evaluated by changes in Electrolyte Leakage Index (ELI) under stress condition. In a study, it was found that initially ELI increased continuously for 2 h and reach to its maximum at the end of this period immediately followed by a decline till 8 h. Initial increase and eventual decrease in ELI suggest induction of cold tolerance and the stability of membranes that plants try to attain after cold shock (Campos et al. 2003; Heidarvand and Maali-Amiri 2013). Degree of unsaturation in FAs also increases in low temperature grown plants. Double Bond index (DBI) is the parameter used to evaluate the level of UFAs. In many species, it was found that monogalactosyl diacylglycerol (MGDG) to digalactosyl diacylglycerol (DGDG) ratio as well as overall phospholipid content decreases under cold stress. In thylakoid membrane DGDG is known to provide high permeability of ions and more stability to lipids and proteins present in membrane (Campos et al. 2003).

Lipoxygenase (LOX) is an enzyme involved in biosynthesis of jasmonic acid and other secondary metabolites involved in antioxidant activity. Increase in LOX activity and DBI is associated with lower levels of membrane damage and ELI. A protein PsbP, oxygen evolving complex (OEC) proteins, stabilizes water-oxidizing complex of PSII and protects Mn cluster from oxidative stress during LT condition. Its level increases till ELI and MDA levels are high. Thus, it plays a cryoprotective role in stabilizing PSII and so electron transfer and energy generation processes for survival of plant. At the same time, an increase in levels of ATP synthase CF1 epsilon subunit was also observed. It is involved in ATP hydrolysis in chloroplast and mitochondria. Increase of epsilon subunit of CF1 indicates high-energy requirement of cells during cold stress. Kinetic study revealed that at an early time point ELI, MDA, and H_2O_2 levels are high due to sudden change in temperature. To combat temperature fluctuation, the levels of longer UFAs, LOX, and ATP synthesis increase. Cytochrome *c* oxidase subunit 6b-1 declined initially but regained its levels after 8 h of cold stress (Heidarvand and Maali-Amiri 2013).

6.8.4 Polyamines

Polyamines are ubiquitous small aliphatic amines, carrying a positive charge at cellular pH. Polyamines viz. putrescine (diamine), spermidine (triamine), and spermine (tetramine) have been reported to play vital role in modulating cellular and physiological functions. Its polycationic nature allows it to interact with other molecules and exists as acid soluble and acid insoluble conjugates (Kasukabe et al. 2004). They can also interact with proteins involved in defense mechanism against various environmental stresses. Polyamines also suppress ROS during cold stress (Cuevas et al. 2008). H_2O_2 production in cold-stressed plants was reduced when these plants were supplemented with polyamine but its production increased when polyamine synthesis inhibitor was applied. This clearly proves the role of PA as ROS scavenger (Zhang et al. 2009). Exogenous supply of polyamines to chilling-sensitive plants has suppressed electrolyte leakage resulting in higher chilling tolerance. Cold-stressed plants also have reduced soluble protein content, which reaches to normal levels after exogenous supplementation of putrescine and spermidine (Zhang et al. 2009). Spermidine and spermine are biosynthetic precursors of β-alanine. In cold stress, the metabolic pathway of β-alanine biosynthesis also get upregulated. Thus, pointing toward the possible role of polyamines in cold condition (Töpfer et al. 2013).

In cucumber, cold-sensitive and -tolerant cultivars show different levels of H_2O_2 and polyamines in cold stress. Cold-tolerant cultivar, contrary to cold-sensitive one, has high levels of free polyamine and low level of H_2O_2. On application of PA synthesis inhibitor, methyglyoxal-bis-(guanylhydrazone) (MGBG), polyamine level decreased and H_2O_2 levels increased to a level similar to that of cold-sensitive cultivar (Zhang et al. 2009).

A study showed that exogenous application of polyamine especially spermidine improved the functionality of enzymes involved in antioxidant Halliwell–Asada pathway (Kubis 2003). Polyamines have been shown to protect plasma membrane and act as antioxidant in other stress conditions also (Kim et al 2002). In oats and transgenic *Arabidopsis* putrescine levels increased at early stage of cold stress but there was no prominent increase in spermine or spermidine levels (Alcázar et al. 2010). Putrescine is a precursor for both spermidine and spermine. Thus, it is hypothesized that spermine and spermidine are under strict regulation (Cuevas et al. 2008). Arg decarboxylase (ADC), involved in synthesis of putrescine, is induced under cold stress. Accumulation of ADC isoforms in *Arabidopsis* under cold stress also indicates increased synthesis of putrescine (Alcazar et al. 2006). Mutations in *ADC* gene cause cold sensitivity in *Arabidopsis* (Cuevas et al. 2008).

6.8.5 Nitric Oxide

Nitric oxide acts as a signaling molecule and triggers downstream pathways to combat various physiological stress and developmental conditions. In *Arabidopsis*, nitric oxide is known to be involved in cold stress response. Studies have found that

NO levels increases in wild type plants in response to cold stress. NO is synthesized by nitric oxide synthase (NOS) or nitrate reductase (NR). NO-synthase-like enzyme was proposed to produce NO by conversion of arginine to citrulline. However, the presence of such a protein is still under question. The other pathway utilizes enzyme nitrate reductase, which assimilate nitrogen by converting nitrate to nitrite (Zhao et al. 2009; Thakur and Nayyar 2013). NR is encoded by gene nitrate reductase NIA that is upregulated under cold conditions. Production of NO in cold stress is entirely dependent only on NR pathway. *Arabidopsis* NR double mutant *nia1nia2* exhibit defective NO production during cold stress and thus results in low freezing tolerance (Zhao et al. 2009). NO activates or inactivates a protein by S-nitrosylation of their thiol group. This leads to regulation of downstream processes (Abat and Deswal 2009). NO is also known to mediate signaling along with ABA, calcium, and hydrogen peroxide. It also plays key role in stomatal opening and closing under stress conditions (Neill et al. 2008).

6.8.6 Proline

Proline is a unique amino acid derived from "pyrrolidine." It acts as small organic cation required to maintain cytosolic acidity and membrane integrity (Janmohammadi 2012; Jouve et al. 2004). Accumulation of proline has been observed in plants tolerant to cold stress, indicting its involvement in cold acclimation (Janmohammadi 2012; Kaur et al. 2011). Proline treatment to cold-sensitive plants reduced electrolyte leakage and stabilized membrane during cold stress. Malondialdehyde (MDA) is the end product of lipid peroxidation and is thus used as a marker for osmotic stress. In cell culture exposed to osmotic stress, MDA levels shoot up but significantly decline after proline supplementation.

The water and chlorophyll levels also increase due to proline treatment in cold-stressed plants (Kaur et al. 2011). Decreased levels of ROS in proline-treated plants describe its role in increased activity of antioxidants. The biochemical assays done by Hong et al. (2000) also supports that proline helps in reduction of free radicals that are formed due to osmotic stress. Even in osmotic stress, proline has been documented to play a role as free radical scavenger (Hong et al. 2000; Jouve et al. 2004).

Another in vitro assay, done to examine free radical scavenging activity of proline, proved that proline accumulation during abiotic stress in plant could suppress ROS produced due to stress condition (Kaul et al. 2008). Cold stress induces lipid peroxidation and methylglyoxal (MG) production that decreases upon exposure to proline and betaine. MG is produced in cells due to abiotic stress along with ROS and is highly toxic to plants (Goulas et al. 2006). It reacts with proteins, carbohydrates, and DNA. Enzymes involved in glyoxalase pathway are stabilized by proline supplementation hence again indicating its role in enhancing antioxidant activity of plant. Exposure of proline also enhanced glutathione-S-transferase and glutathione

reductase activity in plants. These enzymes are majorly involved in detoxification pathways (Goulas et al. 2006).

Cold has its effect on flowering and pod generation as well. Proline depletion in cold-stressed plants increases their sensitivity to cold and leads to flower and pod abortion (Kaur et al. 2011). Proline levels were found to increase in cold-stressed plants till fourth day and decreased as cold persisted, to a level lower than normal. However, flowering and pod generation retained in proline-treated plants. Lower proline content was earlier linked with male sterility also. Moreover, upon exogenous application of proline enhances pollen germination.

Concentrations of proline in cold-stressed plants also regulate the carbohydrate levels (Kaur et al. 2011). Chilling-stressed plants have a lower level of sucrose and trehalose. These carbohydrates are quite important for acclimatization of plants in cold stress as explained earlier. Upon proline treatment, plants were shown to acquire normal levels of carbohydrates aiding in cold acclimatization. As a result of proline-induced carbohydrate levels, under chilling stress these were made available to other parts of plant especially pods and flower. Availability of sugar to pod and flower prevented their abortion (Kaur et al. 2011). Increase in proline level is directly associated with increasing NO level in cold stress. Mutants with reduced production of NO also exhibit lower proline content. Exogenous application of NO scavenger has shown to decrease the level of proline in cold-stressed plants (Zhao et al. 2009).

6.9 Conclusions and Perspectives

Stress disrupts metabolic homeostasis and plants require countless adjustments to acclimate these conditions. Here, we have documented various evidences of several genes and gene networks involved in regulating the cold-mediated responses by several "Omic"-based approaches. With the advent of increasing knowledge yielded from transcriptomic and proteomic-based studies now a shift is being taking place mostly toward metabolomic-based studies to understand the adaptive responses during cold stress. Studies in future are expected to enhance our knowledge on the low temperature responsive transcriptome, proteome, and metabolome from different crops plants. These efforts are essential in order to understand the molecular network of interacting proteins that confers freezing tolerance in crop plants. A concerted approach toward deciphering physiological and metabolic aspects of cold stress is needed to comprehend the phenotype of plants under cold stress. Nevertheless, there remains a major task in connecting these "Omic"-based approaches in different crop plants to develop a holistic ideology to tackle the problem of cold- and freezing-associated damages in the plants.

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Chapter 7
Drought Stress Responses and Signal Transduction in Plants

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Abstract Nature provides all necessary components for healthy growth and development of plants in the form of air, water, light, nutrients, and soil. Any imbalance in the environmental harmony may cause stress to them. Stresses encountered by plants can broadly be categorized into biotic and abiotic stresses. Biotic stresses are mainly caused by pathogens and herbivory, whereas abiotic stresses include the threat imposed by drought, salinity, and extremes of temperature, heavy metals, and pollution. Drought stress is a major cause of yield instability in crops across diverse eco-geographic regions worldwide. A variety of biochemical, molecular, and physiological changes are manifested by plants in response to drought stress. The cellular abscisic acid (ABA) concentration increases on water deficit leading to the activation of a number of stress-responsive genes and the patterns of expression of these genes are very complex, with some genes being induced early while others respond slowly. In general, drought-responsive genes respond to salt and cold stresses as well as to exogenous ABA treatment. However, there are several genes, which express themselves in an ABA-independent manner suggesting that both ABA-dependent and -independent signal transduction cascades exist for drought stress perception, response, and adaptation. Drought stress response and adaptation in plants involves an array of pathways for signal perception, transduction, gene expression and synthesis of proteins, and other stress metabolites. Drought-responsive genes can mainly be classified into two groups. First group constitutes genes whose products provide osmotolerance and protection to plants thus directly functioning in tolerance to stress, while the second group includes genes playing a role in signal transduction as well as regulation of gene expression. This chapter summarizes the complex molecular mechanisms of drought stress response and adaptation in plants, highlighting the transcriptional regulation of stress-responsive

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gene expression. It also focuses on the recent advances in analyzing various stress-responsive pathways with prime emphasis on ABA-dependent and -independent pathways.

Keywords Abscisic acid • Cross talk • Drought • Gene expression • Signal transduction • Transcription factors

7.1 Introduction

Plants are continually challenged by innumerable adverse environmental stresses that affect their growth and productivity. These environmental stresses are of two types, viz. biotic and abiotic. The biotic stresses indicate the stresses caused by living organisms like pathogens and herbivory while abiotic stresses usually comprise moisture deficit or drought, salinity, heat, and low temperature or cold stresses. Irrespective of the type, all these stresses elicit a complex molecular response in plants, starting with perception of stress, initiation of signal transduction, and its manifestation at cellular, physiological, and developmental levels. Of note, these processes rely on the stress duration, genotype, severity of stress, developmental stage, and factors conferring the stress (Bray 1994). Plants are equipped with different strategies to adapt or acclimatize to these environmental stresses, and hence studying these mechanism(s) by which plants perceive stress signals, respond, and adapt to them is of prime importance in biology in order to understand the complex stress regulatory pathways. This knowledge would assist the plant research community to generate elite crop species with enhanced stress tolerance.

The term "drought" comes with several connotations ranging from impaired yields due to too little water according to agronomists, to sudden severe water deficits as defined by molecular biologists (Passioura 2007). In the scenario of global warming, drought is indeed considered to be one of the most important abiotic stresses that adversely affect the plant growth and development, and ultimately questions the food security (Anjum et al. 2011a, b, c; Shao et al. 2009). Limited water supply to roots and/or higher transpiration rate due to elevated atmospheric temperature induces drought stress, which severely impairs growth, yield, and also causes disrupted membrane integrity, reduced pigment content and photosynthetic activity, and changes in osmotic balance (Praba et al. 2009). Plants in turn are equipped with sophisticated stress-responsive mechanisms to circumvent the water deficit stress (Duan et al. 2007; Anjum et al. 2011a, b, c). Therefore, understanding the molecular mechanism of drought stress signal transduction pathway in crop plants is crucial for sustained advancement of rational crop breeding and transgenic approaches to improve stress tolerance in crops of economic importance. This chapter thus aims to summarize various responses of plants to drought stress along with some common characteristics of drought stress signal transduction. It also attempts to analyze some studies on the signaling components.

7.2 Drought Stress Responses

7.2.1 Morphological, Physiological, and Biochemical Responses

7.2.1.1 Growth and Yield

The effects of drought stress largely depend upon severity, duration, and developmental stages. Water deficit stress is more vulnerable at the germination and early seedling growth stages of plants. Reduced or impaired germination and poor seedling stand are some of the early signs of drought stress (Harris et al. 2002; Farooq et al. 2009). A reduction in germination potential, hypocotyls length, and root and shoot length have been reported in several crops (Zeid and Shedeed 2006; Manikavelu et al. 2006; Baloch et al. 2012; Singh and Lata 2013, unpublished). Water stress led to increased root growth in *Helianthus* (Tahir et al. 2002) and *Catharanthus* (Jaleel et al. 2008). Fresh and dry biomasses of crop plants are also greatly affected by water deficit (Zhao et al. 2006). Similarly, Sečenji et al. (2010) reported a decrease in root mass as well as biomass of aerial parts in wheat genotypes during drought stress. In addition, among the physiological processes, the most sensitive process to get affected by water deficit is the cell growth (Farooq et al. 2006). A reduction in turgor pressure due to water deficit limits cell growth while cell elongation of higher plants is inhibited by interrupted water flow from xylem to the surrounding elongating cells (Nonami 1998; Taiz and Zeiger 2006). Further, severe water deficiency imposes reduction in photosynthesis and suppression of leaf expansion, which lead to reduce leaf area (Rucker et al. 1995). A cumulative effect of reduction in growth-related traits, viz. plant height, leaf area, number of leaves per plant, cob length, shoot fresh, and dry weight due to drought stress was reported in maize (Kamara et al. 2003).

Further, several yield determining physiological and developmental processes are also affected by water stress. Disruption in leaf gas exchange properties limit the size of source and sink, impairs phloem loading, nutrient uptake, and dry matter partitioning in plants, thus severely declining yield traits (Farooq et al. 2009). For example, pre-anthesis water stress reduced time to anthesis while a post-anthesis stress decreased the grain-filling duration in several triticale genotypes (Estrada-Campuzano et al. 2008). Reduced grain filling occurs due to decreased assimilate segregation and activities of sucrose and starch biosynthesis enzymes (Anjum et al. 2011c). Drought at flowering stage in pearl millet is more damaging than at vegetative stage (Yadav et al. 2011). Drought stress at tasseling stage in maize leads to considerable decline in yields and yield-related traits in maize (Anjum et al. 2011a). These evidences indicate that dehydration stress directly impairs the growth and yield of several crop plants.

7.2.1.2 Water and Nutrient Uptake

Plant–water relations are largely determined by several physiological characteristics including leaf water potential (LWP), leaf and canopy temperature, relative water content (RWC), stomatal resistance, and transpiration rate (Tr). Water-stressed wheat, rice, and foxtail millet plants had lower RWC as compared to the control plants (Siddique et al. 2001; Lata et al. 2011a). Siddique et al. (2001) reported a decline in LWP, RWC, and Tr with a simultaneous increase in leaf temperature in drought-stressed wheat and rice plants. RWC, turgor potential, transpiration, stomatal conductance, and water use efficiency decreased in drought-stressed *Hibiscus* plants (Egilla et al. 2005). In fact, closing and opening of stomata is badly affected by reduced water availability as compared to other components of plant–water relations. Furthermore, variation in leaf temperature could be a significant factor in regulating leaf water status during water deficit stress (Farooq et al. 2009).

Total nutrient uptake including absorption and translocation is also greatly affected by reduced water availability. Water stress induces an increase in nitrogen (N), a drastic decrease in phosphorus (P), and no significant effects on potassium (K) uptake (Garg 2003). However, several reports indicated that uptake of inorganic ions gets declined under the influence of drought stress in crop plants (McWilliams 2003; Peuke and Rennenberg 2004). Limited availability of energy for assimilation of NO^{3-}/NH^{4+}, PO_4^{3-}, and SO_4^{2-} may also be correlated to the influence of drought on plant nutrition (Grossman and Takahashi 2001). Drought stress thus reduces availability, uptake, translocation, and metabolism of nutrients; however, crop yields can even then be considerably improved by enhancing nutrient-use-efficiency, as there is a significant interaction between soil moisture deficit and nutrient acquisition (Farooq et al. 2009).

7.2.1.3 Photosynthesis and Pigment Content

Water deficit stress has a profound adverse effect on photosynthesis, photosynthetic apparatus, and pigment content (Wahid and Rasul 2005). A considerable decline in net photosynthesis has been reported in drought-stressed maize in contrast to well-watered control plants (Anjum et al. 2011a). With a decrease in RWC and LWP, the foliar photosynthetic rate in higher plants is also observed to decline (Lawlor and Cornic 2002). Severe water stress has also been reported to decline the activity of the most important photosynthetic enzyme RuBisCO, and thus limiting photosynthesis (Bota et al. 2004). Several research groups reported reduced photosynthetic activity under conditions of drought stress because of stomatal or non-stomatal mechanisms (Del Blanco et al. 2000; Samarah et al. 2009; Farooq et al. 2009). However, in sunflower, it has been observed that photosynthesis reduction during dehydration through metabolic damage is more complex than stomatal limitations and is mainly through reduced photosynthetic pigment contents (Reddy et al. 2004). Water deficit-induced loss of chlorophyll content is considered to be the main cause of reduced photosynthesis and the reduction in pigment contents has also been

attributed to acute swelling, appearance of lipid droplets, loss of chloroplast membranes, and the distortion of lamellae vesiculation (Kaiser et al. 1981). This is supported by the reports on reduced concentration of chlorophyll and carotenoids in plant tissues (Kiani et al. 2008) primarily due to drought-induced reactive oxygen species (ROS) production in the thylakoids (Reddy et al. 2004). Similar reduction in chlorophyll content due to drought stress has been reported in several crop plants (Manivannan et al. 2007; Massacci et al. 2008; Jaleel et al. 2008; Kiani et al. 2008; Jain and Chattopadhyay 2010). A changed ratio of chlorophyll "a" and "b" and carotenoids due to water stress has also been reported by Farooq et al. (2009) and Anjum et al. (2011b). These reports justified the drought-inhibition of photosynthesis, which threatens the survival of crop plants.

7.2.1.4 Accumulation of Osmolytes

In order to maintain cell turgor, plants accumulate various types of solutes into the cytosol thus balancing the osmotic pressure (Rhodes and Samaras 1994). The process of accumulation of various solutes such as proline, glycine betaine, glutamate, sucrose, and soluble carbohydrates in the cytoplasm is known as osmotic adjustment, by which the plant maintains leaf turgor under drought stress (Anjum et al. 2011c). These compounds prevent membrane damage and enzyme inactivation at low water activity environment and thereby assist in maintaining normal cellular activities during dehydration (Rampino et al. 2006). Proline is one of the most important osmolytes involved in membrane stabilization. Infact plants exposed to water stress tend to primarily accumulate proline in order to reduce cell injury. Several studies have reported the accumulation of proline under progressive drought stress (Claussen 2004; Rampino et al. 2006; Anjum et al. 2011b; Singh and Lata 2014, unpublished). Proline is a key molecule responsible for stabilization of subcellular structures, removal of free radicals, and buffering cellular redox potential during stress (Demiral and Turkan 2004; Ashraf and Foolad 2007). In addition, proline is also thought to act as a signaling molecule in modulating various cellular events such as mitochondrial function, cell proliferation, cell death, and specific gene expression which help cells in recovering from stress (Anjum et al. 2011b). In several species, higher accumulation of proline in tolerant genotypes during stress is related with stress tolerance of those genotypes (Anjum et al. 2011c).

7.2.1.5 Generation of ROS

Generation of ROS such as superoxide anions, hydroxyl radical, hydrogen peroxide, and singlet oxygen is probably one of the earliest biochemical responses of plants exposed to environmental stresses including water stress. Though ROS is a normal by-product of regular oxygen metabolism, it also acts as secondary messengers to elicit specific defense cell signaling or redox signal transduction during severe environmental stresses. Under drought stress, ROS may react with lipids,

proteins, and nucleic acids to cause oxidative damage and ultimately hampers the normal cellular functions or even result in cell death (Anjum et al. 2011c; Lata et al. 2011a). It has also been reported that ROS can cause widespread protein denaturation, mutation of nucleic acids, peroxidation, and de-esterification of membrane lipids (Bowler et al. 1992). In plants, ROS are generated in chloroplast, mitochondria, and peroxisomes. Excessive ROS generation in chloroplast membrane makes them extremely sensitive to oxidative stress damage (Mahajan and Tuteja 2005). It has been reported that drought-induced overproduction of ROS increases malonaldehyde (MDA) content, which is an indicator of oxidative damage and also a suitable marker for membrane lipid peroxidation caused by ROS (Anjum et al. 2011c; Lata et al. 2011a). Decreased membrane stability indicates the extent of lipid peroxidation and also shows the prevalence of free radicals in affected tissues. Several reports have shown increased levels of lipid peroxidation with drought stress (Yang and Miao 2010; Lata et al. 2011a).

7.3 Molecular Responses

Water deficit or drought stress induces a plethora of molecular, biochemical, and physiological responses in plants. It is a well-recognized fact that tolerance to dehydration stress is a complex phenomenon comprising the combined action of several genes (Farooq et al. 2009). Numerous responses to water deficit stress are controlled by an array of genes with diverse functions whose expression may change (up- or downregulation) under such adverse conditions. In order to compensate water loss from the cell, regulatory processes are initiated to fine-tune cellular metabolism to new cellular conditions (Bray 1993). Simultaneously, inhibited growth and altered developmental pathways lead to changes in gene expression. It is anticipated that genes expressed during water deficit stress promote cellular tolerance against dehydration either through providing osmotic homeostasis or through stress damage control and repair or through growth control and regulating gene expression (Bray 1993; Zhu 2002). Thus, drought stress signaling can be classified into three functional categories: (1) osmotic signaling to reestablish cellular homeostasis; (2) detoxification signaling for repairing and damage control; and (3) cell division and expansion signaling (Zhu 2002). Stress signal perception and transmission, preferably called stress signal transduction is crucial for gene expression regulation.

7.4 General Stress Signaling Pathways

A general signal transduction pathway commences with stress perception by receptor(s). These receptors are plasma membrane proteins, which bind and interact with the extracellular molecules, known as ligands or elicitors. The stress signal is then transduced downstream resulting in the generation of the second messengers

such as ROS and inositol phosphates that can modulate intracellular Ca^{2+} levels. This perturbation is sensed by calcium sensors, which after changing their conformation often initiate a phosphorylation cascade that finally targets stress-responsive genes. These stress-responsive genes could be directly implicated in cellular protection or transcription factors (TFs) that regulate the expression of downstream stress-regulated genes (Mahajan and Tuteja 2005; Xiong et al. 2002). The products of these stress-responsive genes not only help plants to adapt and survive adverse environmental conditions but may also participate in the generation of phytohormones like abscisic acid (ABA), salicylic acid (SA), and ethylene. These hormones act as regulatory molecules in amplifying the initial signal and also initiating a second round of signaling that can follow the same generic pathway or involve on the whole different signaling components. There are also certain components known as accessory molecules, which may not directly participate in stress signaling but may help in modification, delivery, or assembly of various signaling components and thus are crucial for precise stress signal transmission. These accessory molecules include protein modifiers such as enzymes for glycosylation, methylation, myristoylation, and ubiquitination; scaffold proteins and adaptors (Xiong and Zhu 2001).

The products of major stress-inducible genes may be largely classified into two groups: first group comprises proteins that are directly involved in stress tolerance such as water channel proteins and late embryogenesis abundant (LEA) proteins. The second group constitutes regulators of intracellular signaling and stress-inducible gene expression such as protein kinases, phosphatases, *cis*-regulatory elements, and TFs (Hirayama and Shinozaki 2010).

7.5 Aquaporins and Stress Proteins

Aquaporins are intrinsic membrane proteins which simplify and control passive exchanges of water molecules across plant membranes. In plants, they are widely found in plasma and vacuolar membranes. Several studies have been performed on aquaporins and plant–water relations, which showed the vital roles of aquaporins in transcellular water transport. Hence, aquaporins are found to be abundantly expressed in root, where they mediate soil water uptake (Javot and Maurel 2002). The study by Javot and Maurel (2002) had also showed the decrease of root hydraulic conductivity in the presence of mercury, which is reported to be a potential inhibitor of aquaporins, thus hampering overall root water uptake.

Several drought-induced gene products are found to shield the cellular structures from dehydration. Since most of the drought-induced gene products such as stress proteins are water soluble, they provide hydration to cellular structures and thereby confer stress tolerance (Wahid et al. 2007). LEA proteins are the most abundant stress proteins that are linked to both water and cold stress in plants. These proteins are active in seeds that contain high ABA levels (Tunnacliffe and Wise 2007). In plants, six groups of *LEA* genes have been identified of which group 3 and 5 LEA proteins are predicted to play important roles in ion sequestration during cellular

dehydration or water deficit (Bray 1993). Group 1 LEA proteins are predicted to possess enhanced water-binding capability while group 4 LEA proteins may participate in replacing water to protect cellular structures (Bray 1993). Dehydrins are also a group of LEA proteins that are known to accumulate during water deficit stress. Transgenic rice plants expressing barley group 3 LEA genes, *HVA1* were able to maintain relatively high RWC and showed less electrolyte leakage from cells suggesting that this protein may play crucial roles in protecting cell membranes from damage during osmotic stress (Rohila et al. 2002; Babu et al. 2004). This gene when expressed in wheat was also capable of conferring improved growth and higher WUE (Sivamani et al. 2000). Similarly, Group 1 and 2 LEA proteins from wheat provide protection against dehydration stress when expressed in rice (Cheng et al. 2002).

7.6 Protein Kinases in Drought Signaling

Protein phosphorylation, the core theme in cell signaling has also been found to be involved in stress adaptation (Zhu 2002). Several protein kinases are reported to express in response to osmotic stress. Mitogen-activated protein kinase (MAPK) cascades constitute one of the major cellular signaling components in eukaryotic cells and are generally activated in response of hyperosmotic stress (Hirayama and Shinozaki 2010). A large number of MAPKs, MAPKKs, and MAPKKKs have been identified in various plant species, which are shown to function in several various processes thus making complex networks. Transcript levels of many protein kinases including various members of MAPK signaling have been found to be increased under osmotic stress. This ultimately resulted in accumulation of osmolytes that help in reestablishing osmotic balance and protects the cells from stress damage by induction of several stress proteins such as LEA (Zhu 2002; Mahajan and Tuteja 2005).

Sucrose non-fermentation 1 (SNF1)-related kinases are also found to be induced in response to hyperosmotic stresses. Several of these kinases such as *Arabidopsis SRK2D/SnRK2.2*, *SRK2I/SnRK2.3*, and *SRK2E/OST1/SnRK2.6* are strongly ABA responsive are essential for ABA signaling thus establishing the fact that SnRK2-type kinases may play crucial roles in transducing the signals which activate them (Hirayama and Shinozaki 2010).

Calcium-dependent protein kinases (CDPK) are another important group of kinases that are activated in response to osmotic stress and are found to activate calcium signaling (Zhu 2002). In a study in maize protoplast transient expression system showed that a constitutively active CDPK mutant activated the expression of the reporter gene, which was normally responsive to osmotic stress, cold, and ABA while its negative form was capable of inhibiting the stress or ABA induction of the gene (Sheen 1996). CDPK genes, CPK3, and CPK6 regulate ABA activity in guard cells (Mori et al. 2006) while CPK4 and CPK11 positively regulates ABA responses. In addition, they were also able to phosphorylate AREB/ABF TFs in an ABA-dependent manner (Zhu et al. 2007).

7.7 Phospholipid Signaling

Membrane phospholipids not only comprise an active system that produces a large number of signal molecules such as inositol 1, 4, 5-triphosphate (IP_3), diacylglycerol (DAG), and phosphatidic acid (PA) but also serve as important structural elements during stress responses (Zhu 2002). Phospholipid signaling systems are categorized according to the phospholipases that catalyze the formation of lipid and other signal messengers. Among these signaling systems, the phospholipase C (PLC) pathway is well characterized. PLC catalyzes the hydrolysis of phosphatidylinositol 2, 5-biphosphate (PIP_2) into second messengers IP_3 and DAG. IP_3 releases Ca^{2+} from cellular reserves, whereas DAG activates protein kinase C. Drastic increase in IP_3 levels have also been shown in response to hyperosmotic stress in several plant systems (DeWald et al. 2001; Takahashi et al. 2001). In guard cells, IP_3-induced Ca^{2+} increase in the cytoplasm led to stomatal closure and thus enables the cells to retain water (Sanders et al. 1999). An increased cytoplasmic Ca^{2+} could lead to the expression of several osmotic stress-responsive genes (Wu et al. 1997). In fact elevated IP_3 accumulation in *Arabidopsis fry1* mutant has been correlated with, super induction of ABA- and stress-responsive gene transcription (Xiong et al. 2001). Other inositol phosphates such as IP_6 and IP_3 also help in releasing Ca^{2+} from internal stores and thus assist in IP_3 signaling. DAG signaling may be indirect in plants as it can be rapidly phosphorylated to PA.

Increase in phospholipase D (PLD) activity was also evidenced during osmotic stress in various plants (Munnik et al. 2000). PLD is responsible for cleavage of membrane phospholipids to phosphatidic acid (PA) and free head groups wherein PA acts as second messengers in animal cells. PLD was found to be rapidly activated in various plant cells (Frank et al. 2000; Katagiri et al. 2001) and also its activity was higher in drought-sensitive plants than the tolerant ones (El Maarouf et al. 1999). Blocking its activity reduced stress injury and improved stress tolerance, which suggests that its activation results in lipolytic membrane disintegration under stressed condition. On the other hand, application of PA mimics ABA in inducing stomatal closure thus helping to mitigate stress injury (Jacob et al. 1999).

7.8 Role of ABA and Transcription Factors in Osmotic Stress Signaling

ABA carries out a vital role in regulation of many physiological and developmental processes in plants (Lata and Prasad 2011) (Fig. 7.1). It has also been found to be a main physiological signal in inducing drought and high salinity responses (Farooq et al. 2009; Lata and Prasad 2011). The role of ABA is not only in the regulation of developmental pathways but also for controlling many stress adaptation responses like activating genes responsible for osmotic adjustment, ion compartmentalization,

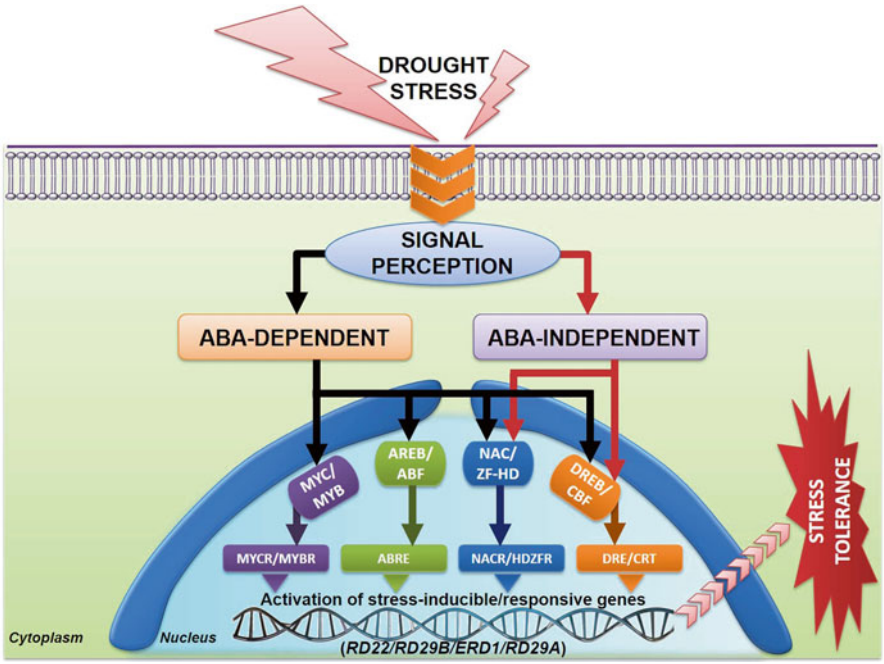

Fig. 7.1 Schematic representation of drought stress tolerance mechanism in plants mediated by several transcription factors (modified from Lata et al. 2011c)

root hydraulic conductivity, regulation of shoot and root growth, limiting transpiration rate and wilting, and ultimately reducing water loss in the plants (Verslues and Zhu 2005; Pospíšilová et al. 2009). It is also involved in modification of gene expression and upregulation of various stress-responsive genes during osmotic imbalance (Ingram and Bartels 1996). Even though many genes are expressed in response to drought stress on exogenous ABA treatment, contrarily some genes do not respond to such treatments. This proposes the existence of two types of pathways: ABA-dependent and -independent signal transduction cascades in crop plants (Hirayama and Shinozaki 2010; Lata and Prasad 2011). ABA-dependent signaling pathway mediates the plant's acclimatization to stress by activating two different regulons: (1) the AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor), and (2) the MYC/MYB regulon. The ABA-independent regulons are: (1) the CBF/DREB (Cold-binding factor/dehydration-responsive element binding) regulon; and (2) the NAC and ZF-HD (zinc-finger homeodomain) regulon (Saibo et al. 2009; Lata and Prasad 2011). Although these different stress response pathways usually function independently, occurrence of pathway cross talks are also possible at some levels.

7.8.1 The AREB/ABF Regulon

The AREB or ABFs belong to the bZIP (basic leucine zipper) transcription factor family which recognizes the ABRE motif thereby activating ABA-dependent gene expression (Uno et al. 2000) (Fig. 7.1). In studies related to ABA-deficient aba2 and ABA-insensitive abi1 mutants, these proteins showed less activity as compared to ABA-hypersensitive *Arabidopsis* era1 mutant (Uno et al. 2000). The ABA-dependent phosphorylation of the AREB/ABF proteins could be a possible mechanism for this response (Shinozaki and Yamaguchi-Shinozaki 2007). The ABFs/AREBs are grouped under group A AtbZIPs (Jakoby et al. 2002), which generally function in ABA signaling during seed maturation as well as under stress conditions (Lata et al. 2011c). Reports indicate that these ABFs play a role in diverse stress signaling pathways, viz. drought, cold, heat, salt, and glucose (Kim et al. 2004; Fujita et al. 2005).

For instance, OsABI5 was found to be induced by ABA and high salinity, and downregulated by dehydration and cold stress in seedlings, while its overexpression led to improved salinity tolerance in rice (Zou et al. 2008; Nakashima et al. 2009). Overexpression of ABF3 and ABF4 led to reduction in transpiration and enhanced drought tolerance (Kang et al. 2002). AREB1/ABF2 is a crucial component of glucose signaling whose overexpression improved drought stress tolerance (Kim et al. 2004). Overexpression of *OsbZIP23* considerably enhanced drought and high salinity tolerance in rice at the reproductive stage (Xiang et al. 2008). Further, upregulation of LEA-class genes and ABA- and dehydration-stress-inducible regulatory genes such as linker histone H1 and AAA ATPase was also evidenced.

7.8.2 The MYC/MYB Regulon

MYC/MYB TFs play active roles in the stress signaling by ABA-dependent pathway and upregulate abiotic stress-responsive genes. *AtMYB2* and *AtMYC2* together act as transcriptional activators in the dehydration- and ABA-inducible expression of *RD22* (Urao et al. 1993; Abe et al. 2003). *AtMYB102* assimilates dehydration, salinity, osmotic, ABA, and wound-signaling pathways (Denekamp and Smeekens 2003). *AtMYB44* confers abiotic stress tolerance by facilitating stomatal closure in an ABA-independent manner (Jung et al. 2008). *AtMyb41* of *Arabidopsis* is transcriptionally regulated under conditions of drought, salinity, drought, and ABA responses.

The overexpression of MYB15 in *Arabidopsis* was found to improve drought and salt tolerance (Ding et al. 2009). Increase in the expression levels of AtMYB2, or AtMYC2 independently or together enhanced ABA sensitivity and improved osmotic tolerance (Abe et al. 2003). *OsMYB4* transgenic lines of *Arabidopsis* exhibited

improved chilling and freezing tolerance with a dwarf phenotype (Vannini et al. 2004), the tomato transgenic showed enhanced tolerance to drought stress (Vannini et al. 2007), while an improved drought and cold tolerance was evidenced in the transgenic apple (Pasquali et al. 2008). Transgenic potato overexpressing *StMYB1R-1* showed higher tolerance to drought stress with no significant effects on other agricultural traits (Shin et al. 2011).

7.8.3 The CBF/DREB Regulon

The CBF/DREB TFs play a significant role in the ABA-independent pathways, inducing the expression of stress-responsive genes. The two main subgroups of DREB subfamily: DREB1 and DREB2 are included in two separate signal transduction pathways under low temperature and dehydration, respectively (Lata and Prasad 2011). In rice, *OsDREB1A* and *Os-DREB1B* were observed to get induced immediately (within 40 min) after cold exposure, but did not respond to ABA treatment (Dubouzet et al. 2003). *Ca-DREBLP1* from hot pepper was rapidly induced by dehydration and high salinity stresses and to a lesser degree by mechanical wounding (Hong and Kim 2005). In another study, *Arabidopsis DREB2A* and its homolog *DREB2B* were induced by dehydration and salinity stresses, but not by cold stress and ABA (Liu et al. 1998; Nakashima et al. 2000). Likewise ABA, mannitol, and cold treatments had meager effect on the expression of *DREB2C* (Lee et al. 2010). *OsDREB2A* accumulated to the highest levels under control conditions and was induced marginally by high temperature, drought, and high salinity treatments (Matsukura et al. 2010). Foxtail millet *SiDREB2* was also evidenced to be upregulated by drought and high salinity treatments (Lata et al. 2011b).

DREB1B/CBF1 or *DREB1A/CBF3* overexpression lines of *Arabidopsis* under a constitutive promoter showed durable tolerance to extreme cold, drought, and high salinity stresses indicating that DREBs/CBFs regulates multiple genes involved in stress tolerance (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). Overexpression of *OsDREB1A* in transgenic *Arabidopsis* and rice plants also exhibited tolerance to low temperatures, high salinity, and drought (Dubouzet et al. 2003; Ito et al. 2006). Improved dehydration tolerance was reported in overexpression wheat lines possessing RD29A::*DREB1A/CBF3* constructs (Pellegrineschi et al. 2004). A constitutive overexpression of *CBF3/DREB1A* and *ABF3* showed enhanced dehydration and salt tolerance in transgenic rice without any phenotypic aberrations or yield penalties (Oh et al. 2005). Overexpression of *OsDREB1F* significantly improved high salinity, drought, and low-temperature tolerance of both rice and *Arabidopsis* transgenics (Wang et al. 2008). These reports support the fact that DREB transcription factors are crucial for regulation of abiotic stress-related genes and thereby play a prime role in imparting stress endurance to plants.

7.8.4 The NAC Regulon

The NAC (NAM, ATAF, and CUC) family is one of the plant-specific TF family whose members are involved in plant developmental programs and disease resistance (Lata et al. 2011c). Many of these genes were also found to respond to various environmental stresses (Lata et al. 2011c; Puranik et al. 2012). *SNAC1*, a stress-responsive NAC is activated primarily in guard cells under dehydration (Hu et al. 2006). *ERD1* (early responsive to dehydration stress 1) promoter analysis revealed that the NAC ZF-HD transcription factors are vital for activation of the ERD1 gene (Tran et al. 2007). *GmNAC2*, *GmNAC3*, and *GmNAC4* were found to be strongly activated by osmotic stress (Pinheiro et al. 2009). OsNAC045 was induced by drought, salinity, low temperature, and ABA in leaves and roots (Zheng et al. 2009). *TaNAC4* was found to be induced by cold, salt, wounding, ABA, ethylene, and MeJA, indicating a considerable cross talk between abiotic and biotic stress conditions (Xia et al. 2010). cDNA microarray led to the identification of several target genes of the AtNAC019, AtNAC055, and AtNAC072 transcriptional activators in the *Arabidopsis* transgenic plants (Tran et al. 2004). These transgenic plants also exhibited enhanced drought tolerance. The *OsNAC6* transgenic plants exhibited the upregulation of various abiotic and biotic stress-responsive genes (Nakashima et al. 2007). The transgenics were also tolerant to dehydration and high salt stresses. Rice plants overexpressing *OsNAC045* showed enhanced tolerance to drought and salt stresses (Zheng et al. 2009). Further, genome-wide analysis of NAC TF family members in foxtail millet and its expression profiling during various abiotic stresses and hormonal treatments revealed a stimulus-specific and time-dependent responses of these TFs (Puranik et al. 2013). The above reports suggest that NAC TFs play a very important role in physiological adaptation of plants for successful growth in stress conditions.

7.9 Functional Genomics Studies on Drought Stress Response and Signaling

Knowledge acquired from the genetic and functional genomic studies of model plants such as *Arabidopsis*, rice, and foxtail millet and also of the plant species that thrive in extreme environmental conditions such as resurrection plant has provided crucial information on drought stress tolerance mechanism(s). Other than model plants, drought tolerance response mechanism has also been elucidated in crops of economic importance such as wheat, maize, and poplar using either gene-by-gene approach or transcriptome, proteome, or metabolome profiling studies (Li et al. 2012; Pérez-Clemente et al. 2013). Typically, a gene-by-gene approach has been used to study plant responses to stress. Overexpression and gene silencing have been major strategies to study stress tolerance mechanisms in different crop plants. Some of the genes, which are involved in drought stress responses and signaling in plants, are listed in Table 7.1.

Table 7.1 Summary of gene(s) involved in drought stress responses and signaling in plants (last 5 year reports were summarized)

Plant	Gene(s)	Gene source	ABA response	Reference
Glycine max and *Nicotiana tabacum*	The endoplasmic reticulum (ER)-resident molecular chaperone BiP (binding protein)	*G. max*	No	Valente et al. (2009)
Oryza sativa	Core-binding factor (*CBF3*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	Salt Overly Sensitive 2 (*SOS2*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	9-cis-epoxycarotenoid dioxygenase 2 (*NCED2*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	Nicotiana protein kinase 1 (*NPK1*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	Molybdenum cofactor sulfurase (*LOS5*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	C2H2-EAR zinc-finger protein (*ZAT10*)	*O. sativa*	Yes	Xiao et al. (2009)
O. sativa	Na(+)/H(+) exchanger (*NHX1*)	*O. sativa*	Yes	Xiao et al. (2009)
N. tabacum	Ethylene response factor 3 (*ERF3*)	*G. max*	Yes	Zhang et al. (2009)
Arabidopsis thaliana	MYB transcription factor (*MYB15*)	*A. thaliana*	Yes	Ding et al. (2009)
O. sativa	β-carotene hydroxylase (*BCH*)	*O. sativa*	Yes	Du et al. (2010)
Solanum lycopersicum	Osmotin	*N. tabacum*	No	Goel et al. (2010)
O. sativa	Ethylene response factor 3 (*JERF3*)	*O. sativa*	No	Zhang et al. (2010)
O. sativa	Ethylene response factor 1 (*ERF1*)	*S. lycopersicum*	Yes	Quan et al. (2010)
A. thaliana	ABA-responsive elements (ABREs) binding factors (*ABF3*)	*A. thaliana*	Yes	Abdeen et al. (2010)
A. thaliana	Phospholipase D (*PLDα1*)	*Setaria italica*	Yes	Peng et al. (2010)
A. thaliana	Ubiquitin-conjugating enzyme gene 2 (*UBC2*)	*G. max*	No	Zhou et al. (2010)
S. lycopersicum	Xyloglucan endo-trans-gluco-sylase/hydrolase (*XTH3*)	*C. annuum*	No	Choi et al. (2011)
O. sativa	RING domain-containing protein (*RDCP1*)	*O. sativa*	Yes	Bae et al. (2011)
O. sativa	Salt- and drought-induced ring finger 1 (*SDIR1*)	*O. sativa*	Yes	Gao et al. (2011)

(continued)

Table 7.1 (continued)

Plant	Gene(s)	Gene source	ABA response	Reference
O. sativa	No apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC] (NAC) [*NAC5*]	*O. sativa*	Yes	Song et al. (2011)
O. sativa	Drought-responsive ethylene response factor (*DREF1*)	*O. sativa*	No	Wan et al. (2011)
Ralstonia solanacearum	MYB transcription factor (PIMP1)	*Triticum aestivum*	Yes	Liu et al. (2011)
A. thaliana	Dehydration-responsive element-binding protein 3a (*DREB3a*)	*Leymus chinensis*	Yes	Xianjun et al. (2011)
S. tuberosum	R1-type MYB-like transcription factor (*MYB1R-1*)	*S. tuberosum*	Yes	Shin et al. (2011)
A. thaliana	Vacuolar H+-pyrophosphatase gene (*AVP1*)	*A. thaliana*	No	Pasapula et al. (2011)
O. sativa	A bZIP transcription factor OsbZIP46 with a deletion of domain D (*OsbZIP46CA1*)	*O. sativa*	Yes	Tang et al. (2012)
N. tabacum	Aquaporin (*AQP7*)	*T. aestivum*	No	Zhou et al. (2012b)
S. lycopersicum	Endoplasmic reticulum (ER)-localized really interesting new genes (RING) E3 Ub ligase (*Rma1H1*)	*Capsicum annuum*	No	Seo et al. (2012)
O. sativa	Heat shock proteins (*Hsp17.0* and *Hsp23.7*)	*O. sativa*	No	Zou et al. (2012)
A. thaliana	C2H2-type zinc-finger protein 1 (ZFP1)	*G. soja*	No	Luo et al. (2012)
A. thaliana	SUMO-conjugating enzyme 9 (*Sce9*)	*Spartina alterniflora*	Yes	Karan and Subudhi (2012a)
A. thaliana	Nascent polypeptide associated complex gene (*βNAC*)	*S. alterniflora*	No	Karan and Subudhi (2012b)
A. thaliana and *Lotus corniculatus*	Dehydration-responsive element-binding protein (*DREB*)	*P. euphratica*	Yes	Zhou et al. (2012a)
O. sativa	Drought-induced lipid transfer protein (*DIL*)	*O. sativa*	Yes	Guo et al. (2013)

(continued)

Table 7.1 (continued)

Plant	Gene(s)	Gene source	ABA response	Reference
T. aestivum	NAC transcription factor (*NAC1*)	*O. sativa*	Yes	Saad et al. (2013)
A. thaliana	Late embryogenesis abundant gene (*LEA*)	*Jatropha curcas*	No	Liang et al. (2013)
A. thaliana	Expansin 4 (*EXPA4*)	*rosa hybrida*	Yes	Lü et al. (2013)
A. thaliana	Acyl-CoA-binding protein 2 (*ACBP2*)	*A. thaliana*	No	DU et al. (2013)
A. thaliana and *G. soja*	Receptor-like cytoplasmic kinase (*RLCK*)	*G. soja*	Yes	Sun et al. (2013)
A. thaliana	Nuclear factor YA (NF-YA)	*G. max*	Yes	Ni et al. (2013)
A. thaliana	Seven In Absentia 2 (SINA2)	*A. thaliana*	Yes	Bao et al. (2014)
A. thaliana	Adenosine diphosphate-ribosylation factors (*ARF*)	*S. alterniflora*	No	Karan and Subudhi (2013)
A. thaliana	Receptor-like kinase (*RLK*)	*Gossypium barbadense*	Yes	Zhao et al. (2013)
O. sativa	DUF966-stress-repressive gene 2 (*DSR2*)	*O. sativa*	Yes	Luo et al. (2013)
A. thaliana	Arabidopsis Response Regulator 22 (*ARR22*)	*A. thaliana*	No	Kang et al. (2013)
A. thaliana	RING E3 ligase (*CTR1*)	*O. sativa*	Yes	Lim et al. (2013)
A. thaliana	Mg-chelatase H subunit (*CHLH*)	*A. thaliana*	Yes	Tsuzuki et al. (2013)
A. thaliana	Calcium-dependent protein kinase (*CPK10*)	*Populus euphratica*	Yes	Chen et al. (2013a)
S. tuberosum	Dehydration-responsive element-binding protein 1 (*DREB1*)	*S. tuberosum*	Yes	Bouaziz et al. (2013)
Gentiana triflora	Dehydrin 1 and 2 (*DHN1* and *DHN2*)	*G. triflora*	No	Imamura et al. (2013)
A. thaliana	Vacuolar pyrophosphatase type I (*EVP1*)	*Eucalyptus globulus*	No	Gamboa et al. (2013)
Zea mays	Molybdenum cofactor sulfurase (*LOS5*)	*Arabidopsis thaliana*	Yes	Lu et al. (2014)
T. aestivum	Ethylene response factor 3 (*ERF3*)	*T. aestivum*	Yes	Rong et al. (2014)

However, functional genomics approach allows large-scale gene function analysis using high-throughput technologies and also helps in establishing function and interaction of gene products at cellular and whole plant level. Also the information generated through next-generation sequencing programs also contribute towards analyzing stress-responsive genes. The large-scale availability of expressed sequence tags (ESTs) and cDNA sequences also compensates for those plant species, which are yet not sequenced. As for example, in *Arabidopsis* several research groups have conducted transcriptome studies to decipher mechanisms regulating stress perception, signaling, and tolerance (Shinozaki et al. 2003; Shinozaki and Yamaguchi-Shinozaki 2007; Rasmussen et al. 2013). The dehydration tolerance mechanism in foxtail millet, a model plant for Panicoid species, has been dissected using transcriptomics approach (Lata et al. 2010). Few of the important transcriptome studies on drought stress response in model plants and different crops have been enlisted in Table 7.2.

One of the basic purposes of studying drought stress response and signaling in crop plants is to generate improved stress-tolerant crops by means of genetic manipulation. The outcome of basic molecular and genetic research using model plants especially *Arabidopsis* have been applied to improve stress tolerance of several other plant species including crop plants. Several agronomic traits are quantitative and are controlled by multiple genes, which may interact with each other. Trait(s) controlled by many genes acting together are known as Quantitative trait locus (QTL). Therefore, QTL analyses among tolerant and susceptible crop species has also received wider attention in recent times as such approaches not only help in identifying stress tolerance QTLs but also enable development of stress-tolerant crops by combining or pyramiding various stress tolerance QTLs (Hirayama and Shinozaki 2010; Pérez-Clemente et al. 2013).

In foxtail millet, a synonymous SNP at the 558th bp position (A/G transition) was identified in the *SiDREB2* gene from dehydration-tolerant (A) and -sensitive (G) cultivars (Lata et al. 2011b). Based on the identified SNP, an allele-specific marker (ASM) for dehydration tolerance was developed. The data on segregation of the ASM along with both the lipid peroxidation (LP) data, and RWC data of 170 foxtail millet accessions were recorded for conducting the single marker regression analyses (Lata et al. 2011b). The regression of LP and RWC on the *SiDREB2*-ASM were highly significant, thus indicating an association between the marker and the traits (Lata and Prasad 2013, 2014). The R^2 values of 0.27 and 0.19 for LP and RWC, respectively, suggested that the *SiDREB2* associated trait contributed to ~27 % and ~20 % of the total variation in LP and RWC among 170 and 122 accessions correspondingly. This suggested that the ASM is tightly linked with LP and RWC, which are important biochemical markers for assessing dehydration-induced oxidative stress tolerance in foxtail millet (Lata and Prasad 2014). Similarly, an SNP associated with drought tolerance in wheat *TaMYB2* was identified and an ASM was developed for the same (Garg et al. 2012). The use of these ASMs might be faster, cheaper, and reproducible than other SNP genotyping methods, thus enabling allele-mining and marker-aided breeding of crop varieties for drought tolerance.

Table 7.2 List of important transcriptome studies on drought stress response in crop plants reported in last 2 years

Crop	Strategy	Platform	Nature of study	Reference
Gossypium herbaceum	Transcriptome sequencing	GS-FLX pyrosequencer	Transcriptome of two *G. herbaceum* cultivars contrastingly differing in their tolerance to drought stress was studied by sequencing the RNA content of leaves from respective cultivars	Ranjan et al. (2012a)
G. hirsutum	Microarray	Affymetrix cotton GeneChip Genome array	Genome-wide transcriptome analysis using microarray was performed in developing fibers of *G. hirsutum* under drought stress	Padmalatha et al. (2012)
G. herbaceum	Microarray; transcriptome sequencing	Affymetrix gene chip; GS-FLX pyrosequencer	Comprehensive transcriptome analysis was performed among four genotypes of *G. herbaceum* exposed to drought stress	Ranjan et al. (2012a, b)
Coffea canephora	Electronic northern; macroarray; 2D gel electrophoresis	Hoefer SE600 Ruby system	Identification and analysis of differentially expressed genes and proteins upon drought acclimation in tolerant and sensitive genotypes of *C. canephora* was performed	Marraccini et al. (2012)
Populus euphratica	Microarray	Affymetrix gene chip	Transcriptomics of *P. euphratica* responsive to long-term drought was characterized using microarray and expression profiling was studied using qRT-PCR	Yan et al. (2012)

(continued)

Table 7.2 (continued)

Crop	Strategy	Platform	Nature of study	Reference
Glycine max	Microarray	Affymetrix Soybean Array GeneChip	Microarray analysis of leaf tissues of soybean plants, which were subjected to drought stress from late vegetative V6 and from full bloom reproductive R2 stages was performed	Le et al. (2012)
Ammopiptanthus mongolicus	Transcriptome sequencing	GS XLR70 Titanium pyrosequencer	Transcriptome sequencing of *A. mongolicus* root was performed and analyzed	Zhou et al. (2012a, b, c)
G. hirsutum	Transcriptome sequencing	Illumina HiSeq 2000	Total RNA from the toot tissues of drought stressed and control plant of *G. hirsutum* was sequenced and analyzed	Bowman et al. (2013)
G. arboretum	mRNA sequencing	Illumina HiSeq 2000	Total mRNA from the leaf, stem (including hypocotyl), and root tissues of drought stressed and control plant of *G. arboretum* was sequenced and analyzed	Zhang et al. (2013)
Lablab purpureus	SSH; cDNA sequencing	Information not available	SSH libraries generated from root tissues of the drought-stressed *L. purpureus* under water–stress and control conditions were sequenced and the ESTs were analyzed	Yao et al. (2013)
P. euphratica	Transcriptome sequencing	454-GS FLX Titanium System	The total RNA of non-stressed control and drought-stressed *P. euphratica* leaves was sequenced and analyzed for identifying the differentially expressed genes	Tang et al. (2013)

(continued)

Table 7.2 (continued)

Crop	Strategy	Platform	Nature of study	Reference
Oryza sativa	Microarray	GreenGene Biotech	Microarray experiments using mRNA from air-dried leaves and roots of rice were performed to investigate the genes involved in acute dehydration response.	Minh-Thu et al. (2013)
Glycine max	Digital gene expression tag profiling (DGE)	Illumina HiSeq 2000	Using DGE, the expression profiles between two soybean genotypes were analyzed for identifying drought-responsive genes	Chen et al. (2013a, b)
Chrysanthemum morifolium	Transcriptome sequencing	Illumina HiSeq 2000	The total RNA of *C. morifolium* exposed to drought stress was sequenced and analyzed	Xu et al. (2013)
Macrotyloma uniflorum	Transcriptome sequencing	Illumina HiSeq 2000	The RNA content of shoot and root tissues of control and drought-stressed samples of drought-sensitive genotype and drought-tolerant genotype was isolated and sequenced	Bhardwaj et al. (2013)
Setaria italica	Transcriptome sequencing	Illumina HiSeq 2000	Transcriptome sequencing of *S. italica* shoot tissue was performed and analyzed	Qi et al. (2013)
Hylocereus undatus	SSH; cDNA microarray	*Custom made*	SSH cDNA libraries were constructed using in vitro shoots of *H. undatus* exposed to drought stress and control. Comparative analysis was performed using microarray	Fan et al. (2014)

(continued)

Table 7.2 (continued)

Crop	Strategy	Platform	Nature of study	Reference
Solanum tuberosum	Transcriptome sequencing	Illumina HiSeq 2000	Transcriptome analysis in drought-stressed potato leaf tissues was performed. A subset of differentially expressed genes associated with drought response was examined using qRT-PCR	Zhang et al. (2014)
Pinus halepensis	Transcriptome sequencing	Illumina HiSeq 2000	Transcriptome sequencing of two phenotypically divergent *P. halepensis* accessions was performed and analyzed	Pinosio et al. (2014)

7.10 Conclusion and Future Perspectives

Drought stress is a major threat for agriculture globally with its direct impact on crop production, quality, and productivity. The situation has become worse with recent global climate change. Stress duration, severity, developmental stage, and genotype of the crop undeniably play crucial roles in understanding how plants respond to water deficit stress. Plants respond and adapt to drought stress by exhibiting various molecular, biochemical, physiological, and morphological responses. Many genes are expressed in response to drought stress, and their products impart stress tolerance to plants. Therefore, identifying the mechanism of plant responses to drought stress at the molecular level is of fundamental importance, since it would proffer the research on improving stress tolerance and productivity of crop plants. This chapter summarizes various responses of plants to drought and role of various signaling components including important TFs, viz. ABRE, MYC/MYB, CBF/DREBs, and NAC, which regulate the expression of various stress-responsive genes (Fig. 7.1). Further, these TFs play crucial roles in providing tolerance and adaptation to plants against multiple stresses in both ABA-dependent and -independent manner. These signaling components and transcription factors can be genetically modified to generate transgenics with higher tolerance to drought and abiotic stresses. Their functional analysis will thus be helpful in providing more information on the complex regulatory networks involved in stress responses and adaptation of plants and the cross talk between different signaling pathways. Additionally, considering these components as candidate genes in plant breeding programs will

provide a better understanding of signal transduction events pertaining to abiotic stresses. Eventually, this would assist in developing crop varieties with enhanced stress tolerance through genetic manipulation.

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Chapter 8
Physiological and Molecular Mechanisms of Flooding Tolerance in Plants

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Abstract Flooding is a crucial factor affecting crop growth and yield in low-lying rainfed areas. Systematic investigation of flooding survival mechanisms in tolerant *species* has deciphered molecular, physiological, and developmental basis of soil flooding (waterlogging) and submergence survival. Flood escape and quiescence strategies of deepwater and submergence-tolerant rice (*Oryza sativa*) plants are regulated by ethylene-responsive factor (ERF) transcriptional activators. Ethylene induces genes of enzymes associated with aerenchyma formation, glycolysis, and fermentation pathway. Nonsymbiotic hemoglobin (NSHb) and nitric oxide (NO) have also been suggested as an alternative to fermentation to maintain lower redox potential (low NADH/NAD ratio). In rice (*Oryza sativa* L.), a calcineurin B-like interacting binding kinase (CIPK; OsCIPK15) is also involved in hypoxia tolerance. Detailed investigation revealed that ERFs are targets of a highly conserved O_2-sensing protein turnover mechanism in *Arabidopsis thaliana*. Transcriptome and metabolome profiling of waterlogging-tolerant plant species reveals survival strategies that may be utilized through crop molecular breeding to develop tolerant cultivars.

Keywords Ethylene • Fermentation • Flooding • Nitric oxide • Waterlogging • Nonsymbiotic hemoglobin • Calcineurin B-like interacting protein kinase

8.1 Introduction

Excess of water in the form of waterlogging (soil flooding) or complete submergence is lethal to majority of the terrestrial plants. Flooding events represent huge variation in duration and extent of inundation resulting in suboptimal levels of oxygen (hypoxia) or complete absence of oxygen (anoxia) affecting plant survival.

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Hampered availability of O_2, CO_2, and light hinders photosynthesis and aerobic respiration, leading to energy and carbohydrate shortage and thus limit growth and alter development (Voesenek and Bailey-Serres 2013). Further, reoxygenation of tissues and organs once floods subside often leads to oxidative stress (Fukao et al. 2011). Plants tolerate low oxygen stress either by altering its metabolism or by modifying its morphology and anatomy. Low oxygen sensing by group VII ERFs has been reported to regulate metabolic adaptation to flooding (Gibbs et al. 2011; Licausi et al. 2011). Use of carbohydrates and ATP is restricted only for processes considered necessary for survival under flooding. Developmental adaptation includes alterations in cellular and organ structure for enhancing the availability of oxygen (Bailey-Serres and Voesenek 2008).

8.2 Physiological and Anatomical Strategies for Flooding Tolerance

Plants face a sudden energy crisis under flooding due to nonavailability of oxygen for sustenance of aerobic respiration. Plants shift to anaerobic, glycolytic, and fermentative metabolism to sustain cell viability. Glycolytic pathway is energetically less efficient, yielding 2-ATP molecules in comparison to 36 molecules of ATP per hexose molecule produced by oxidative phosphorylation. Even glycolytic pathway is limited by the rate of NADH oxidation, as availability of oxidized NAD has a regulatory influence on the continuation of the glycolysis. Lactic acid fermentation involving lactate dehydrogenase (LDH) and ethanolic fermentation involving alcohol dehydrogenase (ADH) are the major mechanisms of NADH oxidation operating in the anaerobic tissues. However, there are certain lacunae with both the systems, while LDH may cause cytoplasmic acidosis, continuous ADH activity may result in toxic levels of ethanol and consequent injury to root cells (Bailey-Serres and Voesenek 2008). Energy-intensive processes like DNA replication, transcription, and cell division are curtailed during anoxia. Protein synthesis is finely regulated by selectively allowing translation of mRNAs encoding proteins involved in anaerobic and reactive oxygen species (ROS) metabolism. Syntheses of anaerobic proteins (ANP) like sucrose synthase, pyruvate decarboxylase, lactate dehydrogenase, and alcohol dehydrogenase are characteristic feature of anaerobic plant roots (Gibbs and Greenway 2003). Cellular carbohydrate reserves like soluble sugars and starch are mobilized during anoxia to support anaerobic metabolism. Anaerobic germination of seeds of rice and some other Poaceae members require slow mobilization of starch reserves (Guglielminetti et al. 1995). Sucrose is converted to UDP glucose and fructose by an enzyme called sucrose synthase (SS). SS was found to be the major enzyme catalyzing sucrose breakdown in anoxic rice seeds (Guglielminetti et al. 1995). Role of SS in anoxia tolerance is demonstrated in crops like maize, rice, pigeon pea, and mung bean (Bailey-Serres and Voesenek 2008; Kumutha et al. 2008a; Sairam et al. 2009a). Root carbohydrate status has often been correlated with flooding tolerance in crop plants (Kumutha et al. 2009). Studies on mung bean

and pigeon pea suggest that tolerant genotypes were able to maintain higher root carbohydrate levels under waterlogging (Kumutha et al. 2008a, b). Waterlogged *Arabidopsis* plants accumulated higher levels of soluble sugars and amino acids as a result of increased starch and fatty acid catabolism (Hsu et al. 2011). Expression and activity of carbohydrate transporters were upregulated in shoots resulting in transport of carbohydrates from shoot to roots. Better phloem loading and soluble carbohydrate partitioning seem to be the basis for waterlogging tolerance of poplar and flooding tolerant oak (*Quercus robur*) as revealed by metabolite profiling (Ferner et al. 2012).

Some recent studies have suggested involvement of nonsymbiotic hemoglobin (NSHb) and nitric oxide (NO) in maintenance of NADH–NAD ratio, and thus in providing anaerobiosis tolerance (Hill 2012; Sairam et al. 2012). Expression and activity of NADPH oxidase increased under waterlogging in mung bean genotypes (Sairam et al. 2011a, b). Anaerobic roots apparently do not have a direct source of NADPH, i.e., photosynthetic light reaction and oxidative pentose phosphate pathway. Alternatively, a NADH kinase might be presumed to be involved in phosphorylation of NADH to NADPH. Consequently, NADH kinase, NADPH oxidase, and NADP phosphatase may provide another alternative route for NADH oxidation and thus continuation of glycolytic pathway (Kumutha et al. 2009; Sairam et al. 2009b).

Lower diffusion of ethylene in water leads to accumulation of ethylene in waterlogged and/or flooded plants and soil. Ethylene regulates physiological and morphological adaptive responses to flooding in plants. One such mechanism is development of soft tissues with large intercellular spaces called aerenchyma. Aerenchyma facilitates diffusion of photosynthetic and atmospheric oxygen from aerobic shoot to waterlogged roots. Primary aerenchyma arising from root cortex has been reported in cereal crops like rice, maize, wheat, and barley (Sauter 2013). Secondary, phellum-derived aerenchyma is observed in flooded roots of legumes like soybean and sesbania (Sauter 2013). In maize, a short span of 24 h of waterlogging is sufficient for production of cortical aerenchyma. Aerenchymas are produced by a programmed cell death process, signaled by ethylene, Ca^{2+}, and ROS (Steffens et al. 2012). Transcriptome profiling of different cell layers of waterlogged maize roots reconfirmed that ethylene, Ca^{2+}, and ROS signaling at root cortex layer induces production of aerenchymas (Rajhi et al. 2011). In rice and waterlogging-tolerant teosinte (*Zea nicaraguensis*), aerenchymas are constitutively formed in roots (Abiko et al. 2012; Steffens et al. 2012).

Specialized roots with poorly developed endodermis, emerging from submerged parts of stems, are called adventitious roots. Flooding-tolerant species produce adventitious roots as an adaptive response, to replace functions of flooded, anaerobic sedimentary root system. Adventitious roots are borne from shoots and without endodermis, hence distance and resistance to oxygen diffusion is less (Sauter 2013). Development of adventitious roots in deepwater rice, tomato, and *Rumex palustris* is an ethylene-dependent process (Sauter 2013). Flooding-adapted *Oryza* sp. has constitutively developed adventitious root primordia buried under nodal tissues (Coudert et al. 2010). Auxin–ethylene interaction leads to emergence of adventitious roots in rice. CRL1, an LBD (Lateral Organ Boundaries Domain) transcription factor acting

upstream of auxin signaling pathway, regulates adventitious root initiation in rice (Inukai et al. 2005). Zhao et al. (2009) reported that WUSCHEL-related homeobox gene WOX11 regulates early development and emergence of adventitious roots in rice. Cell wall and cuticle layers of nodal tissue create barrier for emergence of adventitious roots in waterlogged plants. Epidermal cell death coordinated by ethylene and mechanical signals generated by root primordial facilitate adventitious roots emergence in rice (Steffens et al. 2012). Coordinated regulation of gibberellin and abscisic acid pathways by ethylene directs adventitious root elongation process in rice (Steffens et al. 2006).

Upward or hyponastic growth of leaves and petiole elongation of submerged leaves of semi-aquatic species *Rumex palustris* increases anoxia tolerance by facilitating the leaves to rise above water level. Accumulation of ethylene in submerged leaves leads to ABA insensitivity thereby increasing sensitivity to gibberellic acid (GA)-regulated cell expansion (Pierik et al. 2011). Leaf hyponasty was observed in *Arabidopsis*, following submergence in darkness (Colmer and Voesenek 2009). Flooding-intolerant species like tomato also exhibits leaf hyponasty post submergence, most likely mediated by an ethylene-dependent mechanism (Negi et al. 2010).

8.3 Low Oxygen Sensing and Protein Stability

Mechanism of low oxygen sensing was a mystery until 2011, when two independent groups demonstrated the involvement of N-end rule pathway of targeted proteolysis (NERP) in hypoxia signaling of *Arabidopsis thaliana* (Gibbs et al. 2011; Licausi et al. 2011). Discovery of flooding survival strategies in rice mediated by group VII ethylene response factors (ERF), SUBMERGENCE1A (SUB1A), and SNORKEL 1 (SK1) and SNORKEL 2 (SK2) paved way for identification of homologous genes in *Arabidopsis* (Xu et al. 2006; Hattori et al. 2009). Screening of flood-tolerant landraces of rice revealed the existence of multiple flooding survival strategies in rice. Deep-water rice follows a low oxygen escape strategy (LOES) mediated by ethylene-induced shoot elongation, regulated by ERFs, SK1, and SK2. SK1 and SK2 promote GA-induced stem elongation in rice. Flooding-tolerant rice landrace FR13A follows a quiescence mechanism, wherein GA induced growth; metabolism and other phenological processes are arrested to conserve energy for survival under anaerobiosis. Quiescence mechanism involves downregulation of growth and metabolism mediated by Sub1 locus containing either two or three genes (SUB1A, SUB1B, and SUB1C) belonging to group VII ERFs. Flooding-induced higher expression of SUB1A-1 restricts shoot elongation by regulating GA-signaling repressor SLENDER RICE-1 (SLR1) and the related SLR LIKE-1 (SLRL1) proteins. SUB1A-1 also reduces ethylene synthesis, gene expression of wall loosening enzyme (expansin) mRNAs, and carbohydrate depletion (Xu et al. 2006; Fukao and Bailey-Serres 2008). Five members of group VII ERFs are identified in *Arabidopsis*. Constitutively induced ERFs (RAP2.2,

RAP2.12, and RAP2.13) and hypoxia-responsive ERFs (HRE1 and HRE2) regulate hypoxia tolerance in *Arabidopsis*. Constitutive overexpression of RAP2.12 in transgenic *Arabidopsis* plants resulted in increased postsubmergence survival (Licausi et al. 2011). Manipulation of N-terminal amino acids of RAP2.12 by deletion or addition of peptide tags negatively affected plant growth under normal and hypoxic conditions. N-terminal modification of RAP2.12 downregulated the oxygen-dependent expression of hypoxia marker genes in *Arabidopsis* (Licausi et al. 2011). *Arabidopsis* mutants defective in NERP, lacking either Arginine tRNA protein transferase (ate1, ate 2) or E3 ubiquitin ligase (*proteolysis 6*) demonstrated constitutive overexpression of hypoxia marker genes. N-terminal amino acids of most of the group VII ERFs are conserved, having cysteine (cys2) as the second amino acid. Oxidation of cys2 under normoxic conditions qualify group VII ERFs to be targeted to NERP-mediated proteolysis. Post-translational modification of cys2 is oxygen dependent and hence under anoxia, ERFs like RAP2.12 and HRE2 remain stable. Under normoxia RAP2.12 is plasma membrane localized, which following anoxia gets rapidly relocalized to nucleus for further signaling. Constitutively, active group VII ERFs like RAP2.12 are putative oxygen sensors in plants, and provide a rapid mechanism of flood adaptation. Group VII ERFs are regulated either by low oxygen, ethylene, or by both. NERP-insensitive ERFs regulate ethylene-mediated adaptive responses like production of aerenchyma, adventitious root formation, stem elongation, and hyponastic growth. However, metabolic adaptation of hypoxia tolerance is triggered by oxygen sensing property of NERP sensitive ERFs (Fig. 8.1).

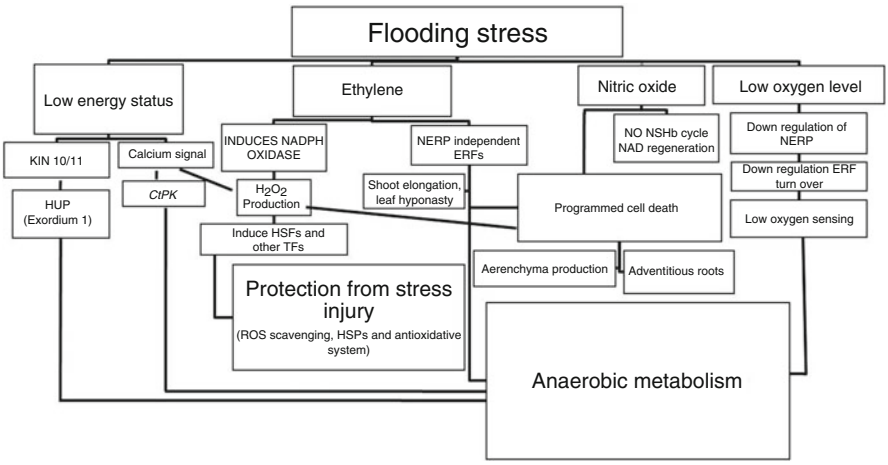

Fig. 8.1 Hypoxia signaling in plants. Flooding rapidly decreases availability of oxygen (O_2) and energy status of plants and induces production of ethylene (C_2H_4) and nitric oxide (NO). These signals turn on downstream signal transduction pathways, which regulates developmental and metabolic adaptation for flooding tolerance

8.4 Nonsymbiotic Hemoglobins and Nitric Oxide Interaction Under Anoxia

Plants contain different classes of hemoglobins (Hb), and the first plant hemoglobin was discovered few decades back (Appleby 1992). Plant Hbs are classified as nonsymbiotic (NSHb) or symbiotic depending upon the plant tissue where they are found (Bogusz et al. 1988). Symbiotic hemoglobins are found exclusively in root nodules, where these functions in controlled transport of oxygen into bacteroids of symbiotic nitrogen-fixing bacteria (Appleby 1992). NSHb are found ubiquitously in plant kingdom and are expressed in seeds, root, shoot, and stem tissues of plants (Hill 2012). There are two classes of NSHb, class 1 NSHbs are induced by low cellular oxygen levels and nutrient toxicity; class 2 NSHbs are induced under cold stress and by cytokinins (Hunt et al. 2001). Taylor et al. (1994) isolated a class 1 nonsymbiotic hemoglobin, from barley. Overexpression of barley NSHb 1 in alfalfa root leads to increase in ascorbate content and higher activities of antioxidant enzymes in control as well as hypoxic roots (Dordas 2009). Expression of NSHb has been reported to be upregulated in response to hypoxia in barley (Taylor et al. 1994), *Arabidopsis* (Hunt et al. 2002), oak (Parent et al. 2008), and rice (Lira-Ruan et al. 2002). Respiratory inhibitors, which limit ATP production, are also as effective as hypoxia in inducing NSHb expression. Rapidly growing tissues like root tips also confront oxygen deficiency and show the presence of NSHb. The exact mechanism by which NSHb renders hypoxia tolerance is being unraveled. Low concentration of NSHb, low dissociation coefficient of oxyhemoglobin complex, and the induction of NSHb expression by low cellular energy levels indicates role of NSHb in stress signaling. Previous works clearly indicate involvement of NSHb in reactive oxygen and nitric oxide (NO) metabolism (Igamberdiev and Hill 2004). Experimental evidences have proved involvement of plant Hbs in catalyzing the conversion of NO to nitrate (Dordas 2009). Nitric oxide is a bioactive signal molecule involved in hormonal and stress signal transduction. NO involved in ROS scavenging, programmed cell death, and aerenchyma formation in plants. Nitric oxide is produced by hypoxia-induced activity of nitrate reductase. Excess of NO is scavenged by oxyhemoglobin form of NSHb in conjunction with NO dioxygenases, converting NO back to nitrate. NSHb is coupled with nitrate reductase, forming the Hb/NO cycle, in which excess NAD(P)H is oxidized (Igamberdiev and Hill 2004). This pathway plays a major role as an alternative of fermentation pathway (Fig. 8.1) in regeneration of NADH in waterlogging-affected mung bean plants (Sairam et al. 2012). Seed and embryo development in plants are also typical examples of hypoxic environment, with young embryos facing low energy levels (Rolletschek et al. 2002). Hypoxia-induced NO production in seeds (Borisjuk et al. 2007) leads to decrease in metabolism, while under normoxia, NO levels decrease and normal metabolism resumes. Manipulation of seed oxygen levels by seed-specific expression of NSHb (Thiel et al. 2011) in *Arabidopsis* led to increased seed metabolic activity.

8.5 Waterlogging, ROS Production, and Antioxidant Mechanism

Accelerated production of ROS is a ubiquitous phenomenon under stress conditions. Abiotic stresses like soil flooding and submergence lead to perturbation of the fine balance between oxidative and antioxidative capacity of plants. Hypoxia-induced increase in redox potential of both plant roots and surrounding soil is ideal for production of ROS. These ROS are necessary for inter- and intracellular signaling, but at high concentrations, they seriously disrupt normal metabolism of plants through oxidation of pigments, membrane lipids, proteins, and nucleic acids (Sairam et al. 2008). Short-term flooding for few hours enhances production of superoxide radicals in soybean roots (Van Toai and Bolles 1991). Accumulation of hydrogen peroxide (H_2O_2) was induced in hypoxia-stressed barley and wheat seedlings (Biemelt et al. 2000; Kalashnikov et al. 1994). Significant increase in lipid peroxidation, superoxide radical production, and membrane injury was observed in waterlogging-stressed, maize, pigeon pea, and mung bean genotypes (Yan et al. 1996; Kumutha et al. 2009; Sairam et al. 2011a). ROS production under soil flooding is owing to the induction of membrane bound NADPH oxidase, as indicated by inhibitor and gene expression studies in pigeon pea (Kumutha et al. 2009).

Detoxification of injurious levels of ROS is mediated by enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), mono-dehydroascorbatereductase (MDHAR), dehydroascorbatereductase (DHAR), glutathione S transferase (GST), and nonenzymatic antioxidants viz., ascorbic acid, glutathione, α-tocopherol, and carotenoids. Upregulation of antioxidant defense system is often correlated with abiotic stress tolerance in crop plants. In *Iris* sp., a 14-fold increase in SOD activity was observed, following hypoxia (Monk et al. 1987). Similarly, in wheat seedlings increased activity of GR and higher contents of glutathione could mitigate post hypoxia oxidative stress (Ushimaru et al. 1997). A recent study comparing transcriptome changes in waterlogging-tolerant and -susceptible maize genotypes, showed upregulation of antioxidant defense and fermentation pathway genes as the basis of waterlogging tolerance (Thirunavukkarasu et al. 2013). There are reports of waterlogging stress-induced increase as well as decrease in antioxidant potential in crop plants (Biemelt et al. 2000; Kumutha et al. 2009). Waterlogging-tolerant pigeon pea genotypes displayed continuous increase in antioxidant enzyme activity over a period of six days of waterlogging, however in susceptible genotypes antioxidant enzyme activities declined after two days of submergence (Kumutha et al. 2009). In anoxia-stressed wheat seedlings, there was either no change or decrease in activities of MDHAR, DHAR, and GR enzymes (Biemelt et al. 2000). ROS production has been implicated in signal transduction for low oxygen stress. Screening *Arabidopsis* seedlings carrying a gene-trap transposon (*DsGus*) led to the identification of mutants with increased ADH-specific activity in response to hypoxia. Mutant phenotype was a result of insertion of *DsGus* in the first exon of a *gene* that encodes Rop (RHO-like small G protein of plants) guanosine triphosphatase (GTPase) activating protein 4

(*ROPGAP4*). Rop signaling is implicated in Ca^{2+} and H_2O_2-mediated signaling for abiotic and biotic stress tolerance of plants. Activation of ROP by GTP under low O_2 induces production of H_2O_2 through a calcium-dependent NADPH oxidase. Accumulation of hydrogen peroxide is crucial for induction of *ADH* and *RopGAP4* expression. *RopGAP4* is needed for negative feedback regulation of ROP level; hence, *ropgap4-1* seedlings succumb to oxidative stress due to excessive accumulation of H_2O_2 (Baxter-Burrell et al. 2002).

Heat shock transcription factors (HSF) have also been proposed to be H_2O_2 sensors in plants. *Arabidopsis* transgenic plants overexpressing *HSFA2* are tolerant to hypoxia in comparison to wild type (Banti et al. 2010). Stress-induced induction of HSFs leads to transcription of genes encoding high-molecular-weight and low-molecular-weight heat shock proteins (HSPs) in plants (Banti et al. 2010). Increase in HSP transcripts in response to low oxygen stress is conserved across various plant *species* (Mustroph et al. 2010). Pucciariello et al. (2012) proposed that NADPH oxidase-dependent ROS pathway regulates HSFs and other ROS-regulated transcription factors in response to flooding stress. HSFs and other ROS-regulated transcription factors are not targets of NERP-dependent proteolysis, hence ROS signaling (Fig. 8.1) is an independent mechanism regulating flooding tolerance in plants (Banti et al. 2010).

8.6 Functional Genomics of Flooding Stress in Plants

The accessibility to sequence information has revolutionized research far ahead of transcriptomics and functional genomics. Transcriptome and proteome analysis of model and crop plants in response to flooding stress has widely been attempted. These studies especially those which compare responses of contrasting genotypes have thrown light on some evolutionarily conserved mechanism of flooding tolerance. Low oxygen-dependent changes in transcriptome (pool of total cellular mRNA) have been analyzed in *Arabidopsis* (Branco-Price et al. 2005; Liu et al. 2005), rice (Lasanthi-Kudahettige et al. 2007), and many more plant *species*. (Christianson et al. 2010; Voesenek and Bailey-Serres 2013) exposed to hypoxia have confirmed that low oxygen stress causes radical changes in gene expression. Apart from a specific set of hypoxia-induced genes, global gene transcription is downregulated under hypoxia. Genes encoding anaerobic proteins (ANPs) involved in sugar metabolism are preferentially expressed. Signal transduction components that activate *RopGAP4*, mitochondrial *alternative oxidase* (AOX), calmodulin, and CAP (calmodulin-associated peptide) were upregulated by hypoxia (Bailey-Serres and Chang 2005). Thirunavukkarasu et al. (2013) compared the whole transcriptome of contrasting subtropical maize genotypes at three stages of waterlogging stress. Genes responsible for programmed cell death that precedes aerenchyma formation was selectively upregulated in HKI 1105 (tolerant) exposed to waterlogging. Calmodulin, a Ca^{2+}-binding protein that was highly expressed only in HKI 1105 interacts with glutamate decarboxylase and helps to maintain cytosolic pH under anoxia. A member of a

flooding-specific gene family, XET A was found upregulated in HKI 1105 during both moderate (253-fold) and severe (16-fold) stresses, but downregulated in V 372 (sensitive). Ethylene-responsive factor-like protein 1, BBM2, AIL5-like, and WRI1 were upregulated in HKI 1105. It was also observed that in the tolerant genotype, auxin receptor genes such as IAA3, IAA14, and IAA16 were upregulated. Cross talk between ethylene and auxin signaling pathways probably enhances the formation of lateral and adventitious roots in waterlogging-tolerant genotypes of maize. Genes belonging to plant hormone biosynthesis and signal transduction were differentially regulated under waterlogging stress, including increases in ethylene, abscisic acid (ABA), gibberellic acid (GA), and auxin (IAA) and a reduction in cytokinin (CK) (Zou et al. 2013). Rice transcription factors Snorkeland Submergence-1A, belonging to group VII ERF (ethylene response factor) have been cloned by map-based cloning (discussed in section3). Apart from rice, extensive studies on quiescence and escape strategies were done in wild species *Rumex acetosa* and *Rumex palustris*, respectively (Hans et al. 2013). *R. palustris* escapes submergence by orientating its leaves in vertical position (hyponastic growth), in an ethylene-dependent manner followed by enhanced elongation rate of young petioles (Cox et al. 2006). In *R. acetosa*, submergence driven accumulation of ethylene suppresses petiole elongation and predisposes the plants towards metabolic rearrangement to minimize carbon use (Pierik et al. 2009). Hans et al. (2013) employed RNA sequencing (RNA-Seq) technology to investigate the molecular basis of adaptive traits of these two species. Upon submergence, there was enhanced expression of amino cyclopropane carboxylate (ACC) oxidase, enzyme catalyzing ethylene biosynthesis in both the species. In *R. palustris*, expression of an EIN3 BINDING F-BOX (EBF) was specifically upregulated. The putative rice ortholog regulates ethylene-induced growth stimulation through preventing negative regulation of GA biosynthesis by ethylene (Kim et al. 2012). The increase in EBF expression is consistent with elongation growth in *R. palustris*. Transcripts encoding ABA biosynthetic enzyme 9-cis-epoxycarotenoid dioxygenase was downregulated in *R. palustris* resulting in lower ABA levels in *R. palustris*. Transcript that encodes an ABA breakdown enzyme ABA-8-hydroxylase was induced in both the species. Transcripts encoding orthologs of two downstream components of ABA signaling (ABA-responsive element binding factor 2 and HOMEOBOX PROTEIN33) were exclusively induced in *R. acetosa*. Maintenance of ABA levels coupled with enhancement in ABA signaling induces metabolic reprogramming of *R. acetosa*. Transcripts regulating auxin transport like transcripts encoding orthologs of a PINOID like (WAG1) that are kinases regulating auxin transport properties of PIN family were upregulated in *R. palustris*. Transcripts of AUXIN (INDOLE-3-ACETIC ACID) 2-11 (AUXIAA2-11) auxin-responsive protein regulating auxin-mediated transcriptional responses were regulated only in *R. palustris*. In *R. palustris auxin* induces cell wall acidification by activating a plasma-membrane proton pump and thereby activates pH-sensitive cell wall-modifying enzymes expansins.

Comparison of whole transcriptome with translatome (mRNAs targeted to translation) revealed highly selective hypoxia-specific protein synthesis (Branco-Price et al. 2008) in hypoxia-stressed *Arabidopsis* plants. Hypoxia-induced translatome

consisted of proteins belonging to anaerobic metabolism and ethylene biosynthesis and responses. Approximately half of the translated proteins were of no known functions and were designated as hypoxia-responsive unknown protein (HUP). Recent developments in low oxygen sensing paved way for the discovery of involvement of group VII ERFs in cellular level low oxygen sensing in *Arabidopsis*. However, there were earlier reports of involvement of various signaling molecules in anaerobic stress signaling in plants. Hypoxia signaling possibly senses changes in levels of cellular energy status, respirable substrates, transient elevations of Ca^{2+}, ROS, and NO (Voesenek and Bailey-Serres 2013). Elevations of cytoplasmic calcium levels following anoxic stress have been observed in maize, rice, wheat, and cucumber plants (Yemelyanov et al. 2011; Subbaiah et al. 1998; He et al. 2012). Proteome analysis of calcium-treated hypoxia-stressed cucumber plants revealed calcium-dependent enhancement in levels of enzymes of primary metabolism and ROS scavenging (He et al. 2012). KIN10 and KIN11 are energy-sensing protein kinases belonging to SnRK1 clade of *Arabidopsis* and regulate carbon utilization under hypoxia (Baena-González et al. 2007). KIN10 positively regulates genes encoding enzymes catalyzing carbohydrate and amino acid catabolism in *Arabidopsis* (Baena-González et al. 2007; Cho et al. 2012). One of the KIN10/11-regulated genes is EXORDIUM-LIKE1, an HUP that is essential for carbon management under low oxygen conditions (Schröder et al. 2011). Calcium signals are transduced via SnRK1 group kinase and calcineurin B-like interacting binding kinase, CIPK15 regulates breakdown of starch, essential for anoxic germination of rice seeds (Lee et al. 2009). Another remarkable molecule regulating hypoxia signaling is nitric oxide (Hill 2012, discussed in section 4). NO homeostasis in hypoxic cells is largely dependent on nonsymbiotic hemoglobins, which are positively regulated by group VII ERF RAP2.12 (Mustroph et al. 2010). NO is a requisite for N-terminal Cys-oxidation and tagging of proteins for turnover in mammals (Hsu et al. 2011). It can be speculated that NO homeostasis under hypoxia may contribute to NERP dependent turnover of the ERFs. Anoxic stress and post-anoxic reoxygenation promotes mitochondrial generation of ROS at complex III (Discussed in Sect. 8.5). Elevated levels of cellular ROS levels ephemerally activate mitogen-activated protein kinases (MAPKs) (Chang et al. 2012). However, MAPK signaling maintains mRNAs selectively excluded from translation during anoxia, but might be essential for survival during post-anoxic reoxygenation.

Regulatory role of non-coding RNAs (nc RNAs) has been elucidated recently in model plants. Sequencing of small RNA libraries of hypoxic and control root tissues of *Arabidopsis* identified 65 unique microRNA (miRNA) sequences and 14 *trans-acting* small interfering RNA (tasiRNA). Putative targets for these hypoxia-responsive miRNA are transcription factors mainly from the MYB, NAC, Homeobox, SPL, ARF, AP2, MADS, and CCAAT-HAP2 families having important roles in plant growth and floral development (Moldovan et al. 2009). Wu et al. (2012) identified long non-coding RNAs (lnc RNA) responsive to hypoxia stress in Arabidopsis. Abundance of lnc RNA AtR8 was decreased by hypoxic treatments and recovered upon reoxygenation. AtR8 was preferentially localized to cytoplasm of root tissues. It is possible that the lnc RNA negatively regulates translation or ANPs and decrease in abundance of these RNAs upregulate translation of ANPs (Wu et al. 2012).

8.7 Conclusions and Future Perspectives

As flooding events depict huge variation in duration and extent of inundation ranging from waterlogging at root level to complete submergence and from few hours to few weeks duration, plant *species* show large variation in flooding tolerance. Identification of Sub1A locus from flooding-tolerant Indian rice landrace FR13A paved way for marker-assisted breeding of this locus into cultivated rice varieties. Current studies shows that under Indian conditions, Swarna-Sub1 can contribute up to 45 % increase in yields compared to current popular varieties under a 10-day period of submergence (Dar et al. 2013). Screening of 86 accessions of *Arabidopsis* and 100 accessions of *Lolium perenne* presents species level variation in flooding survival strategies in plants (Vashisht et al. 2011; Yu et al. 2012). Detailed analysis of the contrasting genotypes aided with transcriptome and proteome profiling and functional validation of candidate genes are required for reaching valuable conclusions. Some of the well characterized genes/proteins may be targeted for improving flooding tolerance by either transgenic manipulation of gene expression or for screening of the germplasm in a need-based manner (Table 8.1). Transcriptome comparison between submergence-tolerant wild *Rorippa species* with *Arabidopsis* revealed that genes of pyrophosphate-dependent pathway of phosphorylation are the candidate genes behind tolerance (Sasidharan et al. 2013). Similarly, transcriptome profiling of *Rumex palustris* and *Rumex acetosa* revealed two distinct mechanisms of survival in this related species. Similar to deep-water rice, *Rumex*

Table 8.1 List of genes/proteins useful for improving flooding tolerance of crop plants either by screening of the germplasm or by transgenic manipulation of gene expression

Mechanism		Gene/protein	References
Ethylene-controlled growth	Submergence-induced ethylene accumulation controls GA-driven cell elongation through ethylene response factors (ERF). In deepwater rice, ERF genes SNORKEL1 (SK1) and SNORKEL2 (SK2) coordinate internode elongation and flood escape	SK1, SK2	Voesenek and Bailey-Serres (2013)
			Xu et al. (2006), Hattori et al. (2009)
	In submergence-tolerant rice lines ERF SUB1A-1, induces SLENDER RICE-1 (SLR1) and SLR LIKE-1 (SLRL1), transcription factors that inhibit GA-mediated growth, thus conserving carbohydrates and limits energy expenditure	SUB1A-1, SLR1, SLRL1	Fukao and Bailey-Serres (2008)
	Five members of group VII ERFs regulate hypoxia tolerance in *Arabidopsis*	RAP2.2, RAP2.12, RAP2.13, HRE1, and HRE2	Licausi et al. (2011)

(continued)

Table 8.1 (continued)

Mechanism		Gene/protein	References
Carbohydrate and energy management	Plants shift to anaerobic, glycolytic and fermentative metabolism to sustain cell viability during flooding stress. Nonsymbiotic hemoglobin (NSHb) and nitric oxide (NO) cycle serve as an alternative to fermentation to maintain lower redox potential (low NADH/NAD ratio). Energy-sensing kinases belonging to diverse classes regulate carbohydrate and amino acid catabolism	Sucrose synthase	Gibbs and Greenway (2003), Kumutha et al. (2008a), Sairam et al. (2009a)
		Pyruvate decarboxylase, lactate dehydrogenase, alcohol dehydrogenase	Bailey-Serres and Voesenek (2008)
		NSHb	Hill (2012), Sairam et al. (2012), Thiel et al. (2011)
		KIN10 and KIN11	Baena-González et al. (2007)
		EXORDIUM-LIKE1	Schröder et al. (2011)
		CIPK15	Lee et al. (2009)
		MAPKs	Chang et al. (2012)
Oxidative stress management	Post-anoxia oxidative stress tolerance correlates with higher rate of survival in tolerant genotypes	Superoxide dismutase	Fukao et al. (2011)
		Glutathione reductase	Kumutha et al. (2009)
Other stress-responsive genes	Transcription factors and non-coding RNAs involved in stress signal transduction	*HSFA2*, HSP	Banti et al. (2010)
			Mustroph et al. (2010)
		AtR8	Wu et al. (2012)

palustris utilizes ethylene-mediated growth modification to avoid submergence. *Rumex acetosa* undergoes complete metabolic reprogramming to tolerate flood prone environments (Hans et al. 2013). Plant species belonging to flood prone ecosystems may serve as valuable models to understand flooding survival strategies useful in crop breeding.

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Chapter 9
Salt Adaptation Mechanisms of Halophytes: Improvement of Salt Tolerance in Crop Plants

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Abstract Soil salinity is one of the most serious environmental factors that affect crop productivity worldwide. Inevitable global climate change leading to rise in sea water level would exacerbate degradation of irrigation systems and contamination of ground water resources, which render conventional agricultural practices impossible due to the sensitivity of most crops to salinity. Breeding for development of salt-tolerant crop plants has been a major challenge due to the complexity and multigenic control of salt tolerance traits. Halophytes are capable of surviving and thriving under salt at concentrations as high as 5 g/L, by maintaining negative water potential. Physiological and molecular studies have suggested that halophytes, unlike glycophytes, have evolved mechanisms, such as ion homeostasis through ion extrusion and compartmentalization, osmotic adjustments, and antioxidant production for adaptation to salinity. Employment of integrated approaches involving different omics tools would amplify our understanding of the biology of stress response networks in the halophytes. Translation of the knowledge and resources generated from halophyte relatives of crop plants through functional genomics will lead to the development of new breeds of crops that are suitable for saline agriculture.

Keywords Functional genomics • Crop plants • Halophyte • Salt tolerance • Smooth cordgrass

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9.1 Introduction

World population is increasing continuously, while there is constant reduction in arable lands due to salinity of the soils (Kafi and Khan 2008). Soil salinity is one of the most serious environmental factors limiting the agricultural productivity and quality of crop plants worldwide (Flowers et al. 2010). No other toxic substance is known to restrict plant growth more than salt (Xiong and Zhu 2002). Salinity degrades agricultural landscape by natural and human interventions (Boesch et al. 1994; Rogers and McCarty 2000), which has been further enhanced by agricultural practices (Zhu 2001).

Significant percentages of arable land are affected by salinity, which renders conventional agriculture impractical because most field crops are salt sensitive. Thus, soil salinity poses a serious threat to global food security and sustainability. Approximately 1,000 million hectares of land is salt affected, which is over 7 % of the world's land area and 20 % of the world-cultivated area (Munns 2005; Jia et al. 2011). Salt-affected land cost over US$12 billion per annum globally (Qadir et al. 2008). Irrigated land, despite being comparatively a small proportion of the total global agricultural area, produces one third of the total food (Munns and Tester 2008). Irrigation systems are particularly prone to saline soil; about 45–60 million ha of irrigated lands were damaged by salt, which is 20–25 % of the total irrigated area in the world (Glenn et al. 1999). Salt-affected land is increasing worldwide through deforestation and ground water irrigation, which raise the water table carrying dissolved salts to soil surface. Thus, vast regions of seacoast and inland lie barren because of inadequate or poor quality underground water available for irrigation (Gul et al. 2009). Salinity-induced crop damage is more acute in resource-poor third world countries mainly located in arid, semiarid, and coastal regions (Allakhverdiev et al. 2000; Munns 2002; Koca et al. 2007; Viégas et al. 2001).

One solution to tackle the salinity problem is to recycle the water near the site of production of salt-tolerant crops, thus reducing the amount of disposed water (Grieve and Suarez 1997). A limitation to this approach is low salt tolerance of agricultural crops and trees (Glenn et al. 1999). Conventional approaches using primary and secondary gene pools in crop plants have yielded salt-tolerant crops; the development has, however, been slow due to complexity and multigenic nature of the salt tolerance traits. On the other hand, reclaiming saline lands for agricultural crops is expensive, but they could be used to grow halophytes (Qureshi et al. 1991). Several halophytes, like salt marsh plants can flourish well in soils with poor drainage. Identifying and making use of desirable traits of suitable halophytic plants not only meet our requirement of salt-tolerant crops but also reduces pressure on arable lands (Khan and Weber 2006).

A salt-tolerant plant is able to maintain its growth under saline conditions relative to its growth under normal nonsaline conditions. High salt concentration disrupts homeostasis in plant water potential and ion distribution at cellular level, leading to degradation of biological molecules, growth arrest, and cell death (Munns and Tester 2008). To sustain future agricultural production, we must be able to develop salt-tolerant food and fiber plants other than salt-sensi-

tive glycophytes, which can successfully grow in salt-affected areas (Rozema and Flowers 2008).

Systems level understanding of plant biology, physiology, genetics, and biochemical approaches to overcome salinity stress responses is required, which can be used for engineering salt tolerance in crops. Genes that are regulated under salt stress can be identified either through transcriptomics (Kawasaki et al. 2001) or proteomics (Salekdeh et al. 2002). Several mechanisms are known to be operating in halophytes to adapt to soil salinity; it is therefore important to study these mechanisms in detail that will allow us for devising strategies for tailoring crops plants to survive and grow under salinity. This chapter provides information on physiological, biochemical, and molecular basis of salinity tolerance in halophytes, which can be used for the development of salt-tolerant crop plant.

9.2 Halophytes: Definition and Classification

Halophytes are considered to be remarkable plants that are much versatile in unrelated plant families during the divergent evolution of angiosperms and that tolerate salt at concentrations considered lethal to 99 % of other species (Trotta et al. 2012). Halophytes are known for several years; however, their definition still remains unclear. Flowers et al. (1986) defined halophytes as the plants with the ability to complete their life cycle at or above 200 mM NaCl, which is possibly encountered in the natural environment. Ayers and Wescott (1989) defined halophyte plants as those that can survive salt content of irrigation water up to 5 g/L total dissolved solids. A true halophyte is considered to remain viable and complete its life cycle at seawater salinity (Rengasamy et al. 2003; Flowers and Colmer 2008).

Aronson (1989) compiled a list of halophytes with 1,560 species under 550 genera belonging to 117 families, which includes only food, forage, fuelwood, or soil stabilization crops. Chenopodiaceae family is the largest with over 275 as halophyte species. The other three superfamilies, Poaceae (grasses), Fabaceae (legumes), and Asteraceae (composites) contained 5 % of their species as halophytes. However, they represent less than 5 % of the species in these families (Aronson 1989). Among the monocot halophytes, Poaceae contains more halophytic genera (45 genera) than any other family, followed by Cyperaceae, which contains about 83 species. Flowers et al. (1977) showed probable relationships of halophytes from primitive (e.g., Laurales, Nymphales) to advanced (Asterales, Orchidales) orders on the basis of their order of flowering. Weiglin and Winter (1991) correlated leaf anatomy with zonation of 13 halophytes along a transect in a salt marsh in Jordan and based on the transectorial difference in photosynthetic pathway, degree of succulence etc. and classified them as euhalophytes (true halophytes), pseudohalophytes (salt avoiders), and crinohalophytes (salt excretors). Few species, i.e., *Salicornia bigelovii* can grow well and set seed even on soil solution exceeding 70 g/L total dissolved solids (TDS, i.e., 1.3 M NaCl) (Glenn et al. 1999). Some crop plants, i.e., sugar beet, date palm, and barley are also considered halophytes as they can be cultivated on irrigation water with 5 g/L TDS (Ayers and

Wescott 1989). On the other hand, most sensitive crops such as rice and bean can be severely affected by as low as 20–50 mM NaCl (Greenway and Munns 1980).

Halophytes have the abilities to tolerate high salt concentrations of the soil by maintaining more negative cellular water potential to absorb water from a soil solution of low water potential. This unique ability is achieved by a number of mechanisms that are found operational in halophytes. Due to their multiple origins, halophytes differ widely in their degree of tolerance as well as adaptability to salt (Ungar 1991). On the other hand, they share a common ability to accumulate large amounts of Na^+ in vacuoles as the major plant osmoticum (Khan et al. 2000; Moghaieb et al. 2004) simultaneously to inhibit K^+ absorption (Zhu 2003). This requires a functional Na^+/H^+ antiport system in the tonoplast and specially adapted membrane lipids to prevent leakage of Na^+ from the vacuole to the cytoplasm. Halophytes have relatively high rates of net Na^+ uptake (1–10 nmol Na^+ g^{-1} fr. root s^{-1}) without injury to the plant. Na^+ influx was first measured in a halophyte, *Spergularia marina*, which showed uptake of $^{22}Na^+$ into whole plants in "steady state" salinity for over 2 h (Flowers and Colmer 2008). Halophytes have much lower Na^+ influx than the glycophyte *Arabidopsis* (i.e., 30 nmol g^{-1} fr. root s^{-1}).

Oil-seed crops, such as *Kosteletzkya virginica* (Ruan et al. 2008); *Salvadora persica* (Reddy et al. 2008); *Salicornia bigelovii* (Glenn et al. 1991); and *Batis maritima* (Marcone 2003); fodder crops, such as *Atriplex* spp. (El-Shaer 2003) and *Distichlis palmeri* (Masters et al. 2007) are some of the agronomically useful halophytes. In spite of having high salt content from 15 to 50 % of leaf dry matter, halophytes generally contain high protein content (10–20 % of dry matter), and are considered as poor energy resource (Le Houérou 1996). Unlike leaves, the seeds of halophytes have a very low salt content, even under saline irrigation. The seeds of most halophytic species germinate usually during spring or during high precipitation, when soil salinity levels are reduced, but they germinate better under saline conditions. Elucidation of the mechanism of seed germination in halophytes under salinity will be extremely beneficial in developing salt-tolerant crops (Aslam et al. 2011). Salt-tolerant biofuel halophytes can also be grown on marginal agricultural land (Qadir et al. 2008) and could be irrigated with brackish water or seawater (Rozema and Flowers 2008). For revegetation and remediation of salt-affected regions to develop sustainable agricultural practice, we also need to rely on halophytes (Peacock et al. 2003). Previous studies also reported that some seagrasses and salt marsh plants have the ability to extract heavy metals from sediments (Cambrolle et al. 2008; Lewis and Devereux 2009). Salt-tolerant plants also improve water conductance, soil fertility (Qadir et al. 2008), and lower the water table (Barrett-Lennard 2002). The monocot halophyte *Puccinellia tenuiflora* can thrive in the saline-alkali soils and has an outstanding nutritional value for livestocks (Yu et al. 2011). It accumulates inorganic ions, proline, betaine, and organic acid for osmotic adjustment (Guo et al. 2010). It is however imperative that the candidate species be assessed to determine the future plants crucial in evolving any economic crop halophyte. The processes and potential applications of halophytes merit much greater emphasis on their research and development for the improvement of salt tolerance traits of crop species.

9.3 Halophytes Versus Glycophytes

Plants have been classified as glycophytes or halophytes based on their ability to grow on high salt medium. Most of the plants cannot tolerate high salt concentrations of the soil and thus cannot be grown on these areas (Parvaiz and Satyawati 2008). These plants are called glycophytes, nonhalophytes, or non-salt-tolerating plants (Xiong and Zhu 2002). Only 2 % of terrestrial plant species have the ability to grow under salinity; such plants are known as salt-resistant plants, salt-tolerant plants, or halophytes (Flowers et al. 1986). The leaves of glycophytes are unable to retain high levels of salt without injury, whereas halophytes prefer to accumulate up to 50 % of shoot dry weight salt in their leaves in order to balance the osmotic potential of the salts outside the plant (Flowers et al. 1986). The optimal growth of a halophyte is observed at soil salinity ranging between 200 and 400 mM NaCl (Khan et al. 2005), where most crop plants would die rapidly. Due to lack of this adaptive mechanism, glycophytes are unable to survive in environments where halophytes thrive. However, there is no correlation detected between Na^+ accumulation and salinity tolerance in glycophytes (Shabala and Cuin 2007). Both halophytes and glycophytes are impacted in a similar fashion under high soil salinity, i.e., delay in the germination and reduction in the seed number (Ungar 1996). Also the fundamental plant metabolic processes, such as photosynthesis and respiration, are equally sensitive to salts (Volkmar et al. 1998). However, halophytes differ from the glycophytes in their ability to survive under a salt shock, i.e., tidal or rainfall events, which allows halophytes to develop a steady growth rate in a saline environment (Niu et al. 1993). Halophytes have a comparative advantage over glycophytes in their ability to determine the nature of transporters involved in the uptake of Na^+ (Wang et al. 2007). Such differences in the mode of Na^+ uptake by different species suggest evolutionary differences among species to thrive under same conditions.

Halophytes when grown in nonsaline (e.g., *S. maritime*) or less saline (i.e., 10 mM, e.g., *Halosarcia pergranulata*) culture solutions accumulate high concentrations of K^+ (Flowers and Colmer 2008). However, K^+ cannot substitute for Na^+ in all halophytes although it was reported to inhibit the growth of halophytes (Ramos et al. 2004; Flowers and Colmer 2008). Similar to the glycophytes, plant K^+ concentrations fall as Na^+ concentrations rise when halophytes are transferred from high K^+/Na^+ ratio (normal solution) medium to low K^+/Na^+ ratio (saline solution). To understand the salt tolerance mechanism and its evolution, it will be important to determine whether visible differences in net $S_{K.Na}$ between species reflect variations in their physiological and biochemical bases of salt tolerance. Still, very little is known about how Na^+ enters halophyte cells and tissues (Cheeseman 1988). However, in glycophytes, two mechanisms are thought to be responsible for Na^+ entry. In first type, (i.e., in rice), if the concentration of external solution is greater than 50 mM NaCl, Na^+ can be carried to the shoot via bypass flow through endodermis and enters the transpiration stream directly to concentrate the leaves. Secondly, (i.e., in wheat), Na^+ leaks into the plant symplastically via the root cortical cells by competitive binding to K^+ transporters or other cation channels (Rubio et al. 1995). This allows

entry of toxic levels of Na^+ into the plant, and depresses the uptake of K^+. Salt-tolerant glycophytes activate pre-existing tonoplast antiporters under salt, whereas halophytes constantly activate vacuolar antiporters, even in the absence of NaCl (Glenn et al. 1999).

9.4 Monocot Versus Dicot Halophytes

Unlike monocotyledonous halophytes, the growth of dicotyledonous halophytes is found to be stimulated by salt. Several (but not all) dicotyledonous halophytes show optimal growth at salinity within 50–250 mM NaCl concentrations (Flowers et al. 1986), while monocotyledonous halophytes generally grow optimally either in the absence of salt or at salinity level of less than 50 mM NaCl concentration (Glenn et al. 1999). Generally, monocotyledonous halophytes show growth retardation at 170 mM salt concentrations. A few halophytes, such as *Plantago maritime* (Erdei and Kuiper 1979), *Atriplex* spp. (Longstreth and Nobel 1979), and *Lasthenia glabrata* (Kingsbury et al. 1976) do not show any increase in their growth at high salt concentrations (300 mM NaCl), but they are still able to complete their life cycles. Tall wheatgrass (*Agropyron elongatum*) is a halophyte relative of wheat, and is one of the most salt-tolerant monocotyledonous species, which grows optimally at salt concentration equivalent to seawater. Relative growth rates (dry mass) of dicot halophytes in 200–360 mM NaCl ranges between 6 and 160 mg g^{-1} d^{-1}, while that of monocot halophytes, it is between 15 and 26 mg g^{-1} d^{-1} (Debez et al. 2006; Harrouni et al. 2003). Osmotic adjustment in dicot halophytes is achieved by means of ions accumulated by the root, which is associated with their succulence. But in monocot halophytes, which have lower water content, sugars may play a significant role. Estimates of the net K^+: Na^+ selectivity (net $S_{K.Na}$) indicates differences between monocot and dicot halophytes. Net $S_{K.Na}$ is calculated as the ratio of K^+ concentration in the plant to that in the medium divided by the ratio of Na^+ concentration in the plant to that in the medium, which ranges between 9 and 60 (Flowers and Colmer 2008). In only Poales the net $S_{K.Na}$ values are of the order of 60, whereas in Alismatales, the average net $S_{K.Na}$ is 16 (Flowers and Colmer 2008). The net $S_{K.Na}$ in monocot halophytes is twice and four times that of dicot halophytes at low and high salinity, respectively. However, many monocot halophytes have Na:K ratios of about one or less (Flowers et al. 1986). The monocots *Triglochin* and *Posidonia* are exceptions of having Na: K ratio and water content alike dicots, which may be the characteristic of Najadales within Monocotyledoneae (Tyerman et al. 1984).

Dicot halophytes are more succulent than the monocot halophytes because of their larger vacuolar volume. However, Juncaginaceae among the monocots are reported to be succulent. Earlier it was thought that normal dicot halophytes accumulate more NaCl in shoot tissues than monocot halophytes, and they were termed as “includers” and “excluders,” respectively (Ahmad et al. 1981). However, further studies on halophytes showed that monocot halophytes use Na^+ uptake into leaves,

similar to dicot halophytes (Rubio et al. 1995; Fricke et al. 1996). But, Na^+ uptake per unit of growth and requirement in monocot halophytes is less as compared to the dicot halophytes because of their lower cell vacuolar volume and leaf water content, so they maintain lower Na^+:K^+ ratio in saline conditions (Glenn et al. 1999). Yet, no detailed analysis of the effects of salinity on monocotyledonous halophytes has been reported. The differences of Na^+ and K^+ usage between mono- and dicotyledonous halophytes and the importance of cereals as crops, it would be pertinent that monocotyledonous halophytes (i.e., species of *Thinopyrum*, *Hordeum*, *Distichlis*, *Spartina*, and *Puccinellia*) be investigated further.

9.5 Physiology of Salinity Tolerance

Salt stress affects crop growth, development, and yield by imposing ion toxicity and imbalance, osmotic stress, nutritional disorders, membrane disorganization, metabolic toxicity, and inhibition of photosynthesis (Munns 2002; Zhu 2001). Two main consequences for plants growing in salinity are physiological drought, created by low osmotic potential of substrate compared to that inside the plant, and specific ion toxicities (Khan et al. 2006; Flowers and Colmer 2008). However, physiological basis of salt tolerance is present in all plants, but plant species show a great variation in salt tolerance ability; 25 mM NaCl can be toxic to certain glycophytes, whereas halophytes can tolerate salt stress level of 500–1,000 mM NaCl (Flowers et al. 2010). It is clear that tight regulation of ion transport from roots to shoots is vital for salt tolerance in halophytes (Munns 2005). The adaptation towards salt stress is an organized physiological adjustment at both cellular and molecular levels, which include ion homeostasis, osmotic adjustment, ion extrusion, and compartmentalization (Zhu 2001). The decline in plant growth at high-salinity level could be due to reduced carbon fixation, i.e., changes in biomass allocation between leaves, stem and root, which alters the balance of photosynthesis and respiration (Lovelock and Ball 2002). Another reason is fall in turgor due to high concentrations of ions in the apoplast (Balnokin et al. 2005; James et al. 2006), or change in cell wall elasticity (Touchette 2006). Other possibilities are related to osmotic adjustment–inability to either distribute or synthesize organic solutes, such as proteins, sugar, amino acids (Britto and Kronzucker 2006; Touchette 2007; Munns and Tester 2008). Accumulation of these compatible solutes is vital for cell osmo-regulation, protection of subcellular structures (Munns 2002), and maintenance of protein structures (Araújo et al. 2006).

Osmotic adjustment plays an important role for providing tolerance in halophytes under salinity (Flowers and Colmer 2008). Decreasing water potential (Ψ) must be established for water to flow through the soil-plant-atmosphere continuum. Thus, with increasing salinity plants must generate increasingly lower Ψ to allow continued water uptake (Touchette et al. 2009). This is achieved by increasing solute concentrations within the plant (Touchette 2007; Flowers and Colmer 2008).

Little, however, is known about how plant–water relations are altered in halophytes with rapid and substantial changes in soil salinity. Changes in tissue elasticity can also play an important role in plant–water relations during osmotic stress (Touchette 2007). The loss of water through transpiration can substantially decrease Ψ in ridged tissues, thus minimizing overall tissue water depletions (Touchette 2006). The general physiology of halophytes has been reviewed occasionally (Breckle 2002; Jithesh et al. 2006; Touchette 2007). The potential of halophytes as donors of tolerance for cereals and other crops has also been reviewed (Colmer et al. 2005, 2006). It is obvious that salt tolerance in halophytes is a complex mechanism and requires a combination of many different processes (Flowers and Colmer 2008). Understanding the physiology of halophyte plants is very important to help solve the problem of salinity in agricultural and horticultural crops.

9.5.1 NaCl Uptake and Sequestration in Halophyte Cells

Plant cells respond to external high salinity both by increasing sodium efflux at the plasma membrane and by the accumulation of sodium in the vacuole (Zhu 2000). Since monovalent ions are toxic at the concentrations required for osmotic adjustment, these (Na^+ and Cl^-) are compartmentalized, predominantly in vacuoles in the halophytes, so that their concentrations in the cytoplasm are maintained within tolerable limits (Subudhi and Baisakh 2011). Due to its low concentration in the cytoplasm, Na^+ is actively pumped into the vacuole from the cytoplasm, whereas Cl^- enter passively via anion channels to balance electrical charge differences across the membrane (Pantoja et al. 1992). However, the extent of ion accumulation and the degree of salt tolerance differ widely among halophytes (Glenn et al. 1996). Tonoplast antiporter activity has been reported in roots and leaves of *Atriplex nummularia* (Hassidim et al. 1990), *Plantago maritime* (Staal et al. 1991), *Atriplex gmelini* (Matoh et al. 1989), and *Mesembryanthemum crystallinum* (Barkla et al. 1995). The vacuolar Na^+/ H^+ antiport appeared to be either constitutive or is found to be activated by high NaCl concentrations. A cytoplasmic Cl^- concentration was found to be from 25 to 150 mM in halophytes (Flowers et al. 1986). However, vacuoles of halophytes can accumulate 200–1,000 mM Cl^- with no expense of cellular energy.

9.5.2 Retention of NaCl in Halophyte Vacuoles

Na^+ is liable to leak back to the cytoplasm from the vacuole due to sharp concentration gradient between the two compartments (Maathuis et al. 1992). Cl^- would leak only when the vacuole remain no longer positively charged with respect to the cytoplasm. Isolated vacuoles of *Suaeda maritima* showed highly saturated fatty

acids and other lipids in tonoplast for minimizing permeability to NaCl (Leach et al. 1990). Further, tonoplast cation channels were also found to be closed at physiological concentrations of Na^+; only a small proportion of tonoplast H^+ATPase activity maintains NaCl compartmentation (Maathuis et al. 1992). Hence, halophytes do not spend much of metabolic energy, and thus have the potential of maintaining high yield during high salinity. In *Aneurolepidium chinense*, a monocot halophyte, membrane protein (*AcPMP3*), localized in the root cap, which acts as a regulator of accumulation of Na^+ and K^+ (Inada et al. 2005).

9.5.3 Salt Inclusion Versus Exclusion

Many halophytes use salt as an osmoticum to balance external medium concentration (Ungar 1991). In halophytes, salt exclusion is the most common mean of surviving under high salt concentrations (Waisel et al. 1986). Halophytes excrete more than 50 % of the salt entering the leaf (Warwick and Halloran 1992). Mangrove plants exclude 99 % of the salts through the roots (Tomlinson 1986), and the casparian strips play a role in salt exclusion from the inner tissues (Flowers et al. 1986). However, there is no obvious correlation between salt exclusion and salt tolerance. In glycophytes, the slow growth rate of the leaves is due to their inability to utilize the salt transported from the root. Majority of crop plants, many dicotyledonous halophytes and most of the monocotyledonous halophytes are excluders, and only a few dicotyledonous halophytes are includers. It is surprising that the domesticated plants selected for salt tolerance by breeders are monocot excluders, while naturally salt-tolerant species are mostly includers (O'Leary 1995).

9.5.4 Na^+/K^+ Discrimination

Due to their competitive ionic interactions, Na^+ and Cl^- can suppress net nutrient uptake or affect membrane integrity. High levels of Na^+ lead to K^+ deficiencies (Tester and Davenport 2003). The ionic stress in plants under salinity is usually due to high Na^+/K^+ and Na^+/Ca^{2+} ratios, and accumulation of toxic Na^+ and Cl^- in tissues, which impairs cellular metabolism (Blumwald et al. 2000; Zhu 2003). Regulation of ion selectivity, especially Na^+/K^+ discrimination is associated with salt tolerance (Volkmar et al. 1998). Because similar uptake mechanisms operate for both Na^+ and K^+, the former can be substituted for the latter (Schroeder et al. 1994). A high level of K^+ in young tissues is associated with salt tolerance in several species (Khatun and Flowers 1995). Generally, monocot halophytes accumulate high levels of K^+ than Na^+ in their leaves (Albert and Kinzel 1973; Albert and Popp 1977). A positive relationship is believed to exist between Na^+ inclusion and salt tolerance in includer halophytes (Clipson and Flowers 1987). In such plants,

K^+ accumulation was observed to be almost 4 % of total cations, and their Na^+/K^+ ratios were as high as 30 at seawater level salinity (Naidoo and Rughunanan 1990). Some nonhalophyte species discriminate against Na^+ more than others by lowering the Na^+ concentrations in their leaf cytoplasm (Hajibagheri et al. 1989). The gene controlling Na^+/K^+ discrimination was identified in *Triticum* that confers increased discrimination and enhanced salt tolerance when introduced through recombination in other species (Dvorak et al. 1994). Na^+/K^+ discrimination, however, is not a requirement for salt tolerance in glycophytes, such as barley and wheat (Volkmar et al. 1998).

9.5.5 Plasma Membrane Antiporters and ATPases

Both halophytes and glycophytes have the ability to export Na^+ from the cytoplasm to the extracellular space using plasmamembrane Na^+/H^+-antiporters, while H^+-ATPases provide the H^+ electrochemical gradient at the plasmalemma (Niu et al. 1995). *Atriplex nummularia* and *Salicornia bigelovii* showed higher activity of root plasmalemma antiporter in plants grown on 400 mM NaCl as compared to control (Hassidim et al. 1990; Lin et al. 1997). NaCl was also found to induce a P-type H^+-ATPase that supply energy for the antiporter (Niu et al. 1995). This also clarifies how halophytes expel Na^+ from the cell. However, the PM-ATPase activity does not primarily determines salt tolerance, and long-term tolerance is due to an increase in the resistance of the root cells to Na^+ entry. Transport of ions across the tonoplast is energized by a proton motive force (PMF) generated by the vacuolar H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase) (Gaxiola et al. 2007). Na^+/H^+ exchange in vesicles was found to be constitutively high and increased with salinization of the plant (Barkla et al. 2002). In *Salicornia bigelovii*, the activity of PM- and V-ATPases along with V-PPase increased upon addition of NaCl to the growth medium (Parks et al. 2002). However, in *Suaeda salsa*, salinity increased the activity of the V-ATPase and Na^+/H^+ antiporter in tonoplast vesicles (Qiu et al. 2007), rather than the V-PPase (Wang et al. 2001). In *Thellungiella halophile*, NaCl increased the Na^+/H^+ antiporter in vesicles but not in plasma membrane (Vera-Estrella et al. 2005).

9.5.6 Accumulation of Osmolytes

Accumulation of metabolically compatible solutes (organic compounds) in the cytoplasm is an important role in salt tolerance mechanism at the cellular level, which balances the osmotic potential of the Na^+ and Cl^- accumulated in the vacuole (Wyn-Jones and Gorham 2002). Halophytes synthesize a range of compatible solutes from quaternary ammonium compounds, methylated sulphonio compounds (sugars, such as sucrose), methylated proline-related compounds

(methyl-proline), betaines (glycinebetaine), and amino acids (proline) to sugar alcohols (sorbitol) depending on phylogeny and functional needs (Hasegawa et al. 2000; Rhodes et al. 2002; Flowers and Colmer 2008). The polyhydric alcohols (polyols) exist in both acyclic and cyclic forms and are believed to be associated with plant salt tolerance (Bohnert and Shen 1999). Commonly found polyols in plants are acyclic forms, such as mannitol, glycerol and sorbitol, and cyclic (cyclitols) forms, such as ononitol and pinitol. Polyols make up a substantial percentage of all assimilated CO_2 by acting as scavengers of stress-induced free oxygen radicals (Bohnert et al. 1995).

A few halophytes accumulate specific compounds: *Melanleuca bracteata* accumulates proline analogue 4-hydroxy-*N*-methyl proline (MHP) and *Spartina* spp. accumulate dimethylsulphoniopropionate (DMS) (Naidu et al. 2000). Such proline analogues increase plant survival under salinity because of their ability to cause regulation, compartmentalization, and production outlay (Bohnert and Shen 1998). However, there is no clear pattern of a particular group of solutes being exclusive to a particular order in other species (Naidu 2003). The concentrations of organic solutes are known to increase several fold (20–80 times) with increasing external NaCl (Ishitani et al. 1996). In a few cases, there is no change in the concentrations of compatible solutes with increasing external salinity, suggesting their constitutive synthesis within the species (Khan et al. 1998). Some organic solutes might redistribute between compartments with increase in salinity, with no significant effect on a whole-tissue basis. However, if confined to the cytoplasm, these solutes can contribute significantly within that volume (Rhodes et al. 2002). This avoids ions toxicity, but the process requires more energy compared to osmotic adjustment with inorganic ions (Ashraf and Harris 2004). Another way of osmotic adjustment in halophyte is by aquaporin water channel, which is involved in intracellular compartmentalization of the water. These aquaporins were found to play important roles in salt tolerance by maintaining osmotic homeostasis and plant cell turgor under salt stress (Maurel 1997).

9.5.7 Other Mechanisms of Salt Tolerance

Secondary mechanisms in halophytes to handle salt stress involve salt glands (Weber 2008), salt bladders (Freitas and Breckle 1992) and succulent tissues (Yeo and Flowers 1986). Salt excretion by salt glands of halophytes is reported to be the fastest ion transport systems in plants (Pollak and Waisel 1979). Salt glands have been described in a few orders, such as Poales (*Aeluropus littoralis* and *Chloris gayana*), Myrtales (*Laguncularia racemosa*), Caryophyllales (*Mesembryanthemum crystallinum* and *Atriplex halimus*), Lamiales (*Avicennia marina* and *Avicennia germinans*), and Solanales (*Cressa cretica*). Salt glands are found on the epidermis of every aerial part but are more concentrated in leaves, and these glands are rich in mitochondria and other organelles but lack a central vacuole (Waisel 1972). The water evaporates

through these salt glands and the salt remains on the leaf surface in crystalline forms, which are blown away through wind or by rain (Lipscshitz et al. 1974). It is a common way of salt avoidance (Waisel et al. 1986). Plants grown under high salinity tend to shed the old leaves as another strategy to avoid the toxic effects of excess sodium salts (Aslam et al. 2011). Ion recirculation from shoots to the roots is also an important salt tolerance mechanism as the toxic ions would have to be carried in the symplasm of the phloem (Munns and Tester 2008).

Succulence dilutes excess NaCl in the leaf tissues (Kramer 1984), but reduction in leaf water content concentrates NaCl in the cell sap, thus supporting osmotic adjustment when halophytes are grown under high salinity (Glenn 1987). Succulence causes increase in cell size, decrease in growth extension, decrease in surface area per tissue volume and higher water content per tissue volume (Weber 2008). The leaves of succulent plants have more and larger mitochondria indicating some extra energy consumption for salt compartmentalization and excretion (Siew and Klein 1969). Succulence is extremely rare in monocots, and only 15 % of monocotyledonous halophytes have salt glands. Halophytes also increase their water use efficiency in response to salt, thereby minimizing the amount of transpired water (Glenn et al. 1997). In *Aster tripolium*, a unique feedback control mechanism of stomatal opening by apoplastic Na^+ in the leaves was identified to regulate maintenance of leaf water potential (Perera et al. 1997).

Seed germination and early seedling vigor, the determinant of population survival and progeny maintenance, are very sensitive to minor environmental disturbance, which in most cases can be lethal (Khan and Gul 2006). The seeds of halophytes have developed strategies to remain dormant until the favorable conditions for germination and early seedling stage are available (Khan and Gul 2006). *Salicornia utahensis* shows the behavior of a true halophyte, where seedling growth at 1,000 mM NaCl was equal to that in control and growth stimulation was observed up to 600 mM NaCl (Gul et al. 2009).

9.6 Breeding Approaches Towards Salt Tolerance

It is not clear how salinity reduces the growth of plants. The accumulation of Na^+ and Cl^- in crop plants reduces their growth and enhances senescence of mature leaves, resulting in a reduction in functional leaf area and yield (Munns and Tester 2008). Therefore, increasing salt tolerance of crop plants is needed for sustaining yield in salt-affected areas. However, complexities of salt tolerance mechanisms always hinder breeding of salt-tolerant crops. So, despite huge efforts ranging from genetic crossing to transgenics towards increasing salt tolerance in crops, only few productive salt-tolerant varieties have so far been developed (Witcombe et al. 2008). Genomic studies on halophytes may help identify specific salt tolerance traits that may be selectively targeted for improvement in crop plants (Huang et al. 2008), which in the long run may lead to the domestication of salt-tolerant genes in economic crop plants. Halophytes respond to salt stress at cellular, tissue, and whole

plant level. Thus, a complete understanding of plant salt tolerance will require studying the mechanisms involved at each level.

The taxonomic diversity of halophytes offers a rich source of germplasm, which suggests a possibility that through wide crosses salt tolerance can be introduced into crop plants (Epstein et al. 1980). Boyko and Boyko (1959) first demonstrated that salt tolerance in crop plants could be increased by crossing them with their salt-tolerant relatives (e.g., wheat × *Agropyrum*). In addition, a universal trait of halophyte may represent its convergent evolution for salt tolerance, and such a trait can be a candidate for transfer from halophytes to glycophytes (Zhu et al. 1997). Few halophytes have desirable crop characteristics that can be introgressed into crop plants through conventional breeding to make them useful for agriculture. Few economically important crops with salt-tolerant, wild relatives are wheat (*Aegelopsis and Thinopyrum*; Gorham and Wyn-Jones 1993), barley (*Hordeum maritimum*; Aronson 1989), tomato (*Lycopersicum cheesmanii* and *L. pimpinellifolium*; Asins et al. 1993), and *Beta vulgaris* ssp. *vulgaris* (*B. vulgaris* ssp. *Maritime*; Rozema et al. 1993). In wheat, few reports are available of transferring salt tolerance between species (Glenn et al. 1999). Transferring + *Knal* gene (for enhanced K^+/Na^+ discrimination) from *Triticum aestivum* to *T. turginsum* through conventional crossing slightly improved salt tolerance of wheat (Dvorak et al. 1994). Wide crosses of wheat with their halophytic relatives provide an alternative approach to raise amphiploids with increased glycinebetaine in the leaves (Islam et al. 2007). However, due to multigenic nature of halophyte salt tolerance, glycophyte-halophyte crosses are yet to be fulfilled (Flowers and Yeo 1995).

Conventional breeding techniques with agronomical parameters related to growth and yield have been used to improve salinity tolerance in crop plants (Ashraf 2002; Ashraf and Harris 2004). Thus far, SKC1 is the only gene that has been used in marker-assisted breeding of salt tolerant rice, although ongoing fine mapping of the salt tolerance QTL "saltol" will lead identification of more markers to facilitate development of salt-tolerant rice cultivars (saltol, Thomson et al. 2010). But, salt tolerance is a multigenic trait in both halophytes and glycophytes (Baisakh et al. 2012). Therefore, conventional breeding for yield has not brought many salt-resistant varieties of the field crops (Witcombe et al. 2008). The probability of combining traits to maximize yield under saline conditions is very low. Thus, it was advocated to understand the physiology of salt tolerance and assemblage of a variety of traits for better results (James et al. 2008). Due to the complexity of the mechanism, transfer of one or two genes has generally not been successful to enhance the tolerance of transgenic plants. However, there are instances where single gene transfers appear to have altered yield (Baisakh et al. 2012; Table 9.1). Study of halophytes can be useful for breeding salt tolerance because: (1) mechanisms of survival and productivity maintenance on saline water can define the required adaptations in tolerant germplasm (Zhu et al. 1997); (2) growing halophytes in an agronomic pattern can be used to find a source of tolerant germplasm for high-salinity agriculture; and (3) halophytes can be used as a direct source of new crops (Glenn et al. 1997).

Table 9.1 Translation of genes from halophyte plants in model and/or crop plants for improvement of salt tolerance

Gene	Halophyte source	Recipient plant	References
Ion transporters			
Na^+/H^+ antiporter	*Thellungiella halophila*	*Arabidopsis*	Wu et al. (2009)
Na^+/H^+ antiporter	*Chenopodium glaucum*	Rice	Li et al. (2008)
Na^+/H^+ antiporter	*Atriplex gmelini*	Rice	Ohta et al. (2002)
Na^+/H^+ antiporter and H^+-PPase	*Suaeda salsa*	Rice	Zhao et al. (2006)
Na^+/H^+ antiporter (*NHX1*)	*Salicornia brachiata*	Tobacco	Jha et al. (2011)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Hordeum brevisubulatum*	Tobacco	Lu et al. (2005)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Halostachys caspica*	*Arabidopsis*	Guan et al. (2011)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Salsola soda*	Alfalfa	Li et al. (2011)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Agropyron elongatum*	*Arabidopsis*	Qiao et al. (2007)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Aeluropus littoralis*	Tobacco	Zhang et al. (2008c)
Vacuolar Na^+/H^+ antiporter (*NHX1*)	*Leptochloa fusca*	Tobacco	Rauf et al. (2014)
Vacuolar Na^+/H^+ antiporter (*NHX2*)	*Suaeda salsa*	*Arabidopsis*	Li et al. (2009, 2012a)
Vacuolar Na^+/H^+ antiporter (*NHX2*)	*Hordeum vulgare*	*Arabidopsis*	Bayat et al. (2011)
Vacuolar-H^+-pyrophosphatase	*Suaeda salsa*	*Arabidopsis*	Guo et al. (2006)
Vacuolar-H^+-pyrophosphatase	*Suaeda corniculata*	*Arabidopsis*	Liu et al. (2011)
Vacuolar-H^+-pyrophosphatase	Halostachys caspica	*Arabidopsis*	Hu et al. (2012)
Vacuolar-H^+-pyrophosphatase	*Kalidium foliatum*	*Arabidopsis*	Yao et al. (2012)
H^+-PPase	*Thellungiella halophila*	Tobacco	Gao et al. (2006)
H^+-PPase	*Thellungiella halophila*	Cotton	Lv et al. (2008)
Tonoplast pyrophosphatases gene (*VP1*)	*Chenopodium glaucum*	*Arabidopsis*	Hu et al. (2009)
AKT-1-type-K^+ channel	*Puccinellia tenuiflora*	*Arabidopsis*	Ardie et al. (2010)
Vacuolar H^+-ATPase subunit c1 (*SaVHAc1*)	*Spartina alterniflora*	Rice	Baisakh et al. (2012)
Vacuolar H^+/Ca^{2+} Transporter	*Suaeda salsa*	*Arabidopsis*	Han et al. (2012)
Tonoplast AQP gene (*TsTIP1;2*)	*Thellungiella salsuginea*	*Arabidopsis*	Wang et al. (2014)
ADP ribosylation factor 1 (*SaARF1*)	*Spartina alterniflora*	*Arabidopsis*, rice	Joshi et al. (2014)

Osmolyte biosynthesis genes			
myo-Inositol *O*-methyl transferase	*Mesembryanthemum crystallinum*	Tobacco	Sheveleva et al. (1997)
myo-Inositol phosphate synthase (*MIPS*)	*Porteresia coarctata*	Brassica, rice	Das-Chatterjee et al. (2006)
myo-Inositol phosphate synthase (*MIPS*)	*Spartina alterniflora*	Tobacco, rice	Baisakh et al. (2009)
myo-Inositol phosphate synthase (*SaINO1*)	*Spartina alterniflora*	*Arabidopsis*	Joshi et al. (2013)
Proline transporter	*Atriplex hortensis*	Arabidopsis	Shen et al. (2002)
Inositol polyphosphate kinase	*Thellungiella halophila*	Brassica napus	Zhu et al. (2009)
Choline oxygenase	*Suaeda liaotungensis*	Tobacco	Li et al. (2003a)
Betaine aldehyde dehydrogenase (*BADH*)	*Suaeda liaotungensis*	Tobacco	Li et al. (2003b)
Betaine aldehyde dehydrogenase (*BADH*)	*Atriplex hortensis*	Tomato	Jia et al. (2002)
Betaine aldehyde dehydrogenase (*BADH*)	*Suaeda liaotungensis*	Maize	Wu et al. (2008)
Antioxidative enzymes			
Glutathione *S*-transferase	*Suaeda salsa*	Rice	Zhao and Zhang (2006a, b)
Gluthathione *S*-transferase	*Limonium bicolor*	Yeast	Diao et al. (2010)
Metallothioneine and UDP-galactose epimerase	*Paspalum vaginatum*	Rice	Endo et al. (2005)
Chloroplastic ascorbate peroxidase (*CHLAPX*)	*Suaeda salsa*	*Arabidopsis*	Pang et al. (2011)
Peroxisomal ascorbate peroxidase (*pAPX*)	*Salicornia brachiata*	Tobacco	Tiwari et al. (2014), Singh et al. (2014)
Chloroplast-located Peroxiredoxin Q	*Suaeda salsa*	*Arabidopsis*	Jing et al. (2006)
Phytoene synthase	*Salicornia europaea*	*Arabidopsis*	Han et al. (2008)
Choline monooxygenase	*Salicornia europaea*	Tobacco	Wu et al. (2010)
β-Lycopene Cyclase (LCY)	*Salicornia europaea*	*Arabidopsis*, Tobacco	Chen et al. (2011)
Chloroplastic monodehydroascorbate reductase	*Avicennia marina*	Tobacco	Kavitha et al. 2010a, b
Stroma ascorbate peroxidase	*Suaeda salsa*	*Arabidopsis*	Li et al. (2012b)
Cytosolic Copper/zinc superoxide dismutase	*Avicennia marina*	Rice	Prashanth et al. (2008)

(continued)

Table 9.1 (continued)

Gene	Halophyte source	Recipient plant	References
Signaling/regulatory pathways			
Serine-rich protein	*Porteresia coarctata*	Finger millet	Mahalakshmi et al. (2006)
Allene oxide cyclase	*Bruguiera sexangula*	Tobacco	Yamada et al. (2002)
Cyclophilin (*CYP1*)	*Thellungiella halophila*	Tobacco	Chen et al. (2007)
CBL-interacting protein kinase (*CIPK2*)	*Hordeum brevisubulatum*	*Arabidopsis*	Li et al. (2009)
Abscisic acid stress ripening-1 (*ASR-1*)	*Salicornia brachiata*	Tobacco	Jha et al. (2012)
Acetylcholinesterase	*Salicornia europaea*	Tobacco	Yamamoto et al. (2009)
Ankyrin repeat protein 1 (*ARP1*)	*Bruguiera gymnorhiza*	*Arabidopsis*	Miyama and Tada (2011)
Cacineurin B-like protein	*Thellungiella halophila*	*Arabidopsis*	Sun et al. (2008)
Transcription factors			
DREB1 (*EREBP*/AP2-type protein)	*Atriplex hortensis*	Tobacco	Shen et al. (2003)
DREB1 (*DREB3a*)	*Leymus chinensis*	*Arabidopsis*	Xianjun et al. (2011)
Zinc finger protein 1	*Thellungiella halophila*	*Arabidopsis*	Xu et al. (2007)
A20/AN1 zinc-finger (Stress Associated Protein)	*Aeluropus littoralis*	Tobacco	Saad et al. (2010)
Zinc-finger-like (ZFL)	*Tamarix hispida*	Tobacco	An et al. (2011)
Late embryogenesis abundant (*LEA*)	*Tamarix hispida*	Tobacco	Qu et al. (2012)
Late embryogenesis abundant (*LEA*)	*Thellungiella salsuginea*	*Arabidopsis*	Zhang et al. (2012)
Basic leucine zipper (*bZIP*)	*Tamarix hispida*	Tobacco	Qu et al. (2012)
MYB-1	*Leymus chinensis*	*Arabidopsis*	Cheng et al. (2013)
bHLH	*Oryza rufipogon*	*Arabidopsis*	Li et al. (2010)
Others			
Uncharacterized universal stress protein gene	*Salicornia brachiata*	*E. coli*	Udawat et al. (2014)
Pathogenesis-related gene (*NPR1*)	*Malus hupehensis*	Tobacco	Zhang et al. (2014)
Unknown function gene (Bg70 and cyc02 homolog)	*Bruguiera gymnorhiza*	*Arabidopsis*	Ezawa and Tada (2009)

9.7 Understanding Molecular Mechanisms of Salt Tolerance in Halophytes

Conventional breeding techniques were reported to be less successful in transferring salt tolerance (Sairam and Tyagi 2004), largely due to the complexity of the physiological mechanisms, strong genotype by environment interaction for yield traits and lack of an efficient marker-assisted selection system (Flowers 2004). Therefore, an integrated strategy involving multiple genes engineering would be required for development and release of salt-tolerant crops (Bohnert et al. 2006).

In recent years, research on molecular mechanisms operating against different types of abiotic stresses is in progress (Aslam et al. 2011). Halophytes show a diversity of growth responses with increasing salinity and the mechanisms behind these remain to be resolved. Although genes/proteins involved in transport of Na^+, Cl^-, and K^+ ions have been identified, how halophytes regulate the constancy of their ion concentrations with the coordinated network of gene regulation requires further investigation. Conventional breeding to improve salt tolerance has been less successful; it is therefore necessary to think on transferring halophyte genes into glycophytes directly. Recent strategy is to genetically modify plants with genes from halophytes to make them more salt tolerant (Aslam et al. 2011). The transgenic salt-tolerant plants were also found to have resistance against multiple abiotic stresses, such as chilling, freezing, heat, and drought (Zhu 2001). Therefore, identification of genes regulated under salt stress in halophytes will have major applications in engineering salt tolerance in crop plants. Although halophytes can adjust to sudden changes in external salinity, but how this change in initial response is sensed is still uncertain (Chinnusamy et al. 2005). Expressed sequence tags (ESTs) have been reported to be an efficient, rapid, and cost-effective strategy to discover novel genes involved in abiotic stress tolerance (Mehta et al. 2005; Baisakh et al. 2008), which helps in understanding the genetic mechanisms that control plant responses to a given ecological condition (Baisakh et al. 2006). Differential expression of transcript-derived fragments under salt stress through complementary DNA-AFLP showed salt stress-induction of genes that may have possible roles in salt tolerance of a monocotyledonous halophyte *Spartina alterniflora* (Baisakh et al. 2006). Comparative transcriptome analysis of *S. alterniflora* showed it to be closer to cereals like maize and rice than other halophytes at both DNA and protein levels.

Halophytes might use the transporters and regulatory networks same as in certain glycophytes, but with different set points. Therefore, comparative understanding of salt tolerance mechanisms in wide/wild relatives of crop plants will be vital for devising strategies to breed salt tolerance. Genetic manipulation of the promoter regions might induce the metabolic pathways related to compatible osmotica, which are blocked in glycophytes (Bohnert et al. 1995).

Undoubtedly, several genes are required for salt tolerance, but it is still interesting to understand the effect of single gene additions or alterations on glycophytes transferred from halophytes (Bohnert and Jensen 1996). Yet, very little is known about the signaling cascades regulating the synthesis of compatible solutes in plants (Chinnusamy

et al. 2005). The molecular basis of salinity-induced accumulation of some organic solutes has already been reported in few halophytes, e.g., inositol in *M. crystallinum* (Vernon et al. 1993), proline in *T. halophila* (Kant et al. 2006), and glycinebetaine in several halophytes. The expression of genes encoding for the first enzyme in the biosynthetic pathway of proline, the α1-pyrroline-5-carboxylate synthetase (P5CS), increased in shoots and roots with increasing salinity, while the gene encoding the first step of the breakdown of proline, proline dehydrogenase (PDH), was found to be decreased (Kant et al. 2006). Overexpression of P5CS in transgenic tobacco plants showed resistance to drought, freezing, and salinity stresses (Hong et al. 2000; Nanjo et al. 2003). Glycinebetaine is another osmolyte, which is synthesized from choline in two steps by enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). Both BADH and CMO expression levels were enhanced during salt stress in *Atriplex nummularia*, *Suaeda aegyptiaca*, *Salicornia europaea*, and *S. maritima* (Moghaieb et al. 2004; Tabuchi et al. 2005) resulting in enhanced expressions of proteins related to glycinebetaine synthesis (Askari et al. 2006). However, in transgenics aimed at increasing glycinebetaine synthesis in plants, such as tobacco and rice that do not naturally contain this solute, precursor (choline) availability hinders glycinebetaine synthesis (Su et al. 2006).

Plants protect their cells from the damage by reactive oxygen species by induction of activities of antioxidative enzymes such as catalase, peroxidase, glutathione reductase, and superoxide dismutase (Mittova et al. 2003). Overexpression of genes, such as mitochondrial Mn-SOD, Fe-SOD, chloroplastic Cu/Zn-SOD, catalase, and glutathione-*S*-transferase (GST)/glutathione peroxidase (GPX) were found to increase the performance of plants under stress (Roxas et al. 2000).

A range of signal transduction pathways including SOS1 and Ca^{2+} has been identified in glycophyte models (Bartels and Sunkar 2005) and these should be further explored in halophytes (Flowers and Colmer 2008). Na^+ efflux from the cytoplasm is mediated by Na^+/H^+ exchange by SOS1 at the plasma membrane and/or NHX1 at the tonoplast level (Apse and Blumwald 2007). SOS1 acts as a sensor for cellular Na^+ and its overexpression through cytosolic Ca^{2+} upregulates ABA concentrations, which leads to an increase in reactive oxygen species (Chinnusamy et al. 2005). SOS1 is present in membrane vesicles of *Thellungiella halophila* shoots and roots and is highly expressed even in the absence of salt (Vera-Estrella et al. 2005). The similarity between the genomes of *T. halophila* and *Arabidopsis* has allowed identification of salinity-induced genes through comparative transcriptome profiling (Wong et al. 2006). The accumulation of Na^+ into vacuoles is mediated by Na^+/H^+ exchanger (NHX; Apse and Blumwald 2007), which was detected in the plasma membrane fraction of *T. halophila* roots (Vera-Estrella et al. 2005). Genes for the Na^+/H^+ antiporter were cloned from *S. salsa* (Ma et al. 2006) and *A. gmelini* (Hamada et al. 2001), and identified in *M. crystallinum* (Chauhan et al. 2000). Some ion-responsive antiporter/channel genes have been reported in monocot halophyte *Puccinellia tenuiflora*; *PutPMP3*-1, and *PutPMP3-2* encoding plasma membrane protein 3 family prevent the accumulation of excess Na^+ and K^+ ions (Zhang et al. 2008a). Similarly, *PutHKT2-1* encoding a high-affinity K^+ transporter facilitates K^+ uptake to maintain a high ratio of K^+/Na^+ in the cells (Ardie et al. 2009). PutAKT1,

a plasma membrane-localized K^+ channel interacts with KPutB1 to alter K^+ and Na^+ homeostasis (Ardie et al. 2011). PutCAX1, a tonoplast Ca^{2+}/H^+ antiporter mediates Ca^{2+}, Ba^{2+}, and Zn^{2+} transport (Liu et al. 2009) and PtNHA1 encoding Na^+/H^+ antiporter functions in the maintenance of low cytosolic Na^+ (Wang et al. 2011). K^+ is an essential element in protein synthesis as it binds tRNA to the ribosomes (Blaha et al. 2000). Homologs of K^+ transporters, such as AKT whose expression changed on salinity stress were identified in the halophyte *M. crystallinum* (Su et al. 2003).

Free polyamines concentrations, which are thought to regulate K^+ fluxes (Shabala et al. 2007), also increase salinity stress tolerance (Shevyakova et al. 2006; Kuznetsov et al. 2007). Osmotic stress induces several proteins in vegetative tissues, such as accumulation of late-embryogenesis-abundant (LEA) proteins (Parvaiz and Satyawati 2008), which indicates their protective role under dehydration stress (Liu et al. 1998). Only a few proteomic investigations have been reported in monocot halophytes (Sobhanian et al. 2010; Sengupta and Majumder 2009). Sobhanian et al. (2010) reported that *Aeluropus lagopoides*, a C_4 monocot halophyte, exhibited enhanced energy production, amino acid biosynthesis, C_4 photosynthetic pathway, and detoxification under saline conditions. However, the wild halophytic rice, *Porteresia coarctata*, shows enhanced energy and osmolytes production, alteration of transcriptional activity and protein modification, as well as change in cell wall components under salinity (Sengupta and Majumder 2009).

A number of transcription factors such as DREBs have been isolated from several plants (Agarwal et al. 2006), but only a few reports are available from halophytic plants like *Physcomitrella patens* (Liu et al. 2007), *Atriplex hortensis* (Shen et al. 2003) and *Salicornia brachiata* (Gupta et al. 2010). Certain genes being highly expressed under salinity but not under normal conditions suggests that their products may act as chaperons in protecting the cellular structure during stress (Xiong and Zhu 2002).

A number of genes isolated from halophytes have been transferred to glycophyte plants through different genetic transformation techniques. Transgenic plants with halophyte genes have shown positive results with their tolerance to salt and other abiotic stresses (Joshi et al. 2013, 2014). A comprehensive list of reports describing development of transgenic plants using halophyte genes is presented in Table 9.1 and hence has not been discussed in detail.

9.8 Stress-Inducible Promoters in Halophytes

A promoter determines the chronological and spatial order of gene expression. The choice of promoters significantly affects the expression of the genes in transgenic manipulation (Munns and Tester 2008). It was shown earlier that high constitutive expression of the foreign gene may be detrimental to the host plant, with increased sterility, retarded development, abnormal morphology, yield penalty, altered grain composition, and transgene silencing (Xu et al. 2006; Kanneganti and Gupta 2008). Strong, tissue-specific, or stress-inducible promoter to direct gene expression to a

required tissue, at a particular development stage and/or in response to a stress may circumvent this problem (Pino et al. 2007). Therefore, identification of stress-inducible promoters must be explored in halophytes for successful exploitation of genes for stress tolerance, (Aslam et al. 2011). It is necessary to use transformation vectors containing specific promoters to ensure the cloned gene expression in individual host cells. Further, it is essential to isolate and analyze the composition and function of the promoter for gene expression, regulation, and vector construction during genetic engineering (Li et al. 2007). At present, promoter analysis is a hotspot in genetic engineering and many promoters have been cloned and some of them were reportedly salt inducible.

Schaeffer et al. (1995) analyzed the sequences of the enhancer and the silencer in the regions of the *CAM* promoter in halophyte *Mesembryanthemum crystallinum*. Yin et al. (2002) obtained a salt-responsive *BADH* promoter from the halophyte *Atriplex centralasiatica* and found basic elements and some motifs, such as TATA-box, CAAT-box, GC-motif, TTCGACA (EIRE), TTATTACAA (MRE), TCCAAG, ACATTACGG, TCATTTCAA (WUN-motif), AGAAAAGTG (HSE), and ACCACGTAAA (ABRE), related to stress. Zhang (2002) isolated *Ped*, a promoter fragment, 243 bp in length with hyperactivity, from *Dunaliella salina* and concluded that this promoter fragment contains a conservative G-box, CAAT-box, and TAAT-box. Isolation and characterization of the promoter of *CMO* gene from *S. liaotungensis* was done to study the relationship between structure and function of this promoter and investigate the molecular mechanism of gene regulation (Li et al. 2007). Kavitha et al. (2010a, b) found the regulatory elements involved in drought, light, cold/freeze, and gibberellic acid response in the promoter of *AmMDAR* gene isolated from *Avicennia marina*. Saad et al. (2010) isolated and cloned the upstream sequences of *AlSAP* translated region (containing the 5′UTR) from the C_4 halophyte grass *A. littoralis*. The *AlSAP* promoter fused to the *gusA* gene in tobacco showed its expression in an age-dependent, multiple abiotic stress-inducible and tissue-specific manners. The promoter of *Thellungiella halophila* vacuolar H^+-pyrophosphatase gene (*TsVP1*) was found to have strong activity in almost all tissues except the seeds and its activity was induced by salt stress in leaves and roots, especially in root tips (Sun et al. 2008).

Yin et al. (2002) isolated ~1.2 kb upstream of *BADH* gene from the halophyte *Atriplex centralasiatica* and characterized the basic elements and some to stress-related motifs, such as, the TATA-box, CAAT-box, GC-motif, EIRE, MRE, WUN-motif, heat shock element (HSE), and ABRE. Deletion analysis showed this promoter to be strongly induced by salt stress and identified two salt-responsive enhancer regions localized between −1,115 to −890 bp and −462 to−230 bp. Similarly, Zhang et al. (2008b) isolated upstream region (1,993 bp) of *BADH* gene from *Suaeda liaotungensis* and identified several putative *cis*-elements in the promoter sequence. These two studies suggested that the smallest promoter fragment (−300 to +62 bp) possessed all the essential *cis*-acting elements and was sufficient for NaCl induction. We have also isolated and characterized a promoter (1,875 bp) of an abscisic acid-responsive gene from *Spartina alterniflora* (*SaAsr1*), which was constitutively expressed. In silico analysis of the promoter revealed a number of *cis* regulatory

motifs, such as DRE-CRT (Dehydration Response Element/C-Repeat), ABRE, CBF (C-repeat binding factors), LTRE (low-temperature-responsive element), ERE (Ethylene-responsive element), and LRE (light-responsive element). Our finding suggested that a minimal promoter of 203 bp, along with two *cis*-acting elements (ABRE, ERE), was enough to drive abundant expression of the reporter gene under a variety of abiotic stresses, such as salinity, ABA, and PEG (Baisakh et al. unpublished). Similarly, in silico analysis of the promoter (1,295 bp) of a cation transport gene, *SaCTP*, from *Salicornia alterniflora* revealed four ABREs in addition to a number of *cis*-regulating, binding motifs, such as DRE-CRT, CBF, LTRE, E-BOX, and MYB (Subudhi and Baisakh 2011). Comparative analysis of the sequences of *Thellungiella parvula* and *T. salsuginea* with *Arabidopsis thaliana*, showed high conservation of the *SOS1* coding region; however, the promoter regions showed conservation only between the two *Thellungiella* species (Oh et al. 2010).

9.9 "Omics" Approaches to Understand Regulation of Salt Stress Responses in Halophytes

In the post-genomics era, comprehensive analyses using functional genomics technologies such as transcriptomics, proteomics, metabolomics, bioinformatics, and high-throughput DNA sequencing have increased our understanding of the complex regulatory networks associated with stress adaptation and tolerance (Urano et al. 2010). Integrated metabolome and transcriptome analyses showed regulation of many important metabolic pathways at the transcriptional level (Mazzucotelli et al. 2008). On the other hand, many metabolic pathways are regulated at the post-transcriptional level, such as by RNA processing, translational, post-translational regulation, or feedback mechanisms (Kaplan et al. 2007). In addition, metabolites, in addition to their functional roles in stress tolerance, act as signaling molecules (Verbruggen and Hermans 2008).

Application of "omics" approaches in understanding salinity stress tolerance mechanisms in halophytes is an important step towards generating crop varieties that can cope with environmental stresses, and knowledge obtained from the study of different halophytes has provided a wealth of information on stress tolerance mechanisms (Hirayama and Shinozaki 2010). Further, omics-based expansive analysis of the differences between halophytes and glycophytes will provide information on the molecular basis of salinity (and other stresses) tolerance attributes of halophytes.

Microarray-based transcriptomic analysis of *Thellungiella halophila* using *Arabidopsis* cDNA microarray identified high level of constitutive expression of six candidate genes in *T. halophile* (Taji et al. 2004; Gong et al. 2005; Du et al. 2008). Mehta et al. (2005) reported that out of 1,841 EST clones from *Avicennia marima* leaf library, 26 novel genes were highly upregulated under salt stress. Through small-scale transcriptomics approach using suppression substractive hybridization (SSH), several novel salt-induced genes were identified in *Bruguiera gymnorhiza* (Miyama et al. 2006; Wong et al. 2007; Ezawa and Tada 2009) and *Aegiceras*

corniculatum, where 30 unique ESTs were reported (Fu et al. 2005). Similarly, 27 % of 1,255 ESTs from both leaf and root tissues of *Spartina alterniflora* represented stress response under salinity (Baisakh et al. 2008).

Genome and transcriptome analyses of halophytes by next generation sequencing (NGS), such as Roche 454 and illumina-Solexa RNA-Seq, can reveal pathways and networks, which can provide clues to the stress behavior of extremophiles (Bressan et al. 2013). Recently, a draft genome sequence of *Thellungiella parvula* provided resources for high-resolution genome-wide comparison of its extremophile attributes with its glycophyte relative, *A. thaliana* (Dassanayake et al. 2011). Through NGS, the authors obtained the de novo assembled genome in 1,496 gap-free contigs with a number of tandem duplications that suggested a possible basis for *T. parvula*'s extremophile lifestyle, which experiences highly saline, poor, degraded and toxic soils, and very high temperature extremes (Oh et al. 2012). Similarly, NGS-based transcriptome analysis of *Porteresia coarctata* under different conditions generated a total of 152,367 unique transcript sequences, and their functional annotation revealed that genes involved in diverse cellular processes including amino acid biosynthesis, hormone biosynthesis, secondary metabolite biosynthesis, carbohydrate metabolism and cell wall structures, contributed to its adaptation under high salinity and submergence conditions (Garg et al. 2013). Transcriptome analysis of a dicot recretohalophyte *Reaumuria trigyna* in response to salinity stress using NGS identified 44 Gene Ontology (GO) terms, 119 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and 25 clusters of orthologous groups (COG) families (Dang et al. 2013). Transcription profiles of these genes showed their relation to ion transport and reactive oxygen species scavenging system being overrepresented under salinity, which most likely allow it to tolerate the stress imposed by semi-desert saline soil.

Metabolomics studies of halophytes can improve our understanding of cellular responses to ion intoxication. Researches with different species have suggested that hormonal regulation modulates induction of the physiological responses in non-adapted species under stress, whereas better-adapted species present a basal metabolic configuration that allows them to tolerate environmental cues (Gagneul et al. 2007; Arbona et al. 2010; Pérez-Alfocea et al. 2011). Comparative analysis of *Thellungiella halophila* with *Arabidopsis* revealed contrastingly different metabolome profiles between the two species. *Thellungiella* contained higher levels of several osmolytes, such as fructose, sucrose, complex sugars, malate, and proline in comparison to *Arabidopsis* even under control conditions (Urano et al. 2010).

A major challenge in the "omics" era remains with the interpretation and integration of large datasets. Highly sophisticated systems biology approaches are required to integrate diverse and massive datasets into intelligible models, which will improve our understanding of the gene regulation networks and their biological relevance under certain conditions (Moreno-Risueno et al. 2010). Another major challenge with "omics" approaches in halophytes is the transfer of such knowledge to crop species. Translational research seems feasible and can potentially enable the reestablishment of regulatory pathways and networks in crops, which will open up avenues for new biotechnological applications in the future.

9.10 Conclusion and Future Perspective

The salt-affected areas in the world are increasing every day due to the degradation of the irrigation system, addition of waste salts, and increasing contamination of underground water sources (Ashraf 2002), which necessitates development of salt-resistant crops to sustain food production to meet the demand of ever-increasing world population (Flowers and Yeo 1995). Glycophyte crops, despite variation in their salt tolerance ability, do not complete their life cycle or are severely affected with respect to their growth and productivity under salinity. To achieve success through conventional breeding, we must identify molecular markers associated with salt tolerance traits to reduce the breeding cycles in development of salt-tolerant crops. Moreover, very few germplasm in the primary gene pool of food crops are available that can reproduce effectively at high salt concentration. While germplasm are available within the secondary gene pool with high salt tolerance, crossbreeding using these resources has not been very successful.

Tertiary gene pool, on the other hand, has several potential species, such as halophytes that have demonstrated, as "salt loving" plants, their capability of thriving under extremely high salinity, thus providing suitable germplasm for identifying and developing new crop systems for saline agriculture (Shabala 2013). One approach is to domesticate halophytes in areas unsuitable for crop establishment. The other approach is to develop salt-tolerant cultivars of conventional crops by introducing halophyte genes directly (Bohnert and Jensen 1996). There are several transgenics that have been developed with halophyte genes and showing high salt tolerance. But, commercial transgenic plants should retain high productivity along with other important traits for agriculture. Hence, transgenic breeding requires identification of stress-related genes and their related promoters.

Despite considerable progress, our understandings of the processes underlying the visible appearances of salt tolerance and salt adaptation of halophytes are still far from complete. There is still a lot to characterize about halophytes and the diversity of mechanisms they employ to cope with salinity. Salt tolerance and adaptation are complex phenomena, which involve multiple physiological, and biochemical mechanisms and numerous genes. Some of the basic questions that need to be addressed are: how halophytic plants discriminate between Na^+ and K^+; how they regulate Cl^- transport; the energetics and regulation of ion transport and compartmentation; the influence of O_2 supply on transport; importance of transpiration in ion accumulation; and control of relative water potential through stomatal aperture, etc. (Colmer and Flowers 2008). To answer these questions, we need to use comparative physiology coupled with genomic, proteomic, and metabolomic approaches that should yield gene regulatory networks and novel stress modules. No single halophyte can be a universal model to address the afore-mentioned questions. Therefore, choosing a right model halophyte is essential in understanding unique genes associated with tolerance/adaptation mechanisms in monocot and dicot halophytes; these may lead to their direct translation into monocot or dicot crops. Fortunately, there are halophyte relatives reported for several crops.

Therefore, research efforts should be made to produce interspecific hybrids, which can serve as genetic resources for mapping of salt tolerance traits and development of crop plants introgressed with halophyte chromosome segments. Concerted efforts must integrate genetic engineering with marker-assisted selection of stress-related genes and QTLs to substantially improve stress tolerance in crop plants. This calls for an international collaboration among research community to identify and exploit need-based halophyte resources as our future generations will rely upon these to a larger extent than now, especially under the inexorable climate change scenarios.

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Chapter 10
UV-B Photoreceptors, Their Role in Photosignaling, Physiological Responses, and Abiotic Stress in Plants

Priyanka Choudhury, Sindhu Kandoth Veetil, and Suneel Kateriya

Abstract Light is a major developmental cue that influences critical aspects of the development, morphology, and metabolism in plants. Hence, the need arose for the evolution of a class of molecule solely responsible for "sensing" light. Photoreceptors are commonly classified based on their chemical nature and photochemistry of their chromophore, and at present, six distinct classes of the photoreceptors are known in the nature. Two putative families have been identified more recently. The electromagnetic spectrum reaching the earth's surface also comprises of ultraviolet radiation, a form of abiotic stress, in the wavelength range from 100 to 400 nm. This chapter mainly deals with the various classes of plant photoreceptors known, the evolution of a UV-B-specific photoreceptor, and the signaling pathways involved to effectively bring about the UV-B-specific stress responses in plants. Importance of "omics"-based approaches would also be discussed for deciphering photoreceptor-mediated cellular signaling and its relevance to stress response in plants.

Keywords Photoreceptors • Chlamydomonas • Uvr8 • UV-B radiation • Abiotic stress response • Photoprotection • Photobehavioral responses and light signaling

10.1 Photoreceptor-Mediated Signaling in Plant

Light is an integral part of plant development. Apart from being the primary source of energy for photosynthesis, it is responsible for the pivotal role of photomorphogenesis. Photomorphogenesis is a series of complex developmental responses, wherein plants assess the quality, quantity, duration as well as direction of light and respond accordingly throughout their life cycle. Plants utilize a variety of photoreceptor molecules and complex downstream signaling networks to sense this light information and transduce the signal (Kami et al. 2010). These photosensors use

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small-molecule chromophores such as flavins, retinals, and tetrapyrroles that undergo photocycles upon light irradiation and are responsible for perceiving the surroundings (Briggs and Olney 2001). When light interacts with these small molecules, they undergo photochemical changes like isomerization, bond formation, photoreduction or cyclic electron transfer, etc. leading to conformational changes in the apoprotein part, which in turn leads to signal propagation and the resulting physiological functions (Fraikin et al. 2013; Jenkins 2014; Losi and Gartner 2012; Sineshchekov et al. 2013). Photoreceptors are classified primarily based on the chemical nature of their chromophore as

1. The light oxygen voltage (LOV)-sensing domain containing photoreceptors senses blue light and use mainly flavin mononucleotide (FMN) as their chromophore. This group includes phototropins, aureochromes, the Zeitlupe (ZTL) family, a hybrid receptor called neochrome, FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1), and LOV KELCH PROTEIN 2 (LKP2) (Briggs 2007; Christie et al. 2012; Imaizumi et al. 2003; Ito et al. 2012; Suetsugu and Wada 2013).
2. Blue light receptors utilizing FAD (BLUF) proteins are blue light receptors as the name suggests and use flavin adenine mononucleotide and/or flavin adenine dinucleotide as their chromophore (Bonetti et al. 2008; Masuda et al. 2004).
3. Cryptochromes with their photolyase homology region (PHR) binding flavin adenine dinucleotide (FAD) and a pterin, methenyltetrahydrofolate as their light sensing chromophore, senses blue light and are divided into plant cryptochromes, animal cryptochromes, and DASH (*Drosophila*, *Arabidopsis*, *Synechocystis, and Human*) cryptochromes (Chaves et al. 2011; Huang et al. 2006; Liu et al. 2011; Moon et al. 2010).
4. Phytochromes are red/far-red photoreceptors that utilize tetrapyrrole-based chromophore (phycocyanobilin and phytochromobilin) (Hughes 2013; Possart et al. 2014).
5. Rhodopsin photoreceptors are visible light absorbing receptors and utilize retinal as their chromophore (Foster et al. 1984; Govorunova et al. 2011; Kateriya et al. 2004).

Another class of photoreceptor, UVR8, which perceives the UV-B region of the light spectrum (280–315 nm) was discovered recently (Kliebenstein et al. 2002; Rizzini et al. 2011). They use their internal tryptophan residues as internal chromophore to sense UV-B (Christie et al. 2012; O'Hara and Jenkins 2012; Wu et al. 2012).

Of the total solar radiation, about 8 % ultraviolet radiation touches down the surface of earth. Out of which 95 % is UV-A (320–400 nm), 5 % UV-B (280–320 nm), and no measure of UV-C (200–280 nm) makes it down past the stratospheric ozone layer. UV-B (280–320 nm) part of the spectrum is of particular interest to all life forms because though this wavelength represents only 1.5 % of the solar radiation reaching earth, it is capable of causing considerable damage to the living systems and anthropogenic activities are only contributing toward increasing the access of this wavelength to the biological systems.

10.2 Effect of UV-B on Plants and Their Damage Control Mechanisms

Exposure to UV-B has a number of detrimental effects on plants, notably, alterations in plant morphology, growth, and development such as reduction in leaf area, reduced stem growth (Cuadra et al. 2004; Dehariya et al. 2011; Frohnmeyer and Staiger 2003), decreased flowering, and alteration in the timing of flowering in certain species of plants. However, variations are seen in the morphological parameters depending on the plant variety and exposure time (Sakalauskaite et al. 2013). UV radiations have negative effects on germinating pollen tubes leading to lowered crop yields (Gao et al. 2004; Koti et al. 2004). UV absorption also causes a net inhibition of photosynthesis in many species of plants caused primarily by reduction in RUBISCO levels and by disruption of PSII (Ferreira et al. 1996; Galmes et al. 2013; Teramura and Sullivan 1994; Vass et al. 1996). The UV-B radiations have the capacity to modify nucleic acids (phototransformation) producing cyclobutane pyrimidine dimers (CPD), pyrimidine-pyrimidinone dimers (Ballare et al. 1996; Landry et al. 1997; Maueler et al. 1994; Takahashi et al. 2011), and aromatic amino acid residues of protein molecules (Vass et al. 1996). The high energy per photon of UV-B causes ROS production and lipid peroxidation (Lidon and Ramalho 2011; Singh and Naseema Beegum 2013; Wang et al. 2010). Photolytic degradation of plant growth regulators like indole-3 acetic acid (IAA) and gibberellic acid are also noted and are one of the molecular reasons for the detrimental physiological effects of UV-B radiation on plants (Jansen et al. 2001; Liu and Zhong 2009). Plants are sessile and are absolutely dependent on sunlight for energy requirements, thus have evolved numerous efficient protective strategies to avoid the harmful effects of the ultraviolet radiations.

Plants exposed to low doses of non-damaging UV-B radiation show UV acclimation by accumulation of UV absorbing flavonoid and phenolic compounds especially in the upper epidermal layers of the leaves (Schmitz-Hoerner and Weissenbock 2003; Warren et al. 2003), and by developing an enhanced antioxidant system (Fini et al. 2011; Wang et al. 2010). In case of damage, a complex set of DNA repair mechanisms such as excision and recombination repairs are adapted (Landry et al. 1997; Ries et al. 2000). Photorepair mechanism is a well established pathway of UV-B-induced CPD repairs (Lario et al. 2013). Accumulation of waxes and polyamines are also defence mechanisms plants adapt to protect against UV-B-induced damages. UV-induced synthesis and accumulation of photoprotective mycosporine-like amino acids (MAA) is observed in the cyanobacteria (Sinha et al. 2001) and green alga Tetraspora sp. CU2551 (Rastogi and Incharoensakdi 2013). This might be a mechanism adapted by some organisms to adapt to harsh environmental conditions with high UV flux. Plants also exhibit enhanced free radical scavenging mechanisms to counter the high-energy radiation stress (Hong-Bo et al. 2008; Tsurunaga et al. 2013; Wang et al. 2010). Plants of the high altitude like *Rheum nobile* have attractive and elaborate bracts. Studies show that these bracts are effective adaptive strategies for protection of pollen against intense UV-B radiations.

10.3 UV-B-Induced Stress Tolerance

UV-B stress response is a universally conserved mechanism in most of the organisms including plants, mammals, yeast, algae, and bacteria. UV response in plants, mammals and yeast in general is a complicated process that involves the activation of stress-induced mitogen-activated protein kinases (MAPK) and the dephosphorylation of these activated kinases by a set of MAPK phosphatases (MKPs) and the associated signaling pathways (Gonzalez Besteiro and Ulm 2013). Working with low fluence UV-B demonstrated photomorphogenic responses like root growth promotion, cotyledon opening, stomatal closure in response to increasing UV-B fluence rates. Regulation of gene expression pathways associated with such different plant process (Kim et al. 1998), UV-B-specific expression of chalcone synthase (CHS) (Christie and Jenkins 1996; Wade et al. 2001), biosynthesis of flavonoid compounds, etc. suggested the existence of a distinct UV-B sensory mechanism independent of DNA damage responses. Comparison of action spectra of UV-induced DNA damage and UV-B-mediated photomorphogenesis showed that action spectrum of DNA damage peaks at 260 nm whereas those photomorphogenic responses peaked at 295–300 nm further emphasizing the involvement of specific UV-B perception pathways in plants (Gardner et al. 2009).

A number of important players that are involved in UV-B-induced photomorphogenic responses namely, CONSTITUTIVELY PHOTOMORPHOGENIC 1(COP1 E3 Ubiquitin ligase) (Huang et al. 2013, 2014), HY5 (bZIP transcription factor), and the UV-B-specific protein UV RESISTANCE LOCUS 8 (UVR8) (Jenkins 2009, 2014) have been identified so far.

10.4 UV-B Sensor (UVR8)-Mediated Gene Regulation and Physiological Responses

Life forms respond to UV radiations by a nonspecific wound/defense signaling mechanism (as a response to DNA damage/ROS) and through a UV-B-specific pathway. Plants use the UVR8 photoreceptor to sense UV-B radiation and perform appropriate acclimation responses (Fraikin et al. 2013; Jenkins 2014; Tilbrook et al. 2013). A UV-B hypersensitive mutant, UV resistance locus 8-1, from a genetic screen for UV-sensitivity showed reduced expression of flavinoid-biosynthesis pathway genes and chalcone synthase gene as well as an increased levels of PR1 and PR5 (pathogenesis-related genes) proteins (Kliebenstein et al. 2002). Mapping this locus showed a 15 base pair deletion in a gene in UV resistance locus 8-1 (uvr8-1). This later led to the identification and implication of UVR8 as a possible UV-B receptor (Rizzini et al. 2011). UVR8 has been implicated in modulating genes involved in a number of responses like defense against herbivores (Demkura and Ballare 2012), accumulation of UV protection molecules, and hormone signal transduction under normal sunlight conditions (Morales et al. 2013). It is also reported to

feed into UV-A/blue light signaling pathways and regulate a number of additional photomorphogenic responses (Casal et al. 2013; Vandenbussche et al. 2014; Xie and Hauser 2012). Low fluence UV-B radiation has beneficial effect on plant defense mechanisms. UVR8 mediates *Arabidopsis* resistance to the pathogenic fungus *Botrytis cinerea* by controlling the expression of the sinapate biosynthetic pathway and increasing the levels of sinapates in leaf tissues (Demkura and Ballare 2012). UV-B stimulation leads to the accumulation of the monomeric form of UVR8 in the nucleus (Christie et al. 2012; Wu et al. 2012) where it interacts with chromatin and CONSTITUTIVELY PHOTOMORPHOGENIC1-SUPPRESSOR OF PHY A (COP1-SPA) core complex (Cloix et al. 2012; Huang et al. 2013, 2014). Cloix et al. in their attempt to decipher how COP1–UVR8 interaction initiates signaling showed that 27 amino acids from the C-terminus of UVR8 are necessary and sufficient for its interaction with COP1 (Cloix et al. 2012). Expression of a UVR8 protein lacking the C27 amino acids was unable to rescue UVR8 function in *uvr8-1* null mutant (Cloix et al. 2012; Huang et al. 2013, 2014).

10.5 Positive and Negative Regulators of UV-B Responses

The E3 ubiquitin ligase, COP1, is a well-known repressor of photomorphogenesis (McNellis et al. 1994) that acts to counterbalance the positive signaling factors that function downstream of phytochrome and cryptochrome receptors by targeting different photomorphogenesis promoting transcription factors for degradation including HY5 in the dark (Osterlund et al. 2000; Saijo et al. 2003). However, the COP1 protein functions as a positive regulator of UV-B-specific UVR8-mediated responses (Huang et al. 2013; Oravecz et al. 2006). UV-B-activated association of UVR8 and COP1 leads to the activation of HY5. UV-B irradiation brings about conformational changes in the UVR8 homodimer converting it into the monomeric form which then interacts with COP1–SPA complex and dissociates it from CULLIN4-DAMAED DNA BINDING PROTEIN 1 (CUL4-DDB1) E3 apparatus. This reorganization converts COP1 from a photomorphogenesis suppressor to activator of photomorphogenesis promoting transcription factor HY5 (Huang et al. 2014).

The UV-B light responses are balanced by a negative feedback loop (Gruber et al. 2010) involving two regulatory proteins, REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP1) and RUP2. rup1, rup2 double mutants showed enhanced UV-B tolerance and a much slower rate of monomer to dimer conversion. Rup1 and RUP2 mediate re-dimerization of UVR8 and disrupt UVR8–COP1 interaction and stops downstream signaling (Heijde and Ulm 2012, 2013). Co-immunoprecipitation experiments suggest that UVR8 interacts with COP1 in the presence of SPA1 (SUPPRESSOR OF PHYA-105 1) protein. Chromatin immunoprecipitation studies showed that UVR8 associates with chromatin (preferentially histone H2B) in the bZIP containing transcriptional regulator HY5 (ELONGATED HYPOCOTYL5) and HYH (HY5 HOMOLOG) promoter region and modulates downstream gene regulation (Huang et al. 2013). UVR8 regulates the expression of

HY5 and HYH both of which have overlapping function (Holm et al. 2002) and mediate light-regulated transcription of all the UVR8 downstream pathway genes (Feher et al. 2011). It also associates with the chromatin of the promoter regions of other genes but not necessarily of all the genes that it regulates as suggested by the ChIP assays. The promoter regions of certain genes regulated by UVR8 like HYH and CHS are not picked up during ChIP assays (Cloix and Jenkins 2008); this however could be due to the limitations of the assay method. Association of UVR8 with chromatin is independent of UV-B stimulation (Cloix and Jenkins 2008). Low fluence rate UV-B radiation induces genes encoding the transcription factors HY5 and HYH and activates the UVR8-mediated pathway (Brown et al. 2005, 2009; Favory et al. 2009). Studies using mutants in the UVR8 pathway sheds light on the signaling mechanism of the UV-B photoreceptor. Studies showed that H_2O_2 and NO accumulated in the stomata upon UV-B exposure through a UVR8, COP1, HY5, HYH pathway and causes stomatal closure in an NO-dependent manner. Recent evidences suggested that there are crosstalk between UVR8 and different photoreceptors. Studies with uvr8, cry1/2, phot1/2, and phya/b mutants showed that ARI12 (potential E13 ligase) is suppressed by UVR8 and Cryptochrome and is unaffected by phototropins and phytochrome A and B up on high fluence rate UV-B exposure (Lang-Mladek et al. 2012; Xie and Hauser 2012). UVR8 and COP1 are required for the entrainment of circadian clock in *Arabidopsis* in response to low fluence UV-B exposure (Feher et al. 2011). UVR8 also regulates the impaired photosynthesis on exposure to UV-B (Davey et al. 2012). Microarray analysis suggested the regulation of over hundred genes involved in multiple pathways and widely different functions by the UVR8 gene (Casati et al. 2011d). The experimental evidences feeding into the UV-B-mediated signaling pathway suggest the existence of a finely tuned mechanism of UV-B detection and response with a number of proteins and pathways interacting. Almost all information on UVR8 functions until date has been obtained from *Arabidopsis* and UVR8 is seen to regulate a large subset of genes, therefore, it would be interesting to understand the function of UVR8 in other evolutionary relevant organisms to understand the mechanism of action of this photoreceptor.

10.6 Structure and Molecular Mechanisms of the UV-B Photoreceptor (UVR8)

The UV-B photoreceptor, UVR8, shows 30 % identity to the human regulator of chromatin condensation protein RCC1 but shows absolutely no similarity in function (Brown et al. 2005; Wu et al. 2011). UVR8 is a seven-bladed β propeller protein that occurs as functionally inactive dimer in the cytosol. Upon UV-B irradiation the dimeric form switches to its active monomeric form, rapidly accumulates in the nucleus (Voityuk et al. 2014; Wu et al. 2012, 2014), and binds chromatin and interacts with an important light-mediated photomorphogenesis regulator molecule, COP1. This COP1–UVR8 interaction controls the transcription of several genes responsible for UV-B-mediated signaling responses. The dimer to monomer

switching is a reversible process (Heijde and Ulm 2013; Heilmann and Jenkins 2013). The dimer interface of UVR8 molecule is rich in aromatic residues and charged side chains. It has seven tryptophans, three phenylalanines, and two tyrosines and forms complex network of salt bridges across the dimer interface (Christie et al. 2012; Wu et al. 2012). The cracking of the *Arabidopsis* UVR8 core domain crystals (residues 12–381) up on UV-B irradiation and the failure of the crystals of the variants W285A and W285F to crack up on prolonged UV-B irradiation showed that the core domain of the protein perceives the UV-B (Wu et al. 2012). UVR8 unlike any other photoreceptor does not have a chromophore-binding domain and does not utilize any external cofactor as chromophore to perceive UV-B radiation. Insights from the crystal structure and mutational analysis identified three tryptophan residues, Trp 285 and Trp 233 and Trp 337 from the Gly-Trp-Arg-His-Thr sequence repeats that occur in blade 5, 6 and 7 that forms a closely packed tryptophan triads as the UV-B sensor of the protein. The Trp 285 and Trp 233 are found to be involved in strong cation–π interactions with Arg 338 and Arg 234, respectively. UV-B perception by these internal tryptophan chromophore results in the disruption of these cation–π interactions, which in turn disrupts the arginine-mediated intermolecular hydrogen bonds and leads to the disassociation of dimeric into the monomeric form (Christie et al. 2012; Wu et al. 2012). The use of tryptophan resides as the UV-B sensor is justified further by the fact that absorption wavelength of tryptophan and UV-B radiation falls in the same range. The molecular mechanism involved in the photoreaction of UV-B-induced UVR8 monomerization was inferred by quantum chemical cluster calculations. UV-B irradiation initiates a transfer of electron from Trp 233 to Trp 285 and further to Arg 338, which is coupled to a simultaneous transfer of proton from Arg 338 to Asp 129 making Asp129 protonated, R338 neutral, and Trp 233 deprotonated. Neutral Arg 338 disrupts salt bridges associated with the residue, which in turn contributes to the disruption of the UVR8 dimers (Voityuk et al. 2014; Wu et al. 2011, 2014). Deciphering mechanistic basis of UV-B-mediated physiological response in plant system would lie in the future.

10.7 "Omics" of UV-B Irradiation on Plants

In order to identify the nodal points in the signaling network and identify the key regulator molecules that elicit physiological responses of plants to UV-B signaling, an integrated and systematic "Omics" studies could be adapted. A number of such studies have been carried out in maize and *Arabidopsis* plants to decipher the signaling pathways involved in detail (Brown and Jenkins 2008; Casati et al. 2011a, b, d; Casati and Walbot 2004). To study the nature of responses to UV-B and to track these responses, Casati et al. 2011d studied and compared the transcriptome of organs that was exposed and those that were experimentally shielded to varying UV-B exposure times. UV-B elicited significant overlapping changes in transcripts of both irradiated and shielded organs after 10 min of exposure. Whereas, upon prolonged exposure to radiation the changes in transcript noted was organ specific,

suggesting that the initial response to UV-B in all organs are through a common pathway with the possibility of mobile molecules being generated and transducing the signals to other organs. In anthocyanin-deficient maize line (shows greater UV-B response), 347 genes were found to be UV-B responsive upon 8 h UV-B supplementation with 285 being upregulated and 80 genes being downregulated (Casati and Walbot 2004). An integrative study by Casati et al. 2011b to monitor the transcriptome, proteome, and metaboliome of irradiated leaves, shielded leaves, and immature maize ears that were carried out over a time course of 1–6 h showed that exposure of a mere 10 % of the leaf area in adult maize plants was sufficient to elicit most of the UV-B response. UV-B radiation increases the expression of the genes of the flavonoid pathway, some phenylpropanoid precursors such as shikimic, quinic, and trans-caffeoylquinic acids exclusively in the irradiated leaves whereas, a few other phenylpropanoid precursors such as cinnamic acid and trans-caffeic acids and also the protein iso-flavone reductase-like 1 are increased in both the irradiated as well as shielded organs. A number of cell wall metabolism pathway enzymes show increased expression up on UV-B irradiation. Comparative studies on microarray data using plants exposed to low fluence (filtered radiation) and high energy radiation (unfiltered radiation) gave insights into the set of genes that are regulated and pathways that are implicated as a stress response to UV-B and those subset that are regulated as an UV-B acclimation response. Another important aspect that needs further studies is to understand the initial steps of the signaling pathway, the molecules/mechanism that are directly influenced after UV-B light perception and how these molecules implicate signaling pathways. Studying the metabolites under various conditions gives an insight into this. Myo-inositol is an important cellular metabolite that is involved in a number of important functions like cell–cell signaling, storage and transport of the plant hormone auxin, stress response and cell death, etc. It is found to rapidly increase in irradiated and shielded leaves and is also found to be capable of mimicking the effect of UV-B irradiation, thus is implicated to be a candidate UV-B signaling compound (Casati et al. 2011c). Some other metabolites that increases immediately up on UV-B irradiation and fall back to their normal levels within a short period are leucine, glutamine, phosphoric acid, fructose, glucose, glycine, glyceric acid, aspartic acid, and mannose (Casati et al. 2011d). Another kinase-like protein also increases in shielded leaves even with just one leaf exposure and could be another possible candidate molecule of UV-B signaling. Most of the protein changes that was detected occurred within 4 h of UV-B exposure and that most of these proteins corresponded to the group of abundant proteins like general metabolism, photosynthetic and heat shock proteins, etc. (Casati et al. 2011b).

Majority of the gene expression profiling studies are carried out on samples grown under laboratory conditions and often the conditions in laboratory does not mimic the illumination conditions of the fields, where a number of other factors act in tandem with illumination condition. It is therefore important to obtain and compare multiple sets of such experimental data under different combinatorial conditions and conclusions need to be drawn with caution.

Studies on *Arabidopsis* show that 96 genes are differentially expressed in wild-type *Arabidopsis thaliana* grown under solar UVA + B radiation from wild-type

plants grown with no UV component. In addition, wild-type plants and *uvr8-2* mutants exposed to solar UVA + B show differential accumulations of transcripts. Transcripts of a number of genes including those involved in biosynthesis of flavonoids, anthocyanins, jasmonic acid signaling pathway, auxin signaling, glucosinolate biosynthesis as well as genes involved in defense responses (WRKY70, MKK4, etc.) and salt stress responses were reduced in *uvr8-2* mutants as compared to the wild-type plants (Morales et al. 2013).

Anthropological influence is only working toward degrading the stratospheric ozone layer and increasing the influx of harmful UV-B radiation. It is therefore very important to thoroughly understand the plants response to UV-B stress, acclimation response and integration of these vast information generated by the "omics" studies into meaningful models.

10.8 Summary and Prospective

It is established beyond doubt that photoreceptors are indispensible biomolecules in nature. In plant system, photoreceptors play a very important role in growth, development, and survival. It is very likely that these molecules communicate to each other in a direct and/or an indirect mode (Fig. 10.1), which is very important for integrating light signal for tuning light-dependent physiological responses. Interaction of UV-B photoreceptors with the other light sensors and/or

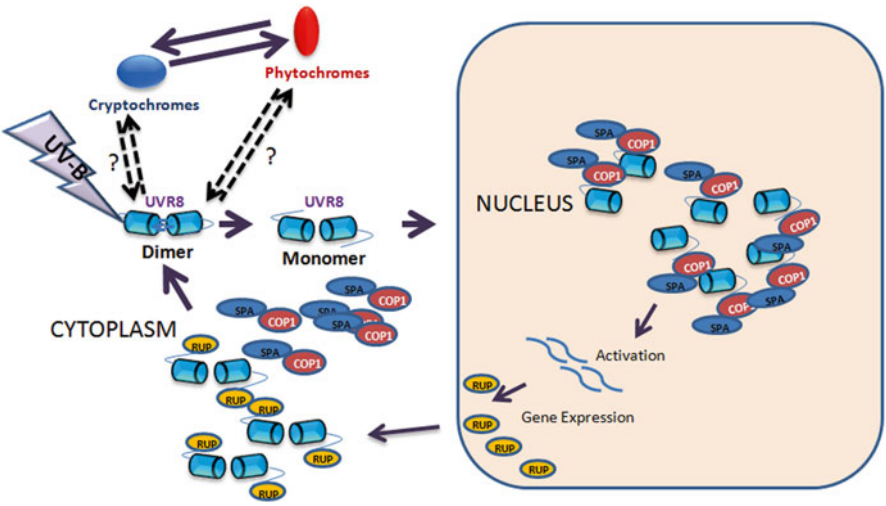

Fig. 10.1 Photoreceptor-mediated protein–protein interaction(s) and signaling in plant. *Solid arrow* represents known photoreceptor-mediated protein–protein interaction and associated downstream signaling. *Broken arrow* shows photoreceptor-dependent possible protein–protein interaction and its role in light-mediated signaling

biomolecules could be studied using genetic, biochemical, and omics-based (transcriptomics, proteomics, and metabolomics/metabonomics) approaches. Developing thorough understanding of the Photoreceptor biology is very important as in future; photoreceptor-based plant biotechnological approaches could be adapted to develop transgenic plants for managing light-triggered abiotic response(s) in these systems.

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Chapter 11
Analysis of Signaling Pathways During Heavy Metal Toxicity: A Functional Genomics Perspective

Gyana Ranjan Rout and Jogeswar Panigrahi

Abstract Abiotic stresses have major limiting factors for plant growth and crop productivity. Plants have different mechanisms to maintain the physiological concentrations of essential metal ions and to minimize exposure to non-essential heavy metals. Some mechanisms are ubiquitous because they are also required for general metal homeostasis, and they minimize the damage caused by high concentrations of heavy metals in plants by detoxification, thereby conferring tolerance to heavy metal stress. Metals in the cell are addressed using a range of storage and detoxification strategies, including metal transport, chelating, trafficking, and sequestration into the vacuole. A large number of genes encoding MAPK pathway components have a major role in cell proliferation and hormone action as well as in stress signaling. Germin-like protein genes were developed by various stresses including metal stress. Functional genomics (integrating genome sequencing, transcriptomics, proteomics, metabolomics, ionomics, and phenomics) allows large-scale gene function analysis with high-throughput technology and incorporates interaction of gene products at cellular and organism level.

Keywords Metal toxicity • Metal transport • Oxidative stress • Metal-binding protein • Signaling pathway

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11.1 Introduction

Agriculture faces various abiotic stresses, especially salinity and drought as major limiting factors for crop productivity. Cold, temperature, heavy metals, and UV radiation are also the abiotic factors affecting crop yields (Qin et al. 2011). Some of the essential micronutrients playing a key role in many metabolic mechanisms can be toxic when the content in tissues is higher than optimal. Abiotic stress is one of the primary causes of crop loss, causing average yield losses of more than 50 % for major crops. Plants can resist abiotic stresses through various distinct mechanisms. Both tolerance and susceptibility to abiotic stresses are very complex mechanisms. Plant traits that are associated with resistance processes are multigenic and thus difficult to control and engineer. Metals like copper, iron, nickel, and zinc are essential for the functioning of physiological and biochemical processes for normal growth and development of plants (Marschner 1995; Broadley et al. 2007). However, elements such as aluminum, cadmium, and lead are considered to be non-essential and generate toxic responses even at low exposure concentrations. An excess of toxic metals commonly has a negative impact on physiological and biochemical processes in organisms, resulting in major risks to the environment and for human health. Plants growing on metal-enriched soils suffer from decreased growth and performance, reducing crop yield. At the molecular level, oxidative stress is widely studied as a key sign of plant stress. This process is commonly described as an imbalance between reactive oxygen species (ROS) and antioxidants. ROS are not only generated from the normal metabolic activity of mitochondria and chloroplasts but also produced during both abiotic and biotic stresses (Neill et al. 2002; Nakata et al. 2002; Patnaik and Khurana, 2001). ROS can undergo further reactions, often catalyzed by metal ions as in the Fenton reaction, and so generate the much more reactive hydroxyl radical, which may be responsible for alterations of macromolecules and ultimately may contribute to cell death (Briat and Lebrun 1999). Sharma and Dietz (2009) reported the close relationship between metal toxicity, redox homeostasis, and antioxidant capacity in plants. Depending on the chemical properties of metals in biological systems, their toxicity is attributed to various mechanisms such as (1) interference with functional sites in proteins; (2) displacement of essential elements, thereby disturbing enzymatic functions; or (3) increased ROS production. High concentrations of heavy metals increase the ROS production in subcellular organelles such as peroxisomes, chloroplasts, and mitochondria, which together constitute the predominant sources of ROS production in plants. Keunen et al. (2011) reported that the mitochondria are key players in cellular redox homeostasis and signaling (Fig. 11.1).

Enzymatic degradation of superoxide is ensured by superoxide dismutases, while that of hydroperoxides is ensured by catalase, glutathione peroxidase, or ascorbate peroxidase (Chaudiere and Ferrari-Iliou 1999; Foyer and Noctor 2005). High peroxidase activity is also reported as a defensive mechanism, which may cause damage or disturb the normal functions of plant cells (Fang and Kao 2000).

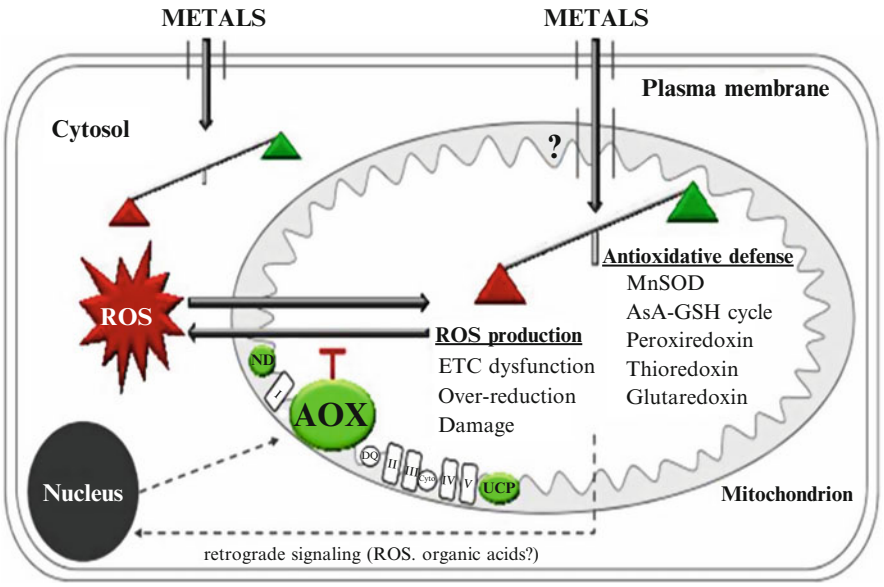

Fig. 11.1 Schematic overview of metal-induced responses in plant cells focusing on mitochondrial effects. Metal exposure has shown to cause mitochondrial electron transport chain dysfunction and over-reduction, thereby increasing mitochondrial ROS production (Keunen et al. 2011)

However, H_2O_2 has been shown to induce cell protection genes and has been shown to act as a diffusible compound, which mediates the regulation of gene expression (Desikan et al. 2001; Vanderauwera et al. 2005). Heavy metal absorption has affected seriously the quality and safety of harvested crop material; hence, an understanding of the biochemical detoxification strategies that plants can adopt against oxidative stress induced by metal ions in plants is key to manipulating metal tolerance in plants (Dixit et al. 2001). Danquah et al. (2014) reported that plants have specific mechanisms that allow them to rapidly perceive and respond to abiotic stresses in the environment. Among the evolutionarily conserved pathways, the ABA (abscisic acid) signaling has been identified as a central regulator pathway of abiotic stress response in plants, triggering major impact in gene expression and adaptive physiological responses. ABA induces protein kinases of the SnRK family to mediate a number of its responses. MAPK (mitogen-activated protein kinase) cascades have also been shown to be implicated in ABA signaling. Seo and Koshiba (2002) reported that except for the conversion of xanthoxin to ABA in the cytoplasm, all the steps for ABA synthesis occur in plastids. The early C5 precursor of ABA, isopentenyl pyrophosphate (IPP), is produced primarily in plastids via 1-deoxy-D-xylulose-5-phosphate (DXP) from pyruvate and glyceraldehyde-3-phosphate (Cutler and Krochko 1999; Nambara and Marion-Poll 2005; Seo and

Koshiba 2002; Wasilewska et al. 2008). This leads to the sequential production of farnesyl pyrophosphate, geranylgeranyl pyrophosphate (GGPP), phytoene, lycopene, and β-carotene. β-Carotene is converted to a xanthophyll, zeaxanthin, which is the first oxygenated carotenoid as reported by Seo and Koshiba (2002). Subsequent steps involve the synthesis of cis-isomers of violaxanthin and neoxanthin that are cleaved to form xanthoxin (the C15 precursor of ABA). Xanthoxin is presumed to migrate from the plastid to the cytosol (Nambara and Marion-Poll 2005) and converted to ABA through abscisic aldehyde, xanthoxic acid, or abscisic alcohol (Seo and Koshiba 2002). Cell-to-cell ABA transport was shown to be mediated by two plasma membrane-bound ATP-binding cassette (ABC) transporters and a family of low-affinity nitrate transporters (Kang et al. 2010; Kuromori et al. 2010; Kanno et al. 2012). Most ABC transporters are integral membrane proteins and act as ATP-driven transporters for a very wide range of substrates, including lipids, drugs, heavy metals, and auxin. Kuromori et al. (2010) isolated AtABCG25, which encodes a half-size ABC transporter protein and is responsible for ABA transport and responses in *Arabidopsis*. Kanno et al. (2012) isolated ABA-importing transporter 1 (AIT1) (which is also known as low-affinity nitrate transporter; NRT1.2) from a modified yeast-2-hybrid screen in which positive clones are capable of inducing interactions between the ABA receptor PYR/PYL/RCAR and PP2C protein phosphatase at low concentrations of ABA. AIT1 was preferentially localized to the plasma membrane of plant cells and was mainly expressed in vascular tissues in cotyledons, true leaves, hypocotyls, roots, and inflorescence stems.

11.2 Molecular Basis of Plant Responses to Heavy Metal Stress/Toxicity

Metals are required by plants in a wide range of concentrations. During the evolution of an angiosperm, the metal requirements are strongly steered by the demands of physiological processes in different organelles, cells, tissues, and whole plants. At the cellular level, the function of the cell and the presence of specific organelles determine the metal content. For example, in photosynthetic active cells, the metal demand of the chloroplasts varies from 15 % of the total cell; Zn in the carbonic anhydrase (Hewitt 1983) to nearly 50 % of the total cell, Cu in the plastocyanin (Hewitt 1983; Lolkema and Vooijs 1986). In mitochondria, however, Cu enzymes are already satisfied with 3–6 % of total cell of Cu (Peng et al. 2005) and Fe enzymes with 2–4 % of total cell of Fe (Hewitt 1983). The original compartmentalization concept (Ernst et al. 1974) has received much support during the past decade. At the cellular level, protection of physiologically active sites in the cell is achieved by a rapid cellular compartmentalization of the metal surplus, especially into the vacuole. The role of metal-binding metabolites in the cytosol is elaborated for Cd- and Cu-tolerant plants (Mengoni et al. 2003). The removal of surplus metals from the cytosol and their transport across the tonoplast are accelerated in metal-tolerant plants (Drager et al. 2004).

11.2.1 Heavy Metal-Induced Signaling in Plants

Metal-induced toxicity is very well reported. Several essential metals like iron, zinc, copper, and manganese participate in controlling the various metabolic and signaling pathways. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metal and is characterized by the synthesis of stress proteins and signaling molecules and, finally, the transcriptional activation of specific metal-responsive genes to counteract the stress (Maksymiec 2007). The signal transduction pathways include the Ca–calmodulin system, hormones, ROS signaling, and the mitogen-activated protein kinase (MAPK) phosphorylation cascade, which converge by activating the stress-related genes. Different signaling pathways may be used to respond to the different heavy metals (DalCorso et al. 2010; Thiele 1992; Skórzynska-Polit et al. 1998). Ca^{2+} signaling features in responses to a number of abiotic stress factors, including temperature extremes, osmotic stress, oxidative stress, anoxia, and mechanical perturbation (Knight 1999). Excess heavy metals modify the stability of Ca^{2+} channels, thus increasing calcium flux into the cell. Intracellular Ca^{2+} is a secondary messenger, which interacts with calmodulin to provide the signal and ultimately to regulate downstream genes involved in heavy metal tolerance, metabolism, and transport (Yang and Poovaiah 2003). The Ca^{2+}–calmodulin system is also involved in the response to other heavy metal toxicity, such as Ni and Pb. Plant hormones are involved in many physiological and developmental processes and play a critical role in the adaptation to abiotic stress through the regulation of hormone synthesis in the presence of heavy metals (Peleg and Blumwald 2011). Heavy metals can produce ROS directly via the Fenton and Haber–Weiss reactions and indirectly by inhibiting antioxidant enzymes (Romero-Puertas et al. 2007). In particular, H_2O_2 acts as a signaling molecule in response to heavy metals and other stresses (Dat et al. 2000). Cuypers et al. (2000) reported a root-to-shoot signaling system that appears to be involved in copper-imposed oxidative stress as well as in the antioxidative defense response (Cuypers et al. 2002). Vitoria et al. (2001) reported the significant role in cadmium-induced increases in catalase, superoxide dismutase, and glutathione reductase activity in both leaves and roots of radish seedlings and also suggested that an oxidative stress signal is sent from roots to leaves. Verma and Dubey (2003) observed that the lipid peroxides in shoots were increased with elevated oxidative stress. The rationale for increased proline in a plant is linked with the ability of proline to quench singlet oxygen (Özturk and Demir 2002), which may arise as a by-product of lipoxygenase in the presence of Cu^{2+} (Arora et al. 2002). Proline can also react directly with the hydroxyl radicals that might result from metal-catalyzed Fenton chemistry, and therefore, increased proline would mitigate the damage from free radicals and leads to a more reduced cellular environment (Siripornadulsil et al. 2002).

Arasimowicz-Jelonek et al. (2012) reported that the roots of 3-day-old yellow lupine (*Lupinus luteus*) seedlings exposed to cadmium (89 mM $CdCl_2$) resulted in programmed cell death starting from 24 h of stress duration, which was evidenced by

TUNEL-positive reaction. Cd-induced programmed cell death was preceded by a relatively early burst of nitric oxide (NO) localized mainly in the root tips. These changes were accompanied by the NADPH oxidase-dependent superoxide anion (O) production. However, the concomitant high level of NO in 24 h of Cd exposure did not provoke an enhanced peroxynitrite formation. But the treatment with the NADPH oxidase inhibitor and NO scavenger significantly reduced superoxide anion and NO production, respectively, as well as diminished the pool of cells undergoing cell death.

11.2.2 Heavy Metal Ion Uptake and Its Translocation

In the biological system, cellular location and metal specificity of most of these transporters in plants are still unknown. The cell wall can play a key role in the immobilization of toxic heavy metal ions by providing pectic sites and histidyl groups, and extracellular carbohydrates such as callose and mucilage, and thus prevents heavy metal uptake into the cytosol. Metal availability and motility in the rhizosphere are influenced by root exudates and microorganisms (Wenzel et al. 2003). Higher plants possess highly effective systems for the acquisition of metal ions and other inorganic nutrients from the soil. One of the major roles of root exudates is to chelate metals and to prevent their uptake inside the cells. The binding of metal ions such as Cu and Zn in the apoplast also helps to control the metal content of root cells (Dietz 1996; Kobae et al. 2006). Cation-binding sites are also present on the root cell wall, and this allows metal exchange, thus influencing the availability of ions for uptake and diffusion into the apoplast (Allan and Jarrel 1989). Plants possess various form of plasma membrane transporters involved in metal uptake and homeostasis. Some of the transporters belong to the heavy metal P1B-ATPase, the NRAMP, and the CDF (Williams et al. 2000). Guerinot (2000) reported that ZIP family is one of the principal metal transporters involved in metal uptake. ZIP family of transporters have also been identified in many plant species and are involved in the translocation of divalent cations across the membranes. Certain ZIP proteins are induced in *A. thaliana* roots and shoots in response to Fe or Zn loading and thus appear to be part of a stress response. Most ZIP proteins are predicted to comprise eight transmembrane domains and have a similar topology, with the N- and C-termini exposed to the apoplast, and a variable cytoplasmic loop between transmembrane domains III and IV that contains a histidine-rich domain putatively involved in metal-binding site (Guerinot 2000). The first ZIP transporter to be characterized was *A. thaliana* IRT1. This was identified by functional complementation of the *S. cerevisiae efet3fet4* double mutant, which is impaired in iron transport (Eide et al. 1996). In *A. thaliana*, IRT1 is expressed in root cells and accumulates in response to iron deficiency, suggesting a role in Fe^{2+} uptake from the soil (Vert et al. 2002). Many metal transporters present at low ion selectivity (Korshunova et al. 1999). The plasma membrane plays an important role in plant response to heavy metals by reducing the uptake of metals into the cell or by active efflux pumping outside the cell. ABC transporters are also involved in metal ion efflux from the plasma membrane as reported by Kim et al. (2007).

11.2.3 Metal Sequestration in the Vacuole by Tonoplast Transporters

Plants respond to high intracellular concentrations of metal ions by using efflux pumps either to export the ions to the apoplast or to compartmentalize them within the cell. The main storage compartment for metal ions is the vacuole, which in plants accounts for up to 90 % of the cell volume (Vögeli-Lange and Wagner 1990). Several families of intracellular transporters involved in this process have been identified in plants and yeast, and they appear to be highly selective. Phytochelatins (PC) are the best-characterized heavy metal chelators in plants, especially in the context of Cd tolerance (Cobbett 2000). PCs are a family of metal-binding peptides with the general structure (c-Glu-Cys)nGly ($n = 2–11$) (Cobbet and Goldsbrough 2002). The cysteine thiol groups allow PCs to chelate metals and form complexes with a molecular weight of 2.5–3.6 kDa (Cobbett 2000). PCs are synthesized in the cytosol and then transported as complexes to the vacuole. Their synthesis is rapidly activated in the presence of heavy metals such as Cd, Cu, Zn, Ag, Au, Hg, and Pb (Cobbett 2000). ABC transporters can transport xenobiotics and heavy metals into the vacuole and two subfamilies (MRP and PDR) particularly active in the sequestration of chelated heavy metals. Some of the CDF transporter family (also called MTPs in plants) are involved in the transport of metal ions from the cytoplasm to the vacuole (Krämer et al. 2007) and to the apoplast and endoplasmic reticulum (Peiter et al. 2007). CDF transporters have been characterized primarily in prokaryotes but are also found in many eukaryotes, where they transport divalent metal cations such as Zn, Cd, Co, Fe, Ni, and Mn (Montanini et al. 2007). The MAPK cascade in plants is a response to both biotic and abiotic stresses (He et al. 1999). It involved three kinases sequentially activated by phosphorylation: the MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK), and the MAPK. At the end of this cascade of phosphorylation, MAPKs phosphorylate different substrates in various cellular compartments, including transcription factors in the nucleus. The MAPK cascade allows the transduction of the information to downstream targets. Four isoforms of MAPK were shown to be activated in alfalfa (*Medicago sativa*) seedlings exposed to Cu or Cd (Jonak et al. 1996), and a MAPK gene is also activated by Cd treatment in rice (Yeh et al. 2004). All these signaling pathways finally converge in the regulation of transcription factors that activate genes required for stress adaptation, particularly in the context of heavy metals.

11.3 Heavy Metal Toxicity-Induced Genes and Proteins and Its Identification

Heavy metal like aluminum (Al) inflicts a wide range of cellular injuries in plants that affect and reduced the root growth, nutrient and water uptake, and productivity. Duressa et al. (2010) reported that several genes might potentially have influence on

soybean aluminum tolerance. Two transcription factors, cell wall metabolism enzymes and a cell proliferation gene, are interesting from the perspective of the physiological and molecular mechanisms of Al tolerance. The first transcription factor, *Cys2His2* zinc finger protein, coregulates molecular response to proton and aluminum toxicities, the major acid soil stress factors (Luchi et al. 2007). The second transcription activator, *ADR6*, is an auxin downregulated gene. Aluminum suppresses the biosynthesis of auxins and transport in root system, which might be one possible mechanism of aluminum-induced root growth inhibition (Kumari et al. 2008). They also stated that *ADR6* is triggered under Al stress acting in a parallel pathway to restore root growth under Al stress. Root cell wall rigidification by Al binding is one of the principal mechanisms of Al toxicity. Contreras-Porcia et al. (2011) reported that 18 selected genes were copper responsive, half of the isolated genes encode putative organellar proteins, the identified proteins are principally involved in antioxidant metabolism and in cellular and organellar repair, and these proteins may act in a coordinated and additive or synergistic manner to ensure copper acclimation and tolerance. Metallothioneins are small cysteine-rich proteins (50–70 amino acids) and are known to play a role in detoxifying heavy metals by sequestration using cysteine residues (Bertini et al. 2000). Zhang et al. (2009) identified that 16 Cu-responsive proteins of low molecular weight will increase our understanding of plant mechanisms of Cu toxicity and tolerance. Two rice-seed embryo proteins, RicMT (OsMT2c) and CYP90D2, were among those most markedly affected by Cu treatment. The level of RicMT increased fivefold, whereas CYP90D2 decreased sixfold. Three proteins, a putative small cytochrome P450 (CYP90D2), a putative thioredoxin, and a putative GTPase, were downregulated by Cu stress. In plants, metallothioneins are thought to play an important role in metal tolerance and homeostasis (Cobbet and Goldsbrough 2002). They reported that C2 domain protein and LysM receptor-like kinases are more responsive to heavy metal stress and leaf senescence. Two possible pathways, i.e., senescence signals by the membrane-bound lysine receptor-like kinase 1 (LysMR 1) by interaction with specific factors and consequently activating downstream signaling cascades. The phosphorylation of kinase domain of the LysMR1 may activate a signaling protein such as a mitogen-activated protein kinase known to be induced during heavy metal stress or phosphatase to trigger a cascade to the nucleus and finally activate a target gene. In the second pathway, once heavy metals are in the cytoplasm, a redox signal may also be generated leading to the production of reactive oxygen species (ROS). It is known that reactive oxygen species such as H_2O_2 activate the membrane Ca^{2+} channels and mediate the influx or release of Ca^{2+} from internal stores. This generates an increase in Ca^{2+} concentration. In addition, ROS activate directly the mitogen-activated protein kinase cascade. Furthermore, the Ca^{2+} signal may activate a C2 domain protein and its Ca^{2+} binding triggers interaction with the transcription factor to form a protein complex and their translocation to the nucleus. Finally, the transcription factor interacts with its corresponding *cis*-acting element on target of the promoter to regulate gene expression (Fig. 11.2).

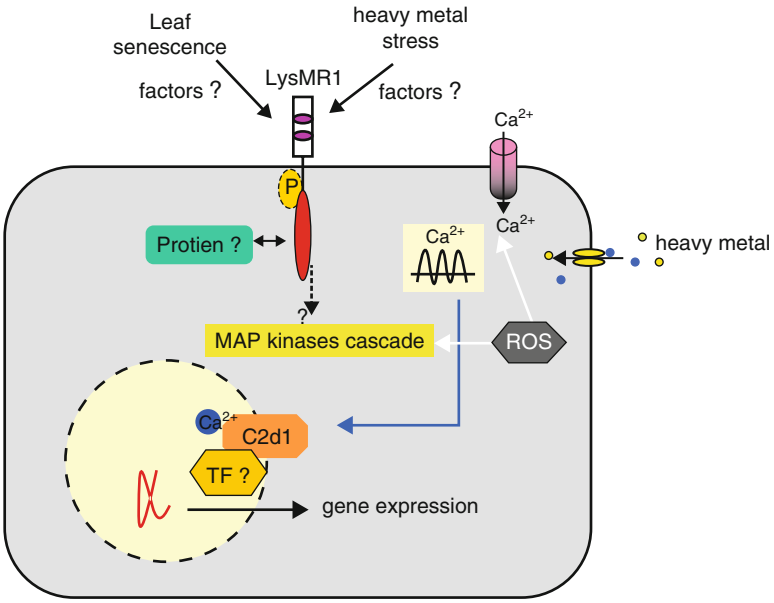

Fig. 11.2 Model for the functional role of LysMR1 and C2 domain protein in plant perception of heavy metal and senescence signals

11.4 Functional Genomics of Heavy Metal Tolerance in Plants: Approaches and Achievements

Crop plants are being constantly exposed to heavy metal toxicity; they respond differentially at hostile environments in concern with several abiotic and biotic stresses, the composition of ions and its concentration, plant habit, and developmental phase of plant growth. These plant responses mostly include a coordinated network of molecular processes such as reduced uptake or increased plant internal sequestration along with multiple metal-detoxifying mechanisms, overexpression of numerous stress-related proteins, glutathione-mediated tolerance pathways, repair systems, and cascade of signaling molecules; as a consequence, the plant became either heavy metal stress tolerant or stress susceptible. The intricacy of heavy metal tolerance response involves the set of molecular regulation, which need to be understood to yield heavy metal-tolerant plant. This would require analysis of the function of numerous genes involved in stress responses imparting tolerance by way of genomic approaches that would help to assign cellular function to each gene. Information about such stress-responsive genes has been obtained largely using conventional approaches. However, the challenge still remains to integrate the function of these genes logically to generate a global understanding of the stress response process (Bohnert et al. 2006; Valliyodan and Nguyen 2006; Vij and Tyagi 2007;

Sheldon and Roessner 2013). Integrating genome sequencing, transcriptomics, proteomics, metabolomics, ionomics, and phenomics allows large-scale gene function analysis with high-throughput technology and incorporates interaction of gene products at cellular and organism level. The complete genome sequence of rice and *Arabidopsis* and emerging sequence information for several other plant genomes, such as *Populus*, *Medicago*, lotus, tomato, pigeon pea, and maize, have enriched the genetic information to employ the tools for the determination of the function of many genes simultaneously (The Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005; Bell et al. 2001; Nakamura et al. 2002; Rensink and Buell 2005; Vij et al. 2006; Ranjan et al. 2012; Singh et al. 2012; Varshney et al. 2012). The use of in silico gene discovery, high-throughput gene expression, altered gene expression by transgenesis, functional characterization of genes of interest via gene-inactivation techniques, and genetic and genomic approaches in understanding the basis of abiotic stress tolerance has been initiated in several plant species (Vij and Tyagi 2007; Sreenivasulu et al. 2007; Sheldon and Roessner 2013). Although these functional genomic tools have been utilized for molecular depiction of abiotic stress response in various plant species, the exploitation of these tools is in its infancy for heavy metal response in plant species. Among the heavy metals reported, cadmium is considered the most phytotoxic and hazardous due to its high solubility, absorption by plants, and radical introgression into the food chain (Lux et al. 2010; Gill et al. 2011; Thapa et al. 2012). Hence, several studies were undertaken in expression genomics, proteomics, metabolomics, positional cloning, and ectopic gene expression and in depicting miRNA target to elucidate the molecular basis of cadmium tolerance in plants.

Gene expression is highly affected under cadmium stress and is up- or downregulated. Hence, the expression of a group of gene(s) during different stages reflects on functional relevance. Expression genomic approaches such as array-based transcript profiling technologies, serial analysis of gene expression (SAGE), differential display and subtractive hybridization, and quantitative real-time PCR (qRT-PCR) are employed to depict high-throughput expression of various genes in control and Cd stress-treated tissues at various developmental stages. It also hypothesized that various genes involve as key players of Cd tolerance, viz., Cd sensors, Cd-induced signaling protein kinases, aquaporins/transporters, *cis*-regulatory elements, transcription factors, hormone biosynthesis, etc., in different plants (Table 11.1).

Proteomics, the systematic analysis of stage-specific protein complements expressed by the genome, is a powerful tool for describing complete proteomes of the cell, cell organelle, and plant tissue (Porubleva et al. 2001; Agrawal and Rakwal 2006) and also for comparing proteomes under different stresses imposed by the environment on plant systems (Vij and Tyagi 2006). The combination of 2-DE and mass spectrophotometry (ESI and MALDI-TOF) proved to be very efficient for proteome characterization (Mann et al. 2001) and were recently upgraded with fluorophores (DIGE) and LC-MS for more precise characterization of proteome. All these techniques were used for proteomic studies in *Arabidopsis*, rice, barley, soybean, barley, and several medicinal plants (Table 11.2).

Table 11.1 Cadmium-responsive genes and transcripts identified using tools of expression genomics

Plants	Name of the gene/proteins	Regulator group	Functions assigned	Tools of expression genomics used	References
Arabidopsis thaliana and *A. halleri*	MT2A, MT2B, MT3	Metallothionein	Hyperaccumulation, sequestration, and ROS scavenging	cDNA microarray (comparative)	Chiang et al. (2006)
	WBC11	ABC transporter			
	P_{1B}-ATPase (HMA3, HMA4)	Efflux transporter			
	CDF (ZAT, MTPa12), IRT3, ZIP3, ZIP6, ZIP9, ZIP12, Nramp3, Nramp5	Uptake transporter			
	ZIP-9, EXP-17, CytP-450, CAM-9, curucilin like lectin, MYB-43,48,124, HSF7, ERF, endochitin, etc.	Protein kinase, metal transporter, calmodulin, energy metabolism, etc.	Hyperaccumulation through signal transduction, sequestration, and transportation to vacuole	qRT PCR and microarray (comparative)	Weber et al. (2006)
A. thaliana and *A. lyrata*	CLE-41, CXIP-4, and other 134 genes	Signaling (kinase), transcription regulators, and proteasome	ROS detoxification, cellular repair, metal sequestration, signal transduction, water transport, etc.	cDNA AFLP	Craciun et al. (2006)
A. thaliana	Cluster of genes for sulfur assimilation, glutathione metabolism, cysteine synthase, phytochelatin	Protein kinase, metal transporter, calmodulin, glutathione, oxidoreduction, etc.	ROS detoxification, cell wall metabolism, signal transduction, water transport, etc.	Whole-genome CATMA microarray, qRT PCR	Herbette et al. 2006
	Ca-dependent protein kinase, ERF2/ERF5				
	ACC oxidase synthase, calmodulin, etc.				
	IRT1 and 42 genes	Transporter	Transportation cross plasma membrane, NO synthesis, and Cd hyperaccumulation	qRT PCR and microarray	Besson-Bard et al. (2009)
	At PDR8	Efflux transporter and ABC transporter	Efflux pump at plasma membrane	qRT-PCR	Kim et al. (2007)

(continued)

Table 11.1 (continued)

Plants	Name of the gene/proteins	Regulator group	Functions assigned	Tools of expression genomics used	References
O. sativa	Ospdr9	ABC transporter	Cd stress tolerance by redox change	DD PCR and RT PCR	Moons (2003)
H. vulgare	HvlysMR1	Receptor-like protein kinase	Signal transduction and leaf senescence	DD RT-PCR, LC-ESI-MS	Ouelhadj et al. (2007)
	Hv C2-binding domain	Phytochelatin	Ca-dependent signaling, ROS detoxification, metal sequestration	qRT-PCR	Tamas et al. (2008)
	Phytochelatin synthase	Metallothionein metal transporter (uptake, efflux, and ABC)			
	Metallothionein, PIP (aquaporin), DHN1,4 (dehydrin), ascorbate peroxidase, glutathione peroxidase, DHAP				
Lotus japonicus	LjPCS2-7R (root)	Phytochelatin synthase	Phytochelatin synthase expression	qRT-PCR	Ramos et al. (2007)
	LjPCS-2-7 N (nodule)				
Lycopersicon esculentum	LePCS1	Phytochelatin synthase	Hyper-tolerance through sequestration and transportation to vacuole	Subtractive hybridization, RT PCR	Ouzoid et al. (2005)
	LeMT2	Metallothionein			
	LeNramp1,2,3	Nramp transporter			
Brassica juncea	19 genes (auxin-responsive GH3, ARF-like GTPase, ARD/ARD, APS reductase, NOP, catalase, zinc finger diacyl glycerol kinase and RAMP4)	Protein kinase, hormone biosynthesis	Dehydration stress signal transduction	Differential display	Dormer et al. (2000)
		Metallothionein, glutathione	Sulfur assimilation		
		Oxidoreduction, etc.	ROS detoxification		
			Metal binding		
	24 genes	Transcription factors	Hyper-tolerance	cDNA AFLP	Fusco et al. (2005)
		Transporters and photosynthetic process			

Solanum nigrum	Calmodulin-5,6,7,8	Calmodulin	Hyperaccumulation, vacuolar transport, ionic sequestration	Differential display	Xu et al. (2009)
	Ascorbate peroxidase like mRNA, catalase mRNA, glutamine synthase	Transporters			
		Cellular metabolism			
Solanum torvum	2,049 tag upregulated (HM chaperones, antioxidative and sulfur assimilation enzymes) and 2,022 tag downregulated (transcription factors and aquaporin); AtFRD-3	Xylem loading	Dehydration stress and ion sequestration through membrane transport	RT-PCR (EST library)	Yamaguchi et al. (2010)
		Transporters		Serial analysis of gene expression (SAGE)	
Datura innoxia	Four CD-specific cDNA	Sulfur transferase gene family	Replenishment of sulfate demand under Cd stress	RT-PCR	Louie et al. (2003)
Camellia sinensis	γ-Glutamyl cysteine synthetase, glutathione reductase, GSH	Glutathione	Oxidative stress	qRT-PCR	Mohanpuria et al. (2007)
			Vacuolar sequestration of metal ion		
Salix carpera	ScMT2B, ScMT3	Metallothionein	Metal homeostasis	cDNA and subtractive hybridization	Konlechner et al. (2013)
	ScSAT1	Serine-o-acetyl transferase	Metal perception signaling		
	ScHMAD1, ScMCT1, ScMT2A, ScZIP6, ScHMA1	Metal transporter/chelator			
	ScPMP1	Metal perception and signaling (kinase)			
	ScWalk1, ScRALFL	Alkalinization			

Table 11.2 Cadmium-responsive proteins identified using tools of proteomics and their putative functions

Plant (tissue)	Tools used	No. of proteins identified	Functions assigned	References
A. thaliana (leaf, thylakoid)	2-DE, MALDI-TOF	20 (up) and 15 (dn)	Proteins related to photosynthesis and energy metabolism; oxidative stress response; protein metabolism; sulfur assimilation	Bashir et al. (2011)
			(Retaining photosynthesis efficiency by stabilizing MCPs)	
A. thaliana (leaf, thylakoid)	2-DE, LC-ESI-MS	20 (up) and 15 (dn)	Photosynthesis and energy metabolism	Brahim et al. (2010)
			Oxidative stress response; protein metabolism	
A. thaliana (leaves)	2-DE, MALDI-TOF	25	Metabolic enzyme (ATP sulfurylase, glycine hydroxyl methyl transferase, trehalose 6-P-phosphatase, glutathione-s-transferase) Latex allergen proteins synthesis	Roth et al. (2006)
			Generation of internal link for sensing sulfur reduction	
O. sativa (roots)	2-DE	27	Carbon metabolism, chaperons, metal uptakers, and transporters	Kim and Lee (2009)
O. sativa (roots and leaves)	IPG 2-DE, MALDI-TOF	18 (root)	ROS scavengers (GST, APX, NADH-ubiquinone oxidoreductase) primarily upregulated to prevent oxidative stress damages	Lee et al. (2010)
		19 (leaf)		
O. sativa (root)	2-DE, MALDI-TOF	21	Transporter-like proteins promoting stress tolerance	Aina et al. 2007
B. juncea (leaves)	2DE/CID	17(up) and 23 (dn)	Photosynthetic gene products, energy metabolism, and Calvin cycle	D'Alessandro et al. (2013)
			Carbonic anhydrase regulated CO_2 homeostasis; RNA binding protein, plastid associated protein, chelating proteins, oxidative stress proteins, and chaperonins	
B. juncea (root)	2-D DIGE, iTRAQ	DIGE-102	O-Acetylserine sulfhydrylase, glutathione-s-transferase, and glutathione conjugate membrane transporter; Cd hyperaccumulation and tolerance	Alvarez et al. (2009)
	Nano LC-MS/MS	iTRAQ-585		
H. vulgare (leaf vacuoles)	iTRAQ, MALDI-TOF-TOF	56	Tonoplast intrinsic protein (CAX1 and ABC transporter) and it transports Cd into the vacuole and led to Cd detoxification of barley mesophyll cells	Schneider et al. (2009)

Glycine max (leaf and root)	IPG 2-DE, nano LC-MS-MS, MALDI-TOF	102 (leaf) 16 (root)	Activation of SOD, APX, and CAT ensures cellular protection from ROS-mediated damage; enhanced expression of molecular chaperones which stabilize protein structure and function, and cellular homeostasis	Hossain et al. (2012a)
G. max (leaf)	IPG 2-DE, nano LC-MS-MS, MALDI-TOF	78	High abundance of HSP-70 helps BABA primed protein folding to maintain normal protein functions; higher abundance of peroxidase indicated BABA potentiated antioxidant defense system to combat Cd stress	Hossain et al. (2012b)
G. max (root microsome)	2-DE, MALDI-TOF-TOF	22	Upregulation of proteins associated with Cd-chelating pathways and increased lignification of xylem vessels lead to low root-shoot translocation	Ahsan et al. (2012)
L. esculentum (leaves)	PG 2-DE, MALDI-TOF, LIFT-TOF-TOF	Low Cd-27	Low Cd activates proteins related to glycolysis, TCA cycle, and respiration	Rodriguez-Ceima et al. (2010)
		High Cd-33	High Cd inhibits the carbohydrate metabolism and respiration and enhanced production of detoxification proteins	
Phytolacca americana (leaves)	2DE, MALDI-TOF	25	Photosynthesis, sulfur and glutathione metabolism, gene expression, and mol. chaperones, 2-cys-peroxidase, oxido-reduction reaction	Zhao et al. (2011)
Kandelia candel (root)	2-DE, MALDI-TOF	53	Upregulated oxidative response, glutathione biosynthesis, enzymes of TCA and PPP cycle indicating prompt anti-oxidant response	Weng et al. (2013)
Catharanthus roseus (root/leaves)	2-DE, MALDI-TOF	19	Impair the photosynthesis process and photosynthetic function; protein biosynthesis, protein folding assembly, protein degradation, cell defense enzymes, redox homeostasis	Kumar et al. (2011)
Populus tremula (leaves)	2D-DIGE, MALDI-TOF-TOF	52 (up) and 73 (dn)	Deleterious effect on protein expression for primary carbon metabolism and oxidative stress response	Keiffer et al. (2008)
			Proteolysis, protein folding, pathogen-related protein, carbon metabolism, photosynthesis, glutathione metabolism, ascorbate and glutamine biosynthesis, oxidoreductase action, lignin biosynthesis, ATP synthase coupled protein transport, hormone (auxin) synthesis, riboflavin metabolism	

Table 11.3 Depiction of the function of cadmium-responsive genes through ectopic gene expression and insertional mutagenesis

Plant	Gene and gene product	Salient finding	References
Transgenic approaches (ectopic gene expression)			
A. thaliana	At APR1 (APS reductase)	Higher tolerance to cadmium	Sakulkoo et al. (2005)
	Hv APX1 (peroxisomal ascorbate peroxidase)	Higher tolerance to Cd stress and more accumulation of Cd	Xu et al. (2008)
	YCF1 (vacuolar transporter)	Pump heavy metals into vacuoles	Tong et al. (2004)
	AtPCS1 (phytochelatin synthase)	Cd detoxification	Lee et al. (2003)
	Os MSR3 (HSP)	Higher tolerance to Cd, bHLH transcription factor expression	Cui et al. (2013)
		More accumulation of phytochelatin, non-proteinaceous thiol, and Glutathione	
Nicotiana tabacum	CUP1 (yeast metallothionein)	ROS scavenging	Krystofova et al. (2012)
		Cd accumulation in root	
	Pv SR2 (stress-related protein)	Higher tolerance of Cd through accumulation and transportation	Xu et al. (2008)
Populus davidiana x P. bollena hybrid	Ta LEA1 (unknown)	Higher DOD, POD, ROS scavenging; lower malondialdehyde leading to higher Cd stress tolerance	Gao et al. (2012)
Insertional mutagenesis approaches (gene knockout)			
A. thaliana	Sn RK2s (SNF1-related protein kinase-2)	ABA-dependent and ABA-independent pathways for osmotic stress response	Fujii et al. (2011)
	At HSP90-3	Cd tolerance by decreased content of phytochelatin and glutathione; inhibited activities of SOD, CAT, and POD; and increased content of malondialdehyde	Song et al. (2012)
O. sativa	LCD (low cadmium)	Cd transport	Shimo et al. (2011)
N. tabacum	Sn RK2s (SNF1-related protein kinase-2)	ROS accumulation	Kulik et al. (2012)

The cadmium-responsive genes are mostly categorized into seven groups such as sensing and signaling, osmolyte biosynthesis, antioxidant protectants, protection of cell metabolism, metal transporter, ion homeostasis, and hormone biosynthesis (Maksymiec 2007; Thapa et al. 2012). Research in genetic transformation for cadmium stress tolerance was limited in the pre-genomics era by the inadequate availability of genes and specific promoters (Zhu et al. 1997). Several studies were made pertaining to gene function assignment for cadmium stress in plants either by overexpression (Sakulkoo et al. 2005; Krystofova et al. 2012; Cui et al. 2013) or suppression of gene expression by gene knockout and gene trap systems (Shimo et al. 2011; Kulik et al. 2012; Song et al. 2012) in three model plant systems, viz., *A. thaliana*, *O. sativa*, and *N. tabacum* (Table 11.3).

It is now possible to study the expression of many genes simultaneously on a genome-wide scale using plant artificial chromosome vectors with respect to their structure and function (Tyagi et al. 2006). Thus, the present trend in cadmium stress biology is to use large-scale genomic data to scrutinize and revalidate the osmolyte biosynthesis, antioxidants, LEA proteins, molecular chaperones, cell membrane proteins, aquaporins and transporters, ion homeostasis, and transcription factors involved in Cd stress tolerance based on transgenic (overexpression and insertional inactivation) approaches (Sreenivasulu et al. 2007). In addition to overexpression and insertional inactivation, another group of tools known as "RNA interference (RNAi)" is quite promising on the depiction of gene to function assignment of complex biological processes even in the regulation/signaling of heavy metal stress response (Jones-Rhoades et al. 2006; Mendoza-Soto et al. 2012; Ding et al. 2013). Among the RNAi tools, microRNAs (miRNAs) are a group of endogenous non-protein-coding small RNAs of 21 nucleotides. These miRNA genes are originated from hairpin precursors by DICER-LIKE1 (DCL1) in plants (Reinhart et al. 2002; Jones-Rhoades et al. 2006). These miRNAs negatively regulate the post-transcriptional processes and translation of specific mRNA targets through RNA-induced silencing complex (RISC) in cells (Bartel 2004). Majority of miRNAs are conserved across the species making it possible to identify putative miRNAs in other species using comparative genomics (Ding et al. 2013). Recently, it has been reported that miRNAs act as crucial regulators of multiple physiological processes, including plant development, signal transduction, and adaption to heavy metal stresses (Sunkar et al. 2006; Zhou et al. 2008, 2012; Huang et al. 2009; Mendoza-Soto et al. 2012; Hartwig 1995, Jagadeesan et al. 2010). Some of the studies on cadmium stress regulatory microRNAs in *O. sativa* (Huang et al. 2009, Ding et al. 2011, 2013), *B. napus* (Zhou et al. 2012), and *M. truncatula* (Zhou et al. 2008) and the putative stress-related functions were annotated (Table 11.4).

With the advent of DNA markers, two major approaches have been used in exploiting the gene pool for imparting cadmium stress tolerance: first, identification of stress-tolerant genes via functional genomic approaches and introduction of stress-tolerant genes into crops of interest and second, identification of DNA markers flanking the QTLs or co-segregating with the genes conferring tolerance to cadmium stress and use in marker-assisted breeding programs (Panigrahi et al. 2013). Although by using functional genomic approaches, regulatory pathways involved in abiotic stress response have been dissected and shown to enhance abiotic stress tolerance in laboratory conditions by activating stress-responsive signal transduction and downstream transcription factor genes in transgenic plants, its success in field conditions are rather poor. Hence, it is equally important to integrate developed knowledge as an outcome of functional genomics into conventional breeding programs via genomic-assisted breeding to develop stable populations conferring both stress tolerance and yield (Sreenivasulu et al. 2007). In this respect, several genes and QTLs responsible for component traits of cadmium stress tolerance have already been identified (Table 11.5), which may be used for molecular breeding programs in the near future. More recently, a concept known to be "genetical genomics" has been developed in combining the advantage of gene expression

Table 11.4 Cadmium-responsive miRNAs and prediction of their target gene function by annotation and/or overexpression in plants

Plants	mi RNA ID	Target gene/function	References
A. thaliana	miR398b	Cytosolic CSD1 and chloroplastic CSD2 (oxidative stress tolerance)	Sunkar et al. (2006)
O. sativa	miR390	TAS3	Ding et al. (2011)
	miR156	SBP transcription factors	
	miR167	Auxin-responsive factors (ARFs)	Huang et al. (2009)
	miR118	Heat shock factor protein-2	Ding et al. (2013)
	mir59	Cadmium tolerance factor, OsWAK45 receptor-like protein kinase, OsWRKY10 superfamily of rice TFs, Ras-related protein RHN1	
	miR1004	Glutathione-s-transferase GSTU6, vacuolar protein sorting protein-72, serine/threonine protein kinase 19	
	miR361	Ubiquitin protein ligase	
	miR1060	Cytochrome P450 74A4	
	miR192	AT binding cassette subfamily2, CPRF-2, F-box domain containing protein, cysteine protease, ubiquitin protein ligase, zinc finger C3HC4-type protein, IAA amino acid hydrolase, etc.	
B. napus	miR156	SBP transcription factors, glutathione γ-glutamyl cysteinyl transferase-2, serine/threonine protein kinase Nek-3	Zhou et al. (2012)
	miR159	ABC transporter, auxin response factors	
	miR164	Monothiol glutaredoxin-S12, chalcone synthase, transcriptional factors	
	miR166, 167	WRKY transcription factor 21, Nramp-1	
	miR168	Cation homeostasis, Ap2-like TFs	
	miR172	AP2-like TFs, ERFs	
	miR396	Ulp1 protease family protein, BHLH TFs, growth regulatory TFs	
Medicago truncatula	miR319	TCP transcription factor	Zhou et al. (2008)
	miR171	SCL transcription factor	

profiling and marker-based fingerprinting of related progenies in a segregating population to analyze *cis*- and *trans*-acting factors and to delineate a trait-related genetic network (Jansen and Nap 2001; Jansen 2003; Sreenivasulu et al. 2007; Kovalchuk et al. 2005; Krämer et al. 1996). In the future, the integration of informations obtained from functional genomic approaches with conventional breeding will hasten the success for various quantitative traits including heavy metal tolerance in general and cadmium tolerance in particular.

Table 11.5 Cadmium-responsive gene(s) and QTLs identified for genomic-assisted breeding

Plant	Gene/QTL (Population)	Trait associated	Markers used	Percentage variation	References
A. thaliana	*RML1*/*CDS2*	γ-Glutamyl cysteine synthetase	Positional cloning	–	
	CAD1	Phytochelatin synthetase	Positional cloning	–	Ha et al. (1999)
A. halleri	*q Cd tol-1,2,3* (BC1)	Cadmium tolerance (cosegregate with HMA4)	*A. thaliana* anchored markers, AFLP	43, 24, 16	Courbot et al. (2007)
O. sativa	*qCDS7*, *qCDR6.1*, *6.2* (DH)	Cd tolerance and accumulation shoot (s) and roots (r)	RFLP, SSR	12.55 12.41 11.72	Xue et al. (2009)
	qcd1, *qcd2*, *qcd3* (RIL)	Cd content	RFLP	9.7, 12.4, 22.7	Norton et al. (2010)
	qcd7 (CSSLs)	Cd concentration	RFLP	–	Ishikawa et al. (2005)
	qGCd7 (RIL)	Grain Cd concentration	RFLP, SSR	35.5	Ishikawa et al. (2010)
Triticum turgidum L. var. *durum*	*qCdU1* (DH)	Grain Cd concentration	STS, ESM	–	Wiebe et al. (2010)
G. max	*Cda1* ($F_{2\text{-}3}$ and RIL)	Seed Cd concentration (colocalize with genes controlling protein kinase, putative adagio-like protein, and plasma membrane H^+-ATPase)	SSR	57.3	Souframanien et al. (2010)
Avena sativa	*Qtl1* (F_2)	Grain cadmium accumulation	RAPD, REMAP, SRAP, SCAR	–	Tanhuanpaa et al. (2007)
Populus deltoides	*qCd1*, *qCd2* (pseudo-backcross pedigree)	Cd tolerance[a]	AFLP, SSR	5.9–11.6	Induri et al. (2012)

[a]Whole genome microarray analysis led to the identification of nine Cd stress-responsive genes (NHL repeat membrane protein, metal transporter, and transcription factors)

11.5 Future Strategies

During the last couple of decades, large amount of data have been accumulated on plant's responses to heavy metal stress, both at the genome and proteome levels. Data mostly include a plethora of regulatory proteins like transcription factors and signaling molecules associated with heavy metal tolerance and its related genes, which have helped to understand the molecular mechanisms associated with plant

survival and crop yields under stressed conditions. But there is an intriguing amount of cross talk and interconnections that are involved in heavy metal stress signaling (Thapa et al. 2012; DalCorso et al. 2010), such as *trans*- and *cis*-acting regulatory elements, and interdependent miRNA-based post-transcriptional and translational gene regulation including alternative splicing and cryptic splicing.

In this review chapter, a large number of functionally characterized genes, either by overexpression or by knockout studies, were introduced into crop plants to provide higher tolerance against heavy metal stress under laboratory conditions in model plant species. It is evident from research outcomes that extensive work is imperative in several fronts including transgenic approaches. In most cases, constitutive expression of stress-tolerant genes is likely to cause unwanted effects. Therefore, it is highly desirable to achieve development and tissue-specific stress-responsive expression of the transgenes by identifying specific promoters. In this regard, genomic tools identified a number of stress-inducible promoters, which can be tested for specificity. Such promoters could also be used to prevent gene silencing when gene pyramiding is sought as a feasible strategy to obtain higher tolerance levels. It has also been reported that plants exhibited higher accumulation of osmolytes, upregulation of an array of stress-related proteins (HSPs, proteinases and PR proteins, and glutathione metabolism-related proteins), and signaling proteins (receptor kinases) upon heavy metal stress that suggest the involvement of diverse networks of stress pathways ultimately leading to metal tolerance.

Due to lack of integrated and coordinated approach on extensive field, tests in the pace of scientific development in genomics, transcriptomics, and proteomics were not done so far. Therefore, we are yet to develop varieties that can overcome the heavy metal constraints and perform better either by tolerance or by phytoremediation. A large number of genomic resources are now available in the form of well-catalogued annotated genome sequence and easily accessible databases. The outcome effort will not only reveal gene function but will also identify the effective combination of genes (Tyagi et al. 2006). Genome-wide strategies have been accelerated by deciphering complex stress-responsive networks and will also help in the identification of key networks and their associated genes, which may be manipulated through either genomic-assisted breeding strategies or genetic engineering. However, there is very less effort paid on the exploitation of marker-aided breeding due to quantitative control of heavy metal stress in plants. Hence, no markers/genes have been identified to quantify the plant under heavy metal stress. There is a sanguinity that the use of recent approaches of functional genomics and genomic-assisted breeding will definitely ameliorate the heavy metal stress tolerance and could generate valuable information for engineering stress-tolerant plants for their ultimate use in sustainable agriculture. On the basis of the foregoing discussion, a strategy has been proposed by integrating tools of system biology, functional genomics, and genomic-assisted breeding to improvise our future effort on the discovery of genes and pathways for the elucidation of heavy metal stress response (Fig. 11.3).

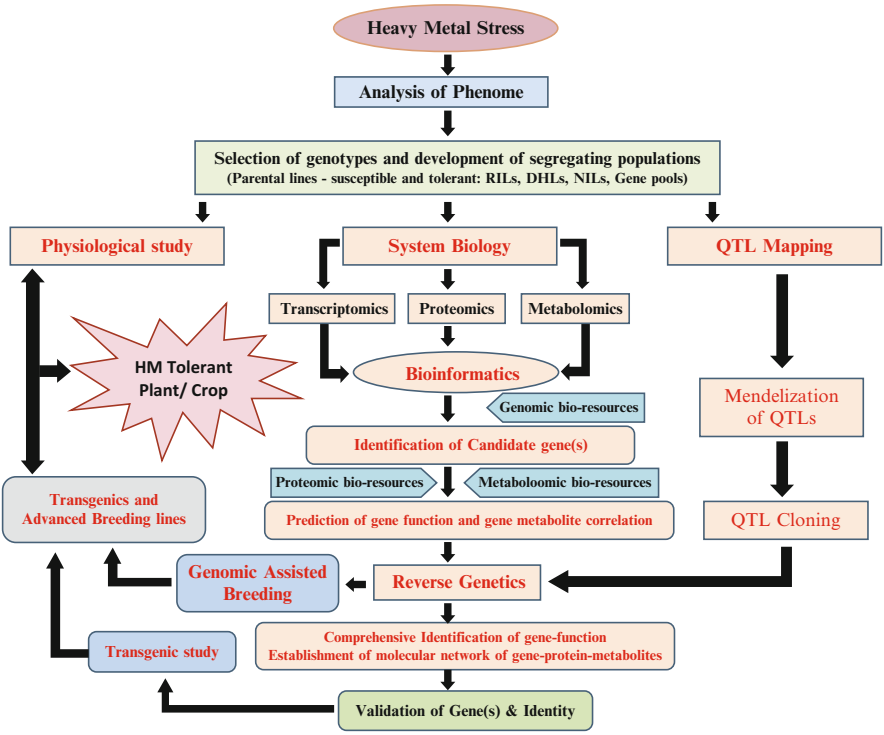

Fig. 11.3 Proposed strategy for development of heavy metal-tolerant plants by integrating functional genomics and genomic-assisted breeding

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Chapter 12
Nitrogen and Stress

Annie P. Jangam and N. Raghuram

Abstract Nitrogen is an essential macronutrient for the plants and fertilizer N-use efficiency is becoming an increasing economic and environmental concern. The nutrient stress conditions of N deficiency and N excess may get exacerbated by other abiotic stresses, which in turn are likely to be worsened by climate change. Exploring their interrelationships is being increasingly facilitated by the growing knowledge of the genome-wide N response as well as other abiotic stress responses in model plants. Nitrate and its more reduced forms are not only sources of plant N nutrition but also signals that govern their own uptake; N, C, and redox metabolism; and hormonal and other organism-wide responses. The signaling mechanisms involved in N response or response to N stress or N-use efficiency are currently far less well understood than those in other abiotic stresses. The purpose of this review is to provide an overview of the current state of knowledge on normal N response and response to N stress, as well as their interrelationships with other abiotic stresses.

Keywords Nutrient • Nitrogen • Nitrogen use efficiency (NUE) • Plants • Stress • Signaling • Integrated nutrient management • QTL • Hormones • Fertilizer management • Climate change

Nitrogen is an important macroelement for plant growth. However, plant can't use atmospheric nitrogen as such and depend on its availability in more reactive forms such as urea, nitrate, ammonium, amino acids, etc. Even legumes depend on symbiotic N-fixing bacteria to convert N_2 into ammonium ions to meet their N requirements. Therefore, the term nitrogen (N) is used in this review to represent a broad range of reactive species of N compounds. In agricultural soils, N compounds and other nutrients have to be constantly replenished as fertilizers/manures to enable repetitive cropping. As N fertilizers are expensive, N-use efficiency becomes an important determinant of crop productivity.

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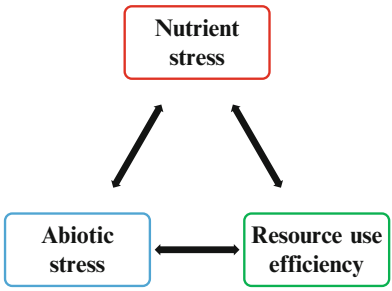

Fig. 12.1 Relationship between nutrient stress, abiotic stress, and resource-use efficiency

Since precision farming techniques to balance the plant nutrient demand with fertilizer supply are not accessible/affordable to farmers in developing countries, the actual availability of N to the crop plant varies from N-deficient state to N-excess state, depending on prevailing fertilizing practices. Both these states can cause nutrient stress to the plant. In addition, loss of reactive N species from the soil–plant system causes widespread environmental stresses, not only through N pollution of ground water and surface water bodies affecting health, biodiversity, and ecosystem services but also through air pollution and climate change (Sutton and Bleeker 2013). In fact, N_2O as a greenhouse gas is 300 times more potent than CO_2 (Galloway et al. 2008), though carbon dominates the entire climate change discussion. Climate change itself causes/exacerbates abiotic stresses to the plant and its N status (Fig. 12.1).

Nitrogen-use efficiency is therefore not only an economic problem of optimizing input costs to the farmer but also an environmental problem of preventing accumulation of reactive N species outside the agroecosystem. Accordingly, the relationship between plant, nitrogen, and stress is twofold: nutritional stress in terms of plant growth/development/productivity due to variation in N availability or climate and N-induced environmental stress (and climate change that in turn affects the plant), which can be exacerbated due to N-inefficient cultivars and/or practices. The primary focus of this book chapter is nutritional stress, but N-use efficiency is a common concern that links both nutritional and environmental aspects of reactive nitrogen.

12.1 Nitrogen Nutrition and N-Use Efficiency

Nitrogen-use efficiency has been defined in many different ways by agronomists, physiologists, and others (Good et al. 2004; Pathak et al. 2008; Hirose 2011). The simplest among them is total biomass or grain yield per unit N fertilizer added (or N available in the soil). Improvement in NUE is possible to some extent by non-plant interventions such as the choice of fertilizer and method/timing of its application and other crop management practices. But only biological avenues for crop improvement are the main focus of this article, whether in relation to NUE or stress resistance. Plants show improved NUE in N-limiting conditions (Kant et al. 2011). In other words, a plant that gives the same or higher agronomic output with lesser N input is considered more N-use efficient than the plant that needs higher N input. But what constitutes

yield, varies from crop to crop, such as grains in cereals or leaves/fruits/tubers in vegetables, and accordingly what constitutes NUE and how to improve it, in each case (see Chardon et al. 2012 for a recent review). This is also often true for stress resistance, if it is measured in terms of the impact of stress on yield, keeping in view the multiplicity of stresses involved. Unfortunately, many of the high-yielding and/or upmarket varieties of the green revolution era were neither designed for N-use efficiency nor stress resistance, whereas the farmers selected traditional cultivars that were more robust to such factors, even if they yielded less. This means that crop improvement strategies for NUE and stress resistance cannot be limited to the narrow germplasm of high-yielding varieties and have to include the wild/traditional varieties.

It is well known that NUE is an inherited, multigenic, quantitative trait. A study of natural variation of N uptake and metabolism in 18 accessions of Arabidopsis under high- and low-N conditions showed that while plants may vary in the way they respond to high- or low-N conditions, their NUE remained similar, indicating that NUE as a trait is exclusively genetically determined (Chardon et al. 2010, 2012). However, the multiplicity of the definitions of NUE, combined with its poor biological characterization at the phenotypic or genotypic level makes it difficult to study the impact of stress on NUE. Nevertheless, any discussion on nitrogen and stress has to be understood in terms of the impact of abiotic or nutritional stress on NUE. For example, elevated CO_2 could enhance NUE in some cases (Shimono and Bunce 2009), while heat or water stress could adversely impact NUE (Harrigan et al. 2009), as does wasteful use of N fertilizer. Understanding the various mechanisms underlying these complex interactions will equip us with the means to maintain crop productivity in a changing climate.

12.1.1 Nitrogen Uptake and Metabolism

Plant nitrogen (N) nutrition is a complex and dynamic process, as the plant has to be able to assimilate various forms/amounts of nitrogen in fluctuating micro- and macroenvironments. This can happen even in fertilized soils, depending on the soil N status, nature of the fertilizer used (organic/inorganic), the frequency of their application, and the action of nitrifying bacteria in the soil. Organic N sources (manures/urea) are broken down by nitrifying bacteria to inorganic compounds such as nitrates and ammonium salts, which are the preferred forms of N uptake for most plants, though amino acids can be used under extremely N-poor and cold conditions.

Plants uptake nitrate primarily through the high-/low-affinity transport systems (HATS/LATS) in the roots and are mostly inducible by nitrate and regulated by its downstream metabolites, hormones, stress, etc., except the constitutive HATS (Tsay et al. 2007). The signaling mechanisms involved in the nitrate regulation of NO_3^- transporter genes need to be elucidated fully, although few genes in Arabidopsis (NRT1.1, NLP7, and CIPK8) have been suggested to play a crucial role (Castaings et al. 2009). NRT1.1 is termed "transceptor" since it is both a transporter as well as a receptor for N signal (Gojon et al. 2011). There are also transporters for ammonium and urea, but their relative contribution to the overall external N acquisition by

plants and their regulation are far less understood, despite the growing interest (Vert and Chory 2009; Näsholm et al. 2009; Bouguyon et al. 2012; Wang et al. 2012). Some aspects of N transport under situations of stress have been mentioned later in this chapter, but one can expect a lot more activity in this area in the coming years.

The N compounds taken up by the roots are distributed throughout the plant, where they enter the cellular nitrate assimilatory pathway. Nitrate is reduced in the cytosol by the enzyme nitrate reductase to nitrite, which is transported to the chloroplast, where it is further reduced to ammonium ions by nitrite reductase. The ammonium ions are incorporated into organic acids to form amino acids, through the glutamine synthase (GS-GOGAT cycle) and various transaminases. This is the primary N metabolic pathway in plants, but secondary N remobilization can occur during senescence, in which cytosolic isoforms of GS and GOGAT also play important roles (Xu et al. 2012). Secondary N remobilization to recycle nutrients from senescing leaves could be of critical importance for grain filling and yield in cereal crops (Kichey et al. 2007). Signaling mechanisms play a key role in N metabolic regulation to optimize the N budget under varying situations of plant N demand and supply.

12.1.2 Signaling in N Metabolic Regulation

Nitrate is not only a nutrient but also a signal for plant metabolic regulation, growth, and development (Stitt 1999). Other forms of N such as ammonium and glutamine have also been shown to have signaling roles, but nitrate remains the best-studied form of N signal, both at the local and systemic levels. Locally, it affects root growth, seed dormancy, and flowering time, but also has systemic effects on an organism-wide basis (Ho and Tsay 2010). For example, nitrate has been shown to induce genome-wide changes in gene expression in model plants such as Arabidopsis (Wang et al. 2003; Scheible et al. 2004), rice (Lian et al. 2006; Cai et al. 2012), maize (Trevisan et al. 2011), and tomato (Wang et al. 2001), involving hundreds, if not thousands, of genes. They include various nitrate transporters, enzymes of nitrate assimilation, carbon and redox metabolism, several protein kinases, cytochrome families, transcription factors, etc. The search for nitrate response elements (NREs) in the upstream sequences of a few nitrate-responsive genes has not yet produced a universally accepted consensus sequence that accounts for most, if not all nitrate-responsive genes found to date (Das et al. 2007; Konishi and Yanagisawa 2010, 2011; Pathak et al. 2011; Krapp et al. 2014). Similarly, several transcription factors are implicated in mediating nitrate response, such as ANR1, DOF1/2, LDB37/38/39, NLP6/7, and SPL9 (reviewed in Krapp et al. 2014), and further research is needed to narrow them down.

It is believed that the combinatorial action of local and systemic signaling determines the ability of a plant to adapt to fluctuating environments (Krouk et al. 2011; Alvarez et al. 2012; Huang et al. 2012). However, the mechanism of nitrate signaling continues to evade scientists for several decades. Signaling is also involved in stress response, whether due to N deficiency/excess or due to other abiotic stresses, impinging on N metabolic regulation. The pathways for stress and nutrient signaling

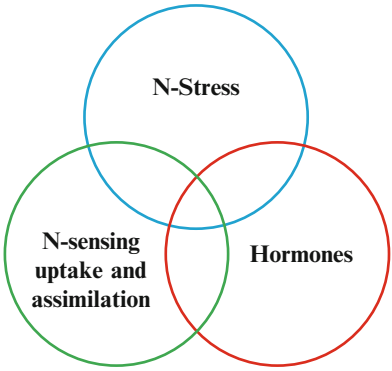

Fig. 12.2 Overlap between N stress, hormones, and N sensing, uptake, and assimilation

may either be separate or shared, depending on the specific stress in question, which will further define whether manipulation of one will impact the response to the other (Fig. 12.2). This is an issue of crucial agronomic relevance, which can only be addressed when the signaling mechanisms connecting N metabolic regulation and stress are better elucidated. Some developments in this regard are elaborated below.

12.2 Nitrogen and Stress

Nitrogen stress is caused by extreme fluctuations in the soil N level or due to the formation of nitroso compounds in the plant as a consequence of other stresses. The normal intracellular nitrate concentration is in the micromolar range and soil N concentration up to multi-millimolar range fall within the nutritional range (and therefore also the tolerance range) of most plants. N-limitation or N-deficiency stress occurs when soil N levels fall below the sub-millimolar range, eventually leading to N starvation. N-excess conditions require N levels to increase beyond 40 mM, though the precise threshold varies depending on the plant, duration of exposure, soil type, organic content, microbial activity, cropping practices, and climate.

Plants respond in many different ways to changes in N provision (Krouk et al. 2010; Kraiser et al. 2011; Kant et al. 2011). Their responsiveness to N availability depends on both genotype and the interaction of genotype with N fertilization level (Gallais and Hirel 2004; Chardon et al. 2010). They can adjust their molecular machinery in accordance with N nutritional status or abiotic stress, often rapidly and sometimes indefinitely (Daniel-Vedele et al. 2010). For example, plants respond to N starvation or deficiency by changes such as increase in the root to shoot ratio by enhancing lateral root growth or suppressing shoot growth or early senescence of leaves (Marschner 1995). Characterization of the machinery responsible for N homeostasis in stress helps to identify appropriate sites of intervention for crop improvement. This machinery includes, but is not limited to, the affinity-based N transport systems such as LATS and HATS, which can be reprogrammed to achieve

N homeostasis at the local or whole plant level. Several studies have shown that the root uptake capacities for nitrate, ammonia, and urea are strongly downregulated under conditions of N excess and upregulated during N starvation or N limitation (Tsay et al. 2007; Nacry et al. 2013). However, going beyond transporters, it seems that the limiting steps in plant N metabolism are different under high and low N levels (Coque and Gallais 2006). Moreover, while studies that report "high" N often do not make clear distinction between N sufficiency and N excess, studies that report "low" N either deal with N limitation or N starvation but not both, making it necessary to discuss them in their own separate context.

12.2.1 Nitrogen Starvation/Limitation

Nitrogen-starved plants show poor development with shunted growth; chlorosis; reduced photosynthesis; poor yield; poor pigmentation due to carbohydrate accumulation, anthocyanin induction, and phenylpropanoid biosynthesis. Studies in which nitrate was supplied to nitrate-starved plants like Arabidopsis and rice (Wang et al. 2000, 2003; Scheible et al. 2004; Lian et al. 2006; Cai et al. 2012) showed the involvement of genes from N/C metabolism, redox metabolism, hormonal response, etc. Many gene families such as cytochrome, protein kinases, and hormone/nutrient transporter were differentially regulated by nitrate in both rice and Arabidopsis (Cai et al. 2012). Various N transporters such as NRT2.1 and NRT2.2 for nitrate; AMT1.1, AMT1.2, and AMT1.3 for ammonia (Tsay et al. 2007); and DUR3 for urea (Kojima et al. 2007) were differentially regulated by N source/availability/concentration. The expression of GLN and GDH genes (Masclaux-Daubresse et al. 2005) were also altered during N starvation. The possible role of NLP7 as a key regulator in N-starved conditions has been suggested recently (Marchive et al. 2013). Studies in Arabidopsis and maize have shown that chronic N limitation elicits a genome-wide response and the genes involved are far more differentially regulated than genes supplied with sufficient N (Bi et al. 2007; Wu et al. 2011).

In the low concentration range such as 1 μM, high-affinity transport systems (HATS) are able to scavenge ions from the soil. During N starvation or limitation, NRT1.1 represses lateral root in Arabidopsis by remobilizing auxin, mimicking the role of an auxin transporter (Krouk et al. 2010). Low concentrations of ammonia have also been known to strongly regulate nitrate transport systems. NRT1.1 mutant studies have shown that a protein kinase CIPK23 phosphorylates NRT1.1 during nitrate limitation, thereby influencing primary nitrate response (Ho et al. 2009). The rate of N uptake in roots is determined by ionic concentration (Tsay et al. 2007), which is influenced by various stress conditions (Segonzac et al. 2007). Recently, it was reported that in maize, N-deficiency stress resembled the response of plants to a number of other biotic and abiotic stresses, in terms of transcript, protein, and metabolite accumulation (Amiour et al. 2012). In rice, two proteins, fibrillin and hairpin-binding protein, have been identified previously as N-deficiency stress-responsive proteins (Song et al. 2010; Amiour et al. 2012).

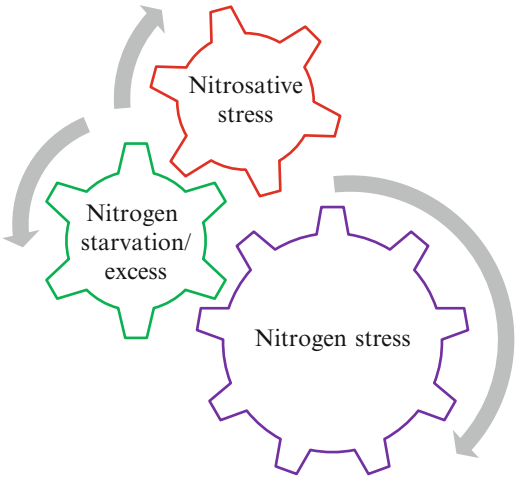

Fig. 12.3 Nitrogen stress caused by nitrogen starvation/ excess and nitrosative stress

12.2.2 Nitrogen Excess

In natural and in well-managed agricultural soils, excess N concentrations are rarely found for long, due to microbial conversions, surface runoff, volatilization, or leaching, apart from plant uptake. For example, although urea application in excess of 100 kg/ha is very common in intensively cultivated areas, its effective concentrations are often <70 mM in agricultural soils (Wang et al. 2008). Reaching far higher concentrations that contribute to nonspecific osmotic stress effects is only possible when other solutes are also high, such as in saline soils. This is also true for the conversion products of urea, viz., nitrate/nitrite/ammonium, whose ionic effects saturate in the millimolar range, and they rarely reach 100-fold levels needed to have any osmotic effect on their own. However, they can influence the pH of the soil temporarily, though the extent and duration of that influence on the soil as well as on the plant depend on the soil type/conditions and the plant itself. In any case, the ionic/pH/osmotic effects are indirect and generic effects that are not specific to N and therefore cannot be strictly considered as N stress. Terms such as N "sufficiency" or "excess" or "high N" have to be understood in this context, as they are often used interconvertibly, mainly to contrast with N limitation/starvation (Fig. 12.3).

Genes from transporter families have shown altered gene expression during chronic N stress probably because plants need to adjust to the varying levels of N available to plants. In the high concentration range, the activity of low-affinity transport systems (LATS) from the large family of transporters for nitrate (NRT1) and peptides (PTR) plays a major role (Tsay et al. 2007). Unlike with the HATS, the LATS-mediated nitrate or ammonia uptake (or influx) does not saturate and shows a generally linear increase with increasing external concentration (Touraine and Class 1997; Nacry et al. 2013). But they could accumulate in plant cells, unless their assimilation can match the uptake. This necessitates a mechanism to regulate

cytosolic nitrate concentration, which is provided by a nitrate-inducible efflux system that prevents excessive accumulation of nitrate in the cell (Miller et al. 2007). An efflux transporter, NAXT1, was recently identified belonging to the NRT1/PTR family of transporters (Segonzac et al. 2007; Chapman and Miller 2011).

Plants also have a capacity to store N in vacuoles as a way of balancing between uptake, assimilation, and translocation to other parts of the plant and minimize losses through efflux/volatilization. Some vegetables grown under excess N conditions have a tendency to accumulate N (Chen et al. 2004; Anjana et al. 2007) that enters our food chain. There is a growing attention toward the adverse health effects of excessive dietary exposure to nitrate and other forms of reactive N, including methemoglobinemia, gastric cancer, respiratory ailments, cardiac disease, etc. (Townsend et al. 2003; Anjana and Iqbal 2007).

12.2.3 Nitrosative Stress

Reactive nitrogen species (RNS) include NO and related molecules such as *S*-nitrosothiols (SNOs), *S*-nitrosoglutathione (GSNO), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and nitrogen dioxide (NO_2). During adverse environmental conditions, these molecules can cause stress to plants, which is designated as nitrosative stress (Fig. 12.3). This can combine with the stress caused by reactive oxygen species (ROS) to form nitro-oxidative stress in plants. Like ROS, the role of RNS signaling has been implicated in many abiotic stresses such as salinity, water stress, temperature stress, and UV radiation. Evidence for RNS signaling in certain abiotic stresses like salinity and heat stress was strictly species or treatment specific, and the literature is inconclusive, if not contradictory (Corpas et al. 2011). In other stresses such as UV-radiation and ozone stress, there is a marked increase in the activity of RNS species, which leads to cell death (Corpas et al. 2011). The role of RNS signaling needs to be studied further to understand the changes in plant physiology under stress.

12.3 Nitrogen in Abiotic Stresses

Nitrogen availability depends on plant–soil–microbe interactions, whereas nitrogen acquisition is basically driven by transpiration, which is in turn affected by temperature and levels of CO_2. Climate change models predict that elevated levels of green house gases (GHGs) like CO_2, CH_4, and N_2O cause increased variability in temperature, humidity, precipitation, wind velocity, and photosynthetically active radiation, all leading to major abiotic stresses such as drought, heat/cold stress, waterlogging, etc. (IPCC 2007; Bloom et al. 2010). All these stresses affect plant phenology and also alter nitrogen availability and its uptake/retention (Fig. 12.4). For example, Borner et al. (2008) observed that the snow depth in

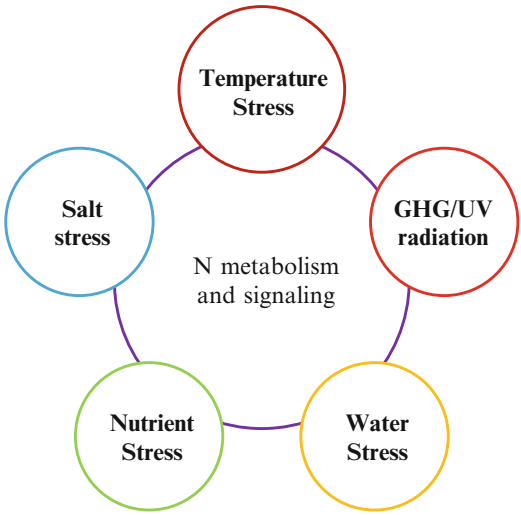

Fig. 12.4 N metabolism and signaling play an integral role in various abiotic stresses

tundra regions affect N mineralization directly. Volatilization of reactive N from the plant further contributes to GHG accumulation, completing the vicious cycle. The role of reactive N in plant stress can be understood in the context of specific stresses as given below or integrated into a network of multiple interacting stresses, as elaborated later in this chapter.

12.3.1 N in Elevated CO_2 and NO_2

Continuous exposure to elevated atmospheric CO_2 may result in stomatal closure, adversely affecting the rate of transpiration and therefore nutrient uptake of the plants, leading to nutrient N deficiency stress. Decrease in activities of assimilatory enzymes such as nitrate reductase (Ferrario-Méry et al. 1997) and RuBisCO (Bloom et al. 2010) was also observed. However, brief exposure to elevated CO_2 showed enhanced activities of NR and GSA in cucumber and sunflower leaves (Aguera et al. 2006). Inhibition of photorespiration-dependent nitrate assimilation (Rachmilevitch et al. 2004) is also observed at higher levels of CO_2. Elevated CO_2 is known to enhance photosynthesis in C3 plants and improve NUE (Shimono and Bunce 2009). The form of N used under various CO_2 concentrations affect the nutrients and their distribution in the plant (Natali et al. 2009). For example, wheat plants supplied with ammonium salts as a source of N were more N responsive under elevated CO_2 concentrations, in terms of nutrient accumulation, yield, and yield components, as compared to those supplied with nitrate (Carlisle et al. 2012 and references therein).

Nitrogen dioxide can be absorbed and utilized by the plants in small quantities for assimilation (Mokhele et al. 2012) and therefore elevated NO_2 causes some increase in intracellular nitrate concentration (Qiao and Murray 1998). On the other hand, reduction of ambient NO_2 level has no effect on the organic N content of the plants or on the amount or rate of N uptake in the plants.

12.3.2 N in Water and Salt Stress

Drought stress alone is projected to double in future, which will lead to loss of yield (IPCC 2007). While photosynthesis can be maintained under fluctuations of water supply (Lightfoot et al. 2007), water deficit can alter the C and N transformations by bringing about changes in the soil–microbe interactions such as reducing the activity of nitrifying bacteria (StClair and Lynch 2010 and references therein). This is evident from the inhibition of nitrogen fixation in legume crops during C and N fluxes under drought (Ladrera et al. 2007; Rogers et al. 2009).

The effect of drought on leaf N status remains uncertain, as it was reported to increase in *Malus domestica* (Jie et al. 2010), decrease in *Prunus persica* (Dichio et al. 2007), and be unaffected in *Quercus* (Li et al. 2013). However, a recent transcriptome study suggests the interactive effects on the genome-wide impact of drought and N limitation in maize (Humbert et al. 2013). It studied 30 conditions involving three major parameters such as organ (leaf, root, or stem), nitrogen supply (optimal or chronic limitation), and water supply (optimal supply, mild water stress by withdrawal for 3 days, severe water stress by withdrawal for 5 days, and recovery from severe stress by rewatering for 2 h or 5 h). The impact of severe stress was more extensive in root and stem than in leaf, in terms of the number of spots/genes affected. The pathways most affected were sucrose and starch metabolism, Calvin cycle, proline, and asparagine biosynthesis. Both photosynthetic assimilation and nitrate assimilation were shown to be downregulated. The effect of water withdrawal and nitrogen limitation on ammonium assimilation was tissue specific; the transcripts for glutamine and glutamate synthases were more in leaf as compared to stem and root. This study also shows that while nitrogen limitation has very little impact on the transcriptome on its own (0.2 % of the spots), even mild water stress makes the plant more vulnerable to N limitation, affecting the expression of a much larger number of genes (Humbert et al. 2013). These observations need to be validated in other plants before wider generalizations could be made. However, studying such interactive effects could be useful to optimize NUE along with water-use efficiency (WUE) (Di Paolo and Rinaldi 2008). On the other hand, heavy use of N fertilizers regardless of the water regime can be detrimental on grain filling and drought tolerance (Humbert et al. 2013 and references therein).

Drought tolerance genes contribute to greater NUE because they improve biomass production over an extended range of soil moisture availability and weather conditions (Harrigan et al. 2009). The traits for drought stress include yield potential, WUE, harvest index (HI), improved transpiration efficiency, and deep root penetration (to access water and nutrients), all of which are relevant to NUE as well.

Flooding is another form of water stress that is expected to increase due to climate change. Waterlogging affects ~10 % of the global land area and an estimated 10 million hectares of land in developing countries. It can cause a wide variety of symptoms that can affect yield either directly or indirectly, through affecting leaf senescence, tiller number, and reduced plant height. While N availability could increase under situations in which floods bring silt and nutrients along, it could also decrease in situations where topsoil and fertilizer N are lost or diluted out. Other parameters such as temperature could also result in interactive effects.

Salinity is one of the major abiotic stresses that lower the yield and usually is accompanied by water stress. The impact of salt stress is dependent on the cultivar/organ/developmental stage and the degree of salt stress. Salinity is known to alter the activities of various enzymes from the N assimilatory pathway like nitrate reductase in leaves than in roots (Mokhele et al. 2012).

12.3.3 N in Heat and Cold Stress

Accumulation of greenhouse gases (carbon dioxide, methane, and nitrous oxide) in the earth's atmosphere is expected to warm up the earth's surface by 1.8–4 °C by the end of this century (IPCC 2007). Rising temperatures of both soil and air could alter the rate of water and nitrogen uptake due to their effect on rate of transpiration and soil moisture respectively (Dong et al. 2001). Elevated temperatures also alter N allocation, reduce foliar N concentration and carbohydrate content (Tjoelker et al. 1999), damage photosynthetic membranes and cause chlorophyll loss decreasing leaf photosynthetic rate, increase embryo abortion, lower grain number, and decrease grain-filling duration and rates resulting in lower grain yield.

Globally, leaf N concentrations have been found to vary along altitudinal and latitudinal temperature gradients across plant species or functional groups (Reich and Oleksyn 2004). Foliar N content increases from the tropics to the cooler and drier midlatitudes due to "temperature-related plant physiological stoichiometry and biogeographical gradients in soil substrate age, as well as cold temperature effects on biogeochemistry at high latitudes" (Reich and Oleksyn 2004).

Cold stress can produce undesirable responses to nitrogen fertilizers that are often applied in high concentrations to increase yield. For example, high nitrogen supply before/during pollen development aggravates the effect of pollen sterility in rice in extreme cold conditions (Gunawardena et al. 2003). Low temperatures are also known to inactivate RuBisCO carboxylase by *S*-nitrosylation (Corpas et al. 2011).

12.3.4 N in UV and Other Stresses

Enhanced exposure to UV is one of the consequences of climate change. Plant N uptake and assimilation are significantly inhibited at high levels of UV-B radiation. Excessive UV light along with nutrient deficiencies can lead to photo-oxidative

stress, which is worsened further in other environmental stresses such as metal toxicity (Lynch and StClair 2004 and the references therein). There is a significant increase in the activity of RNS species under UV-radiation stress as well as under ozone stress, which could lead to cell death (Corpas et al. 2011).

12.3.5 N in Multiple Interacting Stresses

Most of the plant stress studies were done by changing or observing a single variable factor, i.e., changing CO_2, temperature, or water concentrations, but very few studies considered the combinatorial effect of these stresses. Studies show increased concentrations of CO_2 can increase the demand for nutrient, but increase in temperatures can influence the length of growing season and in turn reduce the demand for nutrients (Nord and Lynch 2009; Mittler and Blumwald 2010). Rice plants exposed to increases levels of CO_2 have shown increased sensitivity to cold stress (Shimono and Bunce 2009). Both drought and heat stresses together affect N availability storage and remobilization in pine trees (Rennenberg et al. 2009; Huang et al. 2012). The activity of many nitrate-regulated genes is hypothesized to be regulated by light, and evidence shows that both NRT2 and NR activity is dependent on light as well as nitrogen (Lillo 2004, 2008; Chapman and Miller 2011). Thus, mounting evidence suggests that the effect of various abiotic stresses can lead to unanticipated changes in plant growth and development.

12.4 Conclusions and Prospects

There is a growing demand for developing crops with resilience to climate change, abiotic stress, and N-use efficiency for global food security and environmental sustainability. While studies on individual stresses and the signaling mechanisms involved in the plant's response to them have made impressive progress, integrative studies are needed that can model the complex interactions between various abiotic stresses and the signaling and/or regulatory events involved in them. Similarly, in the area of N-response and N-use efficiency, integration of crop genetics and functional genomics approaches have begun to make rapid strides, but the links between nitrogen and stress remain peripheral, especially at the level of signaling and regulatory interface of stress and N, except in the area of nitrosative stress. This calls for new synergies to be forged between research on abiotic stress and resource-use efficiency in general and N-use efficiency in particular, to identify some common signaling aspects or regulatory targets for developing not only stress-resistant and climate-resilient crops but also N-use-efficient or resource-use-efficient crops.

Crop improvement through QTL mapping and marker-assisted selection/breeding seems to be a promising route in this regard. Extensive studies in various plants have lead to the mapping of many agronomic traits such NUE, yield, biomass, N uptake,

and remobilization (Habash et al. 2007; Fontaine et al. 2009). The possibility of co-localization of multiple agronomically important QTLs is an increasingly attractive avenue to explore in this regard. For example, in tropical maize, QTLs for grain yield and secondary traits were identified under varying N and water supply, some of which were found to be co-localized (Ribaut et al. 2007). More efforts in this direction can be enabled by suitable national policies and intergovernmental cooperation for germplasm exchange and collaboration. The role of public sector may prove to be at least as crucial as that of the private sector in facilitating affordable access to such technologies for the farmers, breeders, and consumers alike.

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Chapter 13
Signaling Pathways in Eukaryotic Stress, Aging, and Senescence: Common and Distinct Pathways

Ritika Das, Amita Pandey, and Girdhar K. Pandey

Abstract Aging has been often described as a set of intricate changes occurring in an organism leading to a decline in its physiological functions, ultimately promoting disease and death. Senescence is also a term commonly associated with wear and tear of an organism that occurs with age. Aging as a process has long been thought to be a random process without any strict molecular basis. However, discovery of various conserved signaling pathways controlling longevity has provided strong proof for aging to be under the control of a programmed pathway. Multiple signaling cascades such as insulin signaling and TOR (target of rapamycin) pathway have been shown to be critical regulators of lifespan and aging-related processes across species. Improved longevity has also been associated with increased stress resistance suggesting cross talk between longevity and stress signaling pathways. Plant aging and senescence differ from that of other eukaryotes in terms of a broader range of lifespan observed across plant species that could be explained by different modes of nutrient accumulation and means of reproduction. Different signaling cascades such as those involved in sugar sensing and nutrient sensing in general have been found to be playing an important role in plant longevity. Existence of similar homologues of these proteins in animal kingdom that perform similar roles in aging-associated functions suggests some degree of conservation in pathways controlling aging across plant and animal kingdom. TOR pathway is one such signaling pathway, which is a well-known regulator of lifespan in animals and has been recently shown to be important for plant longevity as well. Besides nutrient signaling, different classes of hormones have also been implicated in plant stresses and senescence suggesting the existence of a complex interplay between these different physiological and environmental signals in regulating plant aging. With the advent of functional genomic approaches such as whole genome microarray, proteomics and ChIP-based (chromatin immunoprecipitation) sequencing have been utilized to understand the molecular mechanisms underlying the aging process. In this

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chapter, we attempt to summarize such findings that are relevant to aging and senescence in different organisms such as animal and plant model system.

Keywords Aging • Senescence • Stress • FOXO (forkhead box O) • TOR (target of rapamycin) • Microarray • RNAi

13.1 Introduction

Aging and senescence reflect physiological changes that occur in every organism's lifespan, ultimately resulting in morbidity and death. The biological basis of aging has been a debated question for a long time that science has struggled to answer in a satisfactory way. Over the years, many ideas and theories have been advocated to provide explanations for aging and longevity in different species. However, in the last decade, multiple studies have yielded interesting insights into the aging process and its molecular mechanism. With increased age comes the increased risk of diseases such as cancer, neurodegenerative disorders such as Alzheimer's disease (AD), and diabetes. According to the world health organization report, the world old population is expected to be triple by 2050, increasing costs of medical and social care for the elderly.

Thus, understanding the biological basis of aging and how we can alleviate age-associated health risks is of prime importance. Besides profound progress made in the field of animal aging, some of the basic mechanisms and pathways involved in plant aging and senescence are also beginning to be understood. Understanding the underlying mechanisms of plant stress and aging would be particularly beneficial in generating stress-resistant/stress-tolerant crop varieties that give better yield in terms of productivity that would be helpful in eradicating hunger and malnutrition from different parts of the world. In addition, elucidation of signaling pathways that cross talk to regulate senescence and nutrition could provide avenues for engineering plant species capable of detoxification of various pollutants in soil and water, thereby promoting a healthier ecological state for other living organisms.

13.2 Aging: An Inevitable Byproduct of Living or a Programmed Pathway?

One of the basic questions concerning aging is, what is it? How does an organism age? And how could genetic or environmental perturbations act as factors to affect the debilitating consequences of aging? Over the years, multiple ideas/theories were proposed in animals mostly to explain aging, such as oxidative damage theory of aging and genomic instability theory. All such theories can be broadly grouped into the programmed theory of aging and error theory of aging as described below (Jin 2010). The basic distinction between the two theories is that while the programmed theory believes aging to be a phenomenon under regulation of well-defined genetic and cellular pathways, the error theory views aging as an outcome of damage accumulation that happens due to less faithful repair pathways as an organism grows old.

13.3 Programmed Theory of Aging

Programmed theories of aging propose existence of specific cellular pathways whose dysfunction with age promotes disease and death.

Hormonal control of aging: In animals, endocrine and hormonal signals have long been believed to be important in aging that has been validated with the discovery of IGF (insulin growth factor) signaling as a conserved mechanism controlling lifespan across species (Jin 2010). IGF signaling comprises of insulin ligands binding to the insulin receptor that triggers a signal transduction cascade terminating with phosphorylation of FOXO transcription factors. Studies in human centenarians have revealed different polymorphisms in IGF genes, affecting levels of insulin in the plasma and sensitivity of the receptor to insulin suggesting negative role of this signaling pathway in aging (van Heemst 2010; Alcedo et al. 2013). FOXO activation upon inhibition of insulin signaling is believed to produce metabolic shift, tolerance to cellular stress, and suppression of inflammation. The complexity of IGF signaling in aging increases in mammals with multiple IGF receptors and various isoforms of FOXO that exhibit variation in tissue distribution and subcellular localization. Also, existence of growth hormone (GH) signaling in vertebrates and its cross talk with IGF signaling provides an additional layer of complexity in hormonal control of aging. Growth hormone secreted by the pituitary gland promotes release of IGF from the liver and other distant tissues. However, besides regulating IGF synthesis directly, GH also participates in other signaling cascades such as the JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway and MAPK (mitogen-activated protein kinase) pathway that could also have implications for aging and associated diseases such as cancer (Lanning and Carter-Su 2006).

Immunological theory of aging: Immunological theory predicts "immunosenescence" or decline in immune function as a causative agent for aging (Sidler et al. 2013). The adaptive immune response comprising of B and T lymphocytes protects organisms from different pathogens and cancer. Decline in T cell number is associated with decrease in thymus size (a process referred to as thymic involution) that occurs with age, promoting morbidity and disease in the elderly. Besides dysfunction in production of T cells, other aspects of the immune system such as effectiveness of antibodies to combat infections are also known to decline. Defective immune system has been linked to various age-associated diseases such as Alzheimer's disease, cancer, and inflammation (Jin 2010; Rozemuller et al. 2005; Cornelius 1972).

Genetic instability as a cause of aging: This theory predicts accumulation of somatic DNA damage as a causative agent for aging. It is known that DNA repair capacity of an organism declines with age that leads to increasing accumulation of deleterious mutations promoting disease and death. This damage increases more significantly in nondividing mammalian cells causing them to malfunction that ultimately affects the whole organism (Jin 2010; Campisi 2000).

13.4 Error Theory of Aging

The error theory of aging as the name suggests hypothesizes the occurrence of malfunction of different cellular processes with age that increases the rate of error, promoting damage accumulation that causes aging and senescence. Different ideas have been put forward that fall under the category of the error theory as described below.

13.4.1 Rate of Living Theory

The rate of living theory first proposed by Raymond Pearl suggests that the metabolic rate of an organism is a key determinant of longevity as observed in temperature differences in longevity in *Drosophila* (Loeb and Northrop 1916). The variation in metabolic rate among mammals does indeed as observed in some cases show inverse relationship with lifespan (Hulbert et al. 2007). However, differences in metabolic rate predict maximal lifespan in only 26 % of the evaluated cases hinting toward the fact that other factors also play a role in determining aging (Hulbert et al. 2007). This theory was further modified as an antagonism between TOR pathway and stress resistance mediated by FOXO (Rollo 2010). Although lower temperature is known to slow down metabolic rate and thus influence the aging process, many observations have been inconsistently made with this theory. For example, mammals such as humans have 5–10 times higher metabolic rate than endothermic vertebrates of same size but actually live longer again pointing toward the insufficiency of this theory in explaining the lifespan variation observed in diverse species (Hulbert et al. 2007). This theory posits that higher metabolic rate amounts to higher energy expenditure that is associated with short maximum lifespan. However, this correlation between metabolic rate and lifespan has not been observed in many cases as evident from studies that found mice with higher BMR (basic metabolic rate) actually live longer than those that have lower metabolic rate (Speakman et al. 2004). Also, large-sized breeds of dog that have higher metabolic rate actually live longer than smaller-sized breeds (Speakman et al. 2003). The missing link in this theory has come from the analysis of lipid composition in different species that has led to the idea that lipid content in the membrane can (a) influence the metabolic rate (by determining the fluid nature of membrane) and (b) determine the degree of damage caused to DNA, protein, and other biomolecules by lipid peroxidation (Hulbert et al. 2007). Consistent with this idea, short-lived mammals with small body size have higher polyunsaturated fatty acids (PUFA) in their membrane as compared to long-lived mammals with large body size that makes them more susceptible to the damaging effects of lipid peroxidation and damage by free radicals (Pamplona et al. 1999).

13.4.2 Free Radical Theory

The free radical theory of aging first proposed by Harman in 1956 states that free radicals and reactive oxygen species (ROS) cause increasing oxidative damage to cells and result in decline in physiology and hence aging in organisms (Harman 1956). Initial studies found this idea to be true as concluded from studies showing increase in free radicals with age. However, other studies done in recent times that showed benefits of TOR pathway and physical exercise by increase in oxidative stress challenged the original free radical theory of aging (Afanas'ev 2010). Moreover, research showing that free oxygen and nitrogen radicals can actually function as signaling molecules and participate in enzymatic/gene processes has renewed the idea of free radical theory of aging as an important modern theory of aging (Afanas'ev 2010). Superoxide ions ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) are physiological-free radicals generated in biological processes that are inactive by themselves but can act as precursors of highly reactive hydroxyl and peroxy species that damage biomolecules (Afanas'ev 2010). The original free radical theory proposed that any form of free radical in organisms would shorten lifespan and promote aging, while reducing it would be beneficial. However, with many experimental studies done in recent times, it has become increasingly clear that ROS (reactive oxygen species such as hydroxyl, superoxide, and peroxide ions) and RNS (reactive nitrite species such as nitric oxide and peroxynitrite) also serve as important signaling molecules critical for cellular processes and homeostasis under normal as well as pathological conditions (Afanas'ev 2010). This suggests that regulation of free radical production might be the key to healthy aging rather than activating or completely abolishing it. Different cellular enzymes such as NADPH oxidase, xanthine oxidase, superoxide dismutase (SOD), and NO synthase play an important role in ROS regulation and, thus, might be critical regulators of ROS and RNS signaling with age (Afanas'ev 2010). Increased longevity in Ames dwarf (AW) mice that also showed increased resistance to oxidative stress was associated with decreased ROS levels in the serum (Choksi et al. 2007). Aged rats showed increased superoxide production from NO synthase and xanthine oxidase as compared to younger rats (Jacobson et al. 2007). Knockdown of Nox4 (NADPH oxidase) in human umbilical vein increased their replicative lifespan suggesting that damage to human endothelial cells with age is due to oxidative damage (Rodriguez-Manas et al. 2009). Thus, all the studies and observations made recently support Harman's theory of free radical in aging. With greater knowledge and understanding of signaling cascades, different pathways have been found to be regulating ROS that could also have implications for aging. *p66shc*, *FOXO3a*, and *sirtuin* have been shown to be regulating ROS that is believed to impact aging. FOXO3a belongs to subclass O of FOXO family of transcription factors shown to control p53 activity via ROS formation. ROS also activates FOXO3a via phosphorylation that promotes cellular senescence (Purdom and Chen 2003). Klotho is a membrane-localized protein that has been shown to inhibit FOXO3a phosphorylation and thereby promote its nuclear localization. The nuclear-localized FOXO3a can then bind to the MnSOD (superoxide dismutase) promoter and decrease

ROS production that prevents cellular senescence associated with ROS damage (Yamamoto et al. 2005). Upregulation of Sirt (silent information regulator) proteins, Sirt1 and Sirt3, can also positively impact aging as demonstrated by reduced cardiac dysfunction and lower expression of senescence markers (Alcendor et al. 2007). Intermediate overexpression of Sirt1 reduced ROS production together with FOXO3a. Thus, multiple longevity pathways have been demonstrated to influence aging partly via ROS/free radical regulation.

And no theory alone at present is sufficient by itself to explain all causes and effects of aging, and it is highly probable that more than one theory may hold true and a significant overlap may exist between the different proposed ideas and pathways to impact aging. Different hypothesis-driven experiments at both genomic and single gene level could help test and validate the above-described theories. In this book chapter, we are trying to emphasize on the knowledge developed till now in different organisms for aging and senescence and how it is influenced by environmental stresses.

13.5 Aging Pathways in *C. elegans*

C. elegans has been a pioneering model organism in the field of aging with the discovery of insulin-like signaling as a potent regulator of lifespan (first identified in worms) that is conserved across species. The 1 mm long soil nematode offers a cheap, fast, and a comparatively easier system to understand the complex aging process. With well-defined cell lineage, a transparent body, lack of genetic redundancy, and ease of gene knockdown via RNAi feeding, many critical regulators of aging and longevity have been identified in *C. elegans*, highlighting its importance as a valuable organism for aging studies. Different genome-wide approaches have identified genetic and cellular mechanisms acting downstream of insulin-like signaling, calorie restriction, and sirtuins in regulating lifespan and aging. This section describes the past and current findings made in *C. elegans* that have shaped our understanding of aging.

13.5.1 Insulin-Like Signaling as a Potent Regulator of Lifespan

Partial loss-of-function mutations in insulin-like growth factor receptor gene (*daf-2*) in worms result in more than twofold increase in longevity (Kenyon et al. 1993; Kimura et al. 1997). This observation first made more than two decades ago has revolutionized the field of aging research, proving aging to be a tractable, genetically defined problem rather than a random event. Moreover (as later described), the insulin signaling pathway has been found to be conserved across species such as fly and even humans, supporting the basis for research in simple model system such as *C. elegans* (summarized in Fig. 13.1). The insulin signaling activated by insulin-like ligands (such as *ins-7*) results in dimerization and autophosphorylation

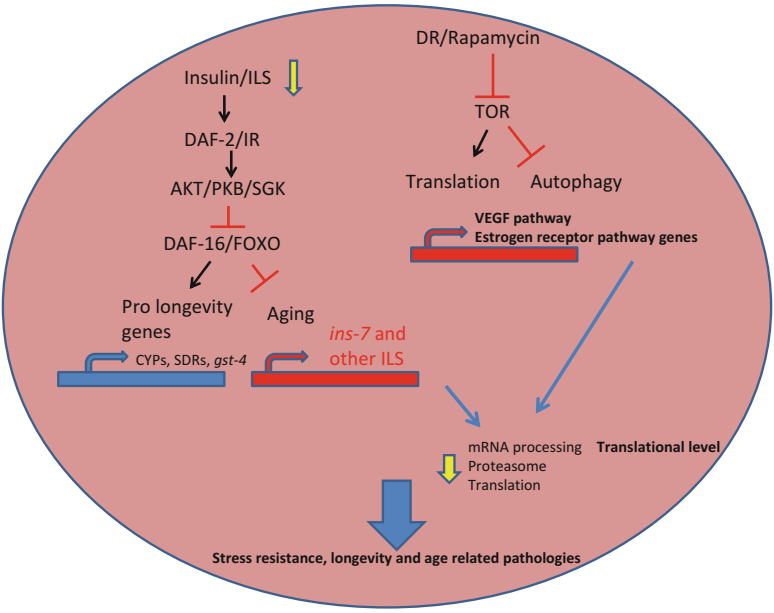

Fig. 13.1 Pathways regulating aging in invertebrate and vertebrate organisms. IGF (insulin growth factor) and TOR (target of rapamycin) are the major pathways that have been implicated in animal aging from *C. elegans* to humans. Genetic screens combined with gene and protein expression analysis have revealed various downstream effectors of these aging pathways that also cross talk with stress-associated functions. As shown in this model, insulin signaling-mediated repression of FOXO class of transcription factors inhibits pro-longevity genes related to stress and metabolism. Inhibition of TOR pathway by rapamycin and dietary restriction promotes reduced protein synthesis while increasing autophagy. All of these downstream cellular processes are known to enhance stress resistance and longevity and provide protection from age-associated pathologies. *ILS* insulin-like peptides, *AKT*/*PKB* protein kinase B, *SGK* serum and glucocorticoid kinase-1, *DR* dietary restriction, *IR* insulin receptor, *VEGF* vascular endothelial growth factor

of tyrosine residues on the receptor followed by sequential activation of other downstream kinases such as PI3K/PDK/AKT that ultimately results in inhibition of nuclear localization of forkhead transcription factor (FOXO) DAF-16 in worms. Upon inhibition of this pathway, the negative inhibition of DAF-16 is relieved that results in constitutive nuclear accumulation of this transcription factor that then activates a battery of different genes and pathways involved in aging, metabolism, stress resistance, development, and immunity and confers increased lifespan in worms. In addition to DAF-16, other transcription factors such as HSF-1 (heat shock transcription factor 1), SKN-1 (SKiN head/homologue of mammalian Nrf proteins), and HIF-1 (hypoxia inducible factor 1) have also been found to be playing an important role downstream of *daf-2* in mediating lifespan extension and stress resistance (Antebi 2007; Leiser et al. 2013). The next obvious questions as details of insulin-like signaling pathway emerged were, which genes may act downstream of the *daf-2* pathway in providing longevity assurance, and are there other pathways/genes that could act independent of this pathway that also enhance or

inhibit longevity? The answers to such questions have been greatly facilitated by functional genomics involving the ease of gene inactivation via bacterial RNAi feeding in worms and development of other approaches such as DNA microarrays that have been used on a large scale in understanding the intricate process of aging across species.

13.5.2 Use of DNA Microarray Reveals DAF-16-Dependent Genes Involved in Longevity and Stress Response

Study published by Murphy et al. (2003) was the first report carrying out a detailed analysis of genes acting downstream of DAF-16 in controlling worm aging. Microarray analysis performed in *daf-2* and *daf-2*; *daf-16* double mutants revealed a host of genes that were upregulated in *daf-2* or downregulated in *daf-2; daf-16* double mutants, and those showing at least fourfold expression change were considered as significant by the authors who further grouped them into categories based on their expression levels in different genotypes and function. Class I genes were the ones that were induced by *daf-2* mutations but reduced in *daf-2daf-16* double mutant and were believed to be promoting longevity. Class II genes were the ones that showed an opposite expression pattern with reduced expression in *daf-2* mutants but increased expression in *daf-2* (RNAi); *daf-16* (RNAi) double loss-of-function worms and were speculated to be anti-longevity. Interestingly, a host of these genes involved in hormonal signaling (*ins-7* and other insulin-like peptides), antimicrobial function, and stress response (such as catalase, superoxide dismutase, and metallothionein) were found to be differentially regulated in a DAF-16-dependent manner suggesting their important role in longevity-associated functions. Consistent with this idea, knockdown of several heat shock protein genes and oxidative stress response genes decreased *daf-2* mutant longevity by 10–20 %. In addition to enhanced lifespan and stress resistance, *daf-2* mutants also display increased resistance to bacterial infections that were correlated with increased expression of some antibacterial genes downstream of *daf-2*/*daf-16* axis such as *lys-7* and *8* (lysozymes), and saposin-like gene *spp-1* with antibacterial functions belonged to the class I category of genes. In addition to class I genes, several class II genes such as aminopeptidase, carboxypeptidase, and F box/cullin/Skp (*skr-8* and *9*, *pes-2*) involved in ubiquitin-mediated protein degradation were found to increase longevity of both *daf-2* and wild-type worms upon RNAi, providing further validation for genes identified via microarray approach and their role in longevity and aging.

Additional microarray studies by McElwee et al. (2004) identified genes commonly regulated in dauer larvae stage (an alternative hibernation stage reached by worms in the early stages of development under conditions of adverse environmental stress such as starvation and high temperature) and *daf-2* adult animals. Insulin-like signaling in worms acts as one of the regulators of dauer formation; *daf-2* mutants arrest as dauer at 25 °C suggesting important role of this signaling in development besides longevity. The important question addressed by the authors using

oligonucleotide array revealed a shared transcriptional signature between dauers and *daf-2* mutants that were similarly altered in both situations and involved genes functioning in detoxification. Three classes of genes that showed upregulation in both *daf-2* and dauer larvae stage were CYPs (cytochrome P450s), SRDs (short-chain dehydrogenases/reductases), and UGTs (UDP glucuronosyltransferase). CYPs are detoxification enzymes believed to be acting in metabolizing lipophilic substrates like steroid hormones. Fifteen CYPs showed some degree of induction, four of which were either daf-2 or dauer larvae induced, and six SDRs were identified to be upregulated in both dauer and *daf-2* mutants forming a second class of detoxification genes that act to reduce carbonyl functional group in aldehydes and ketones using NADH. Six UGTs are upregulated in *daf-2* and dauer conditions that have also been shown previously to be induced in *daf-2* mutant worms and have possible effect on dauer development (Murphy et al. 2003; Wang and Kim 2003). As reported by the authors, CYPs and SDRs perform phase 1 detoxification of drugs, which involves functionalization reaction of the chemical groups, while phase 2 refers to reactions that change the solubility of drugs enabling secretion such as in the case of UGTs. Further in silico analysis by the authors appeared to suggest that these TRPs contain signal peptides, which hint that they could be acting in secretion of lipophilic substances in the intestinal tissue. Taken together, these studies suggested an important role played by detoxification enzymes in longevity assurance acting possibly via helping the organism to get rid of various endogenous toxic compounds that accumulate with age, thus promoting healthy aging. In addition, it showed some degree of overlap between dauer and *daf-2* transcriptional signature, linking the dauer phenotype and stress resistance to longevity.

13.5.3 Quantitative Proteomic Approach Reveals Altered Protein Metabolism in Long-Lived daf-2 Mutants

Multiple studies have analyzed the transcriptional output of insulin signaling in longevity mediated via *daf-16* using microarray and other genome-wide approaches such as feeding via RNAi to identify additional regulators of longevity. However, what precisely happens in these long-lived mutants at the translation/protein level has been largely unanswered. A recent study published by Stout et al. (2013) attempted to address the proteomic status of *daf-2* mutants using a mass spectrometric approach involving post-lysis labeling of peptides with unique tags. Forty percent of the identified proteins were found to be altered in *daf-2* mutant worms, and 22 % of these showed an increase in abundance, while 19 % had decreased levels compared to N2 (wild type) worms suggesting that insulin signaling mutation does evoke changes at the proteome level that may be important in longevity. Overall, protein translation, mRNA processing, and proteasome activity were novel pathways that were found to be downregulated in *daf-2* long-lived mutants. Decrease in small and large ribosomal subunits, elongation, and initiation factors were observed suggesting an overall decrease in translation. In addition, decrease in expression of

protein degradation machinery genes such as *eel-1* (E3 ligase) and *pas-3* (20S proteasome activity) that form the core proteasome complex was observed. The mRNA processing genes such as *cgh-1* (ATP-dependent RNA helicase) also showed downregulation of −1.64-fold (as compared to wild type). This reduction in mRNA was concomitant with reduction in total mRNA levels in *daf-2* as compared to wild type as determined by quantification of total mRNA levels in *daf-2* and wild type (N2). On the other hand, upregulation of proteins in stress protective pathways (thioredoxins) and metabolic pathways (phosphoenolpyruvate carboxykinase) was observed that was consistent with previous studies at both genomic and proteomic level (Murphy et al. 2003; Dong et al. 2007). The most interesting finding of this study was that there is reduced de novo protein synthesis in *daf-2* mutant animals, which differs from earlier studies using methionine labeling to show equal levels of protein synthesis in *daf-2* vs. wild-type worms (Hansen et al. 2007; Dong et al. 2007). However, a detailed kinetic analysis done by Stout et al. (2013) showed that this unchanged level is actually due to a decrease in translation coupled with decrease in protein degradation activity in *daf-2* mutant worms. As a further biological validation of processes identified in this study to be important for longevity, knockdown of various genes in wild type actually produced a lifespan phenotype proving them to be having longevity-related functions. For example, loss of *rpl 17* and *28* via RNAi significantly extended longevity in wild-type worms by 10–20 %.

13.6 Dietary Restriction and Aging in Worms

Reducing food intake to an extent that does not cause starvation or malnutrition is known as dietary restriction (DR), and this process is shown to alleviate age-associated pathologies in a variety of species as well as extend longevity in multiple species including *C. elegans* (Mair and Dillin 2008). The protocol for DR regimen varies significantly between species but does produce the common effect of lifespan extension, which could possibly be due to shared or distinct genetic pathways via which DR is acting (Mair and Dillin 2008). TOR/AMPK (Target of rapamycin/AMP activated kinase) is an amino acid/energy-sensing pathway, via which DR is believed to be acting. TOR/AMPK signaling acts as a sensor of nutrition (amino acid)/AMP/ATP ratios within the cell to promote/restrict cellular growth depending upon nutrient abundance. TOR is a serine threonine kinase, conserved in all eukaryotes, and acts with two complexes in mammals, rapamycin sensitive (Raptor) or rapamycin insensitive (Rictor), to promote translation and inhibit autophagy (Sarbassov et al. 2005; Inoki and Guan 2006; Vellai et al. 2003). Inhibition of TOR in worms via knockdown of *daf-15* (raptor homologue) is known to produce lifespan extension, which is supported by longer lifespan observed in *daf-15* heterozygous mutants. In *C. elegans*, loss of other downstream TOR components such as *rsks-1* (worm homologue of S6 kinase) that regulate translation also enhances longevity (Hansen et al. 2007; Pan et al. 2007). Evidence for role of TOR in DR-mediated lifespan extension has come from studies showing no further

lifespan extension in *eat-2* mutant worms (displaying slow pharyngeal pumping and acting as genetic mimic of DR conditions) upon TOR RNAi (Hansen et al. 2007). However, the same result is conflicted by another study reporting additive lifespan extension of *eat-2* mutants with loss of TOR (Henderson et al. 2006). Thus, the involvement and role of TOR in DR-mediated longevity in *C. elegans* is yet to be empirically proved. AMPK is also a potent regulator of worm longevity that inhibits TOR under low energy (high AMP/ATP ratio) to downregulate energy-consuming cellular processes such as translation (Mair and Dillin 2008). Consistent with its role in nutrient sensing and aging, overexpression of AMPK has been shown to extend lifespan in worms (Apfeld et al. 2004). This section discusses some of the functional genomic and proteomic approaches taken to understand DR-mediated longevity in worms that also hint toward the involvement of TOR as a mechanism for this pathway.

13.6.1 Microarray Reveals Role of Fat Metabolism Genes Under Dietary Restriction in C. elegans During Development

Dietary restriction (DR) studies have mostly been performed in adult animals where significant longevity has been associated with limiting food conditions compared to condition when worms are fed ad libitum. Study done by Palgunow et al. (2012) reported an increase in triglyceride to protein ratio in worms that are subjected to developmental DR (dDR). Further, microarray profiling of these worms revealed altered expression of lipolysis and lipogenesis genes, consistent with fat phenotypes observed under dDR. The differentially expressed genes under moderate and stringent dietary restriction conditions were divided into set I (comprising of 263 genes in L4 larvae) and set II (2,736 genes in adult worms) with 124 genes showing similar alteration in both stages under both types of DR conditions. A number of genes involved in fatty acid metabolism were altered such as those involved in mitochondrial and peroxisomal beta-oxidation. The *acs-2* (acyl-CoA synthetase), an acyl-CoA oxidase, and an acyl-CoA dehydrogenase were found to be upregulated. Several lipases such as triacylglycerol lipase were downregulated, whereas another lipase (*lipl-6*) was upregulated. *fat-5* (encoding a desaturase) and *far-3* (a retinol-binding protein) were upregulated under DR with *far-3* showing specific upregulation in L4 stage. Interestingly, a host of previously identified genes acting in longevity, stress response, and immune regulation were also regulated by dDR. In conclusion, the study showed that increase in lipid droplet formation observed in intestine and hypodermis under DR conditions is accompanied with altered expression of several lipases and other fat metabolism genes. The biological basis of increase in lipid droplet could be explained by the fact that lipids stored in these droplets are less accessible to lipases and provide a slow energy store under DR conditions, which could further enhance longevity by influencing the whole metabolic status of the worm.

13.6.2 ChIP Sequencing Reveals PHA-4 Binding Sites During Development and Environmental Response

PHA-4/FOXA transcription factor in *C. elegans* has functions in both development and DR-mediated longevity as described above. However, precise genes that are regulated by PHA-4 in mediating its role in nutrient sensing and other associated functions have been largely unknown unlike *daf-16* whose target genes have been extensively studied. Study published by Zhong et al. (2010) established and utilized ChIP sequencing approach to identify PHA-4 transcriptional targets. To achieve this, PHA-4:GFP:3X FLAG tag strain was used to identify PHA-4 binding sites during embryogenesis when pharynx development occurs and starved L1 conditions. A total of 4,861 genes were identified as PHA-4 targets in embryos and 4,621 genes were identified in the L1 stage. Further, GO (gene ontology) categorization of genes identified in the study revealed enrichment of developmental processes genes that are bound by PHA-4 during embryogenesis, while L1 stage showed large number of genes functioning in metabolism and defense responses. For example, autophagy genes such as *lgg-1* and *bec-1* were strongly enriched in gene set identified in starved L1s supporting previous studies showing requirement of upregulated degradation process such as autophagy in starvation-induced increased longevity (Hansen et al. 2008).

13.7 eIF4G and Its Role in Lifespan Extension via Posttranscriptional Regulation of Stress-Responsive Genes

eIF4G is a translation initiation factor that acts as a scaffold for cap-dependent eIF4 cap-binding complex for initiation of protein synthesis (Rogers et al. 2011). It acts as a bridge between eIF4E that binds 5′ methylated cap to poly A-binding protein (PABP) resulting in mRNA circularization. It also interacts with eIF3 to recruit 40S ribosome for translation (Rogers et al. 2011). Inhibition of *C. elegans* eIF4G, *ifg-1* results in lifespan extension, which most probably acts via inhibition of protein synthesis. Interestingly, inhibition of *ifg-1* during development results in larval arrest, but its knockdown via RNAi during adulthood results in significant lifespan extension suggestive of antagonistic pleiotropy that is based on the idea that genes important in early development have deleterious functions later in adulthood (Curran and Ruvkun 2007; Pan et al. 2007). To gain further insight into what might be happening downstream of *ifg-1* in controlling longevity, translation initiation was measured in wild-type vs. *ifg-1* RNAi-fed worms, and a translation index (TI) was calculated. Of the total of ~18,000 genes examined, 412 genes were found to be having higher TI in *ifg-1* loss-of-function worms, and 310 genes had reduced TI showing increased and decreased translation initiation rates, respectively. The identified genes were classified as per gene ontological enrichment analysis, and two of the overrepresented categories included stress response and cellular homeostasis genes such as *hsp-70*, *daf-21* (*hsp-90*), *hsp-6*, and *fasn-1* (fatty acid synthase). Taken together, these studies

provided a comprehensive insight into differential translation regulation acting downstream of *ifg-1* pathway that in some ways could also reflect alterations occurring at the proteomic level under dietary restriction conditions providing enhanced tolerance to stress and increasing longevity. Future studies using similar methodology in different dietary restriction mutants and pathways such as TOR, *rsks-1*, and *eat* mutants could help dissect differences between them, helping to unravel greater complexities of different conditions known to mediate longevity via similar mechanisms.

13.8 Genome-Wide RNAi Approach Identifies Additional Longevity and Stress-Associated Genes in *C. elegans*

As mentioned before, the ease of achieving gene knockdown in worms using RNAi offers a great advantage in performing large-scale screens for scorable phenotypes such as longevity and stress responses. RNAi library targeting >80 % of the *C. elegans* genome has been employed by most *C. elegans* research groups as a reverse genetics approach in identifying genes involved in a variety of cellular processes including aging (Kamath et al. 2003). Many studies done over the past decade have utilized this approach to identify gene activities that regulate aging and related processes, some of which are described below.

13.8.1 Genome-Wide RNAi Reveals Metabolism, Oxidative Phosphorylation, and Protein Turnover Genes in Longevity

An unbiased genome-wide RNAi screen done by Hamilton et al. (2005) identified 89 gene inactivations that extend longevity in wild-type worms by 5–70 %. Longevity genes identified in this study belonged to different functional categories such as metabolism, ETC (electron transport chain), and GPCRs (G protein coupled receptors). Metabolism genes such as those involved in carbohydrate, alcohol, and purine metabolism were uncovered. Of the genes involved in oxidative phosphorylation, genes in Complex I, III, and V were found to increase longevity, which could be hypothesized to be acting in longevity via lowering ATP production and reducing ROS production. The authors also looked at mitochondrial unfolded response reporter *hsp-6*::*gfp* to identify those ETC genes that modulate lifespan via possible alteration of mitochondrial function. Around seven of ETC genes were found to be inducing expression of *hsp-6*::*gfp*, suggesting their involvement in mitochondrial stress. Four candidate genes identified from the screen were GPCRs that could potentially act to control longevity via their functions in chemosensory neurons that modulate animal behavior in response to different environmental stimuli by regulating downstream signaling (Hamilton et al. 2005). A host of genes involved in

protein degradation were also identified such as ubiquitin ligase, hydrolases, and proteases suggesting protein turnover pathways to be playing an important role in lifespan regulation. Additional genes identified belonged to transcription factor category (*ceh-18*, *spt-4*) and other chromatin modifying proteins bearing PHD/SET domains. One of the major drawbacks of the study as noted by the authors was that it failed to find genes acting to enhance longevity in neurons since neurons are resistant to RNAi in the strain used for the study. An additional factor in missing the genes that could be acting in longevity might be because of RNAi feeding approach at the L1 stage. Potentially, this might be the possible reason for missing the genes, which could have a deleterious effect when knocked down during development but possibly involved in longevity. Further, genetic interaction experiments done in *daf-16* and *sir2.1* mutants (known downstream effectors of longevity) revealed some degree of overlap, with nine genes showing requirement of both for increasing lifespan. Seventeen gene inactivations required only *daf-16* and two required only *sir2.1* for promoting longevity. In summary, the study reported many genes acting to inhibit longevity and could perhaps be under cooperative control of known longevity pathways such as *sir2.1* and *daf-16*. Further analysis of novel class of genes identified in this study at a single gene level could yield interesting insights into their precise functions in the aging process.

13.8.2 RNAi Approach Identifies Cytoprotective Pathway as a Major Output of Various Longevity Pathways in C. elegans

Enhanced resistance has also accompanied increased longevity observed with mutations in different pathways to different environmental stresses such as heat, oxidative damage, irradiation, and xenobiotic toxins. However, how cytoprotective pathways correlate to longevity has largely been an unexplored question. Shore et al. (2012) studied this precise question using a battery of stress response reporter strains carrying GFP fused to stress-inducible promoters specific to a certain stress such as *hsp-6* (mitochondrial unfolded protein response reporter), *hsp-4* (ER stress reporter induced by toxins such as tunicamycin), *sod-3* (constitutively induced in *daf-2* mutants), and *gst-4* (induced by oxidative stress agents such as paraquat). Fifteen hundred gene inactivations by RNAi (previously identified to be important in longevity) yielded 73 genes that upon knockdown via RNAi impaired the activation of various cytoprotective stress response pathways as measured by GFP fluorescence quantification of above-described reporters using various stress stimuli. These genes were identified to be acting in a variety of processes such as protein degradation (*ufd-1*, *cul-1*, and *let-70*), transcription (*mdt-26* AND *elt-2*), deacetylation (*sdc-2*, *had-1*, and *lin-40*), and phosphorylation (*wnk-1*, *kin-1*, and *nekl-2*). Sixteen of these gene inactivations exhibit specificity to one of the four stress response pathways tested by the authors. For example, *lin-40*, a histone deacetylase, prevents *psod-3*::*gfp* induction by almost 17-fold. *nekl-2*, a serine/threonine kinase involved in Ras

signaling, was also an interesting candidate since it reduced *pgst-4*::*gfp* induction by almost 50 % that was even stronger than that observed with *skn-1* RNAi, which is a master regulator of oxidative stress response genes. Other genes such as *elt-2* and *let-70* reduced expression of all four cytoprotective stress response reporters suggesting their possible involvement in a broad range of stress response pathways.
In summary, identification of genes that are required for longevity as well as induction of stress highlights the importance of these genes in acting to preserve the damage response of the organism by acting as a buffer to get rid of cellular damage that occurs normally with age but is restricted to a greater degree in long-lived worms by virtue of induction of cytoprotective mechanism. It would be interesting to study in future the precise nodes of regulation by these genes and how they act to provide this protection and whether their overexpression is sufficient to enhance longevity or stress tolerance or both.

13.9 Sirtuins in Worm Aging

SIRT2 protein belongs to a family of conserved NAD^+ protein deacetylase that was found to be showing increased longevity first in yeast (Kaeberlein et al. 1999) followed by similar studies in fly and *C. elegans* (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Resveratrol is one of the known small molecules derived from plants, which act as an activator of mammalian SIRT1 and yeast Sir2p (Howitz et al. 2003). Resveratrol-mediated lifespan extension in *C. elegans* has been shown to be *sir2* dependent (Viswanathan et al. 2005). Microarray analysis of worms treated with resveratrol to identify downstream genes involved in resveratrol-/Sirt2-mediated lifespan extension revealed induction of genes encoding prion-like glutamine/asparagine-rich protein and those involved in ER stress and unfolded protein response. Loss of one such gene *abu-11* (involved in ER stress response) decreased lifespan extension mediated by *sir-2.1* overexpression in worms, while overexpression of *abu-11* was sufficient to extend lifespan suggesting that ER stress genes activated downstream of *sir2.1* and resveratrol are positive regulators of longevity.

13.10 Micro RNAs in Worm Aging

miRNAs first discovered in worms are small (15–25) nucleotide long noncoding RNA species that control important functions such as gene expression and development (Stefani and Slack 2008). Some indication for role of miRNAs in aging came from studies in *C. elegans* showing a function for *lin-4* and its target *lin-14*, development timing genes, in modulating aging (Boehm and Slack 2005). Subsequent studies identified other miRNAs that control the aging process. A study by Lencastre et al. (2010) used deep sequencing approach to identify miRNAs differentially expressed with aging in wild type as well as long-lived *daf-2* mutant worms. Several miRNAs such as miR-71, miR-253, miR-34, miR-238, and miR-239a/b were found

to be strongly upregulated with age, while others such as miR-70, miR-253, miR-238, miR-34, and *let-7* showed downregulated expression with age. In support of these findings in higher organisms, *let-7* was found to be involved in age-induced senescence in mouse neural stem cells (Nishino et al. 2008). Specific miRNAs had altered expression in *daf-2* mutants such as miR-62, miR-252, and miR-237, showing the greatest upregulation in young *daf-2* mutants when compared to wild-type worms of the same age (Lencastre et al. 2010). Further experiments by the authors showed some of these miRNAs to be actually important in lifespan as well as stress resistance. Specifically, miR-71, miR-238, and miR-246 mutants had reduced lifespan whereas miR-239 deletion produced significant extension in lifespan. Different stress assays were also performed in these mutants, and *mir-239* mutants exhibited enhanced oxidative and heat stress resistance, while *mir-71* mutations resulted in increased sensitivity to both of these stresses. Additional genetic pathway analysis by the authors could predict plausible role of these miRNAs in acting via IGF-1 and DNA damage response checkpoint pathway (Lencastre et al. 2010).

Some studies at the single gene level have supported previously described findings of miRNA acting in worm aging, for example, *mir-71* acts in neurons to promote germline-mediated longevity (Boulias and Horvitz 2012). In addition, *mir-71* expression in neurons was sufficient to induce nuclear localization of DAF-16 in the intestine, suggesting a cell non-autonomous mode of action. Another study by Shen et al. (2012) showed the role of *let-7* miRNA in aging and found its expression to be upregulated in specific larval stage (L2–L3 transition) by steroid hormone receptor (DAF-12) signaling. *let-7* was shown to target *lin-14* and *akt-1/2* kinase to promote DAF-16-mediated longevity.

13.11 Aging in Drosophila

Similar to studies in *C. elegans*, multiple pathways such as insulin signaling, calorie restriction (CR), and sirtuins have been found to be important in fly aging as well (Zahn and Kim 2007). Fly as a model system offers similar advantages for aging studies such as cheap, easy maintenance, short lifespan, and complete genome sequence availability. However, one distinction in terms of its additional advantage compared to *C. elegans* is the ability to dissect different tissues and organs to determine similarities and differences in various organs/tissues during aging process. In this section, some studies reporting transcriptional profiling of aging pathways in *Drosophila* are described.

13.11.1 Microarray Study Reveals Genes in Normal and DR-Mediated Aging Processes in Flies

A study by Pletcher et al. (2002) revealed 885 genes with differential expression in young and old flies, while comparison of normal fed vs. calorie-restricted flies showed 827 genes to be different in expression. Expression of genes belonging to stress response and oogenesis changed with age, while those involved in cell growth,

metabolism, and reproduction were downregulated with calorie restriction. Specifically, genes involved in DNA replication, DNA repair, formation of replication fork, chromosome condensation, chromosome segregation, and other cell cycle processes were downregulated under low-calorie conditions. Protein metabolism and ubiquitin degradation pathway genes had lower expression under dietary restriction conditions. In contrast, genes upregulated under low-calorie conditions did not fall into specific categories with genes performing broad range of functions in RNA processing, RNA metabolism, cell communication, abiotic stimulus, and radiation response showing increased expression under CR.

Age-dependent profiling also revealed a number of genes that showed altered expression. For example, many of the genes encoding proteins localized to the mitochondrion and those involved in ETC (ubiquinol cytochrome-c reductases) were downregulated with age. PGPRs (peptidoglycan recognition proteins) involved in biotic stimulus perception showed 2–3-fold increase with age, while other proteins involved in defense response encoded by genes such as *Defensins* and *Relish* had a constant expression throughout its lifespan suggesting mechanisms that could be important in maintaining steady expression of these genes and importance of immune response pathways in aging flies (Pletcher et al. 2002).

Landis et al. (2004) found support for oxidative theory of aging upon comparing flies grown under 100 % oxygen with aged flies that revealed similar pathways regulated under both conditions. DNA microarray comparison of young (10-day-old) vs. old (61-day-old) flies with that of 3-day-old flies (treated with 100 % oxygen for 7 days) revealed upregulation of heat shock protein, antioxidant, purine biosynthesis, and innate immune response genes with both age and oxidative stress. This observation supports the correlation between aging and stress pathways. *hsp-70*, *hsp-22*, and *hsp-23* exhibited upregulation with age, e.g., *hsp-70* induction in response to oxidative stress treatment was dependent on Cu/Zn SOD, and mutations in this enzyme resulted in increased expression of *hsp-70*. Immune response genes regulated by both oxidative stress and age included *Defensin*, *Attacin A*, and *PGRP-LC*. The entire purine biosynthetic pathway, with genes such as *Ade3* and *Nmdmc*, showed increased expression under both conditions. In contrast, pyrimidine biosynthesis genes did not show upregulation probably because of the fact that purines are more susceptible to oxidative damage and therefore have greater need for replacement. Another plausible reason could be increased requirement of ATP/NADP (H) with age and oxidative damage. Downregulated genes with age and oxidative stress condition included proteases, lipases, and proteasome subunits, reflective of the fact that with increased age, there is a reduction in protein synthesis and turnover in flies.

Besides the above-described similarities, differences in gene expression were also found between aged and oxidative stress-treated samples. Genes involved in energy metabolism showed broad downregulation with age. Specifically, oxidative phosphorylation (42 genes), TCA cycle (13 genes), and ATP synthetase (9 genes) were reduced suggesting of breakdown of mitochondrial function with age. GFP reporter and *lacZ* reporter constructs for *hsp-22* and *hsp-70* and immune response genes such as *Drosomycin* revealed a change in expression of these genes with age and stress, providing validation for some of the target genes identified in this study using GeneChip array.

13.11.2 Tissue-Specific Changes in Gene Expression with Age

Girardot et al. (2006) performed microarray studies in head, thorax, and whole flies to identify both common and specific pathways acting in these different tissues. In flies, thorax and head represent sites where age-related degeneration is known to occur and therefore could reveal gene expression changes important in the aging process. Overall, the authors found significant number of stress-responsive genes that showed alteration with age supporting the notion that these two processes may indeed be related and have possible cross talk with each other. Consistent with this idea, genes that showed changes with age were also previously shown to be responsive to stress treatments such as paraquat, hydrogen peroxide, and tunicamycin (Girardot et al. 2006). Besides these similarities, opposite trends were observed in age- and stress-related changes. For example, significant proportion of stress downregulated genes was found in both age upregulated and age downregulated category (Girardot et al. 2006). Also, many of the early bacterial infection-induced response genes were repressed with age. These observations suggest that aging cannot be simply viewed as an increased expression of stress response and immune response genes.

Several genes (234) were specifically downregulated in the thorax when compared to the head and whole body. Significant downregulation of genes involved in mitochondrial function, ATP synthesis, and MnSOD (superoxide dismutase, *sod2*) suggested that impairment of oxidative stress response and mitochondrial activity occurs in thoracic region and muscles compared to other tissues. Of the genes that fall in the upregulated category, an enrichment of genes involved in cell morphogenesis, immune response, components of endoplasmic reticulum, and proteasome function was observed. As discussed by the authors, many of these changes reflect an activation of JNK signaling pathway in aging flies. For example, induction of *Jra* (JUN homologue in flies) in thorax is an indication of this fact. Also, previous SAGE studies showed many stress-responsive genes regulated by JNK signaling to be upregulated with age that were also found to be enriched in thorax in this study (Girardot et al. 2006).

Expression studies in the fly head showed downregulation of genes involved in synaptic transmission. These genes could be grouped in three different categories; the first category included genes involved in neurotransmitter metabolism such as choline acetyl transferase and dopamine *N*-acetyl transferase. The second category included genes involved in different steps of neurotransmitter release such as synaptic vesicle fusion (*unc-13*, *comatose*), presynaptic fusion (*Csp*, *rab3-GAP*), and reformation of vesicles after fusion via endocytosis (*AP-50*, *AP-20*). The last category of genes included those encoding receptor ion channels such as two nicotinic acetylcholine receptors and three ionotropic glutamate receptors. Upregulated gene category in the head involved those similar to whole flies such as immune function and amino acid metabolism. Taken together, the expression studies in the head revealed a large downregulation of genes involved in synaptic transmission and function.

Of the above-described genes, functional relevance of SOD2 was described by Kirby et al. (2002), who showed that RNAi-mediated ablation of mitochondrial SOD2 produces increased mortality and sensitivity to oxidative stress in flies.

This increased oxidative stress disrupts enzymatic components of TCA cycle and mitochondrial respiratory chain, producing early onset of mortality in young flies but has no effect on development (Kirby et al. 2002). This function of SOD2 was found to be true by other groups as well (Paul et al. 2007), where deletion of SOD2 caused accelerated senescence in olfactory behavior and DNA damage in sensory neurons producing neurodegeneration in flies.

13.11.3 TOR Pathway-Mediated Regulation of Aging in Flies

TOR signaling-mediated benefits in lifespan based on nutrients were first uncovered in flies (Katewa and Kapahi 2011). The TORC1 complex, which is rapamycin sensitive, controls multiple processes such as protein synthesis, transcription, translation, and autophagy, while TORC2 mediates cell growth and proliferative effects depended on AKT signaling (Um et al. 2006; Jacinto et al. 2004). Different components of TOR complex have been shown to be involved in aging and stress-related functions in *Drosophila* (Katewa and Kapahi 2011). Inactivation of dTOR or activation of upstream negative regulators of TOR (dTSC1 and dTSC2) produces a 30 % increase in lifespan (Kapahi et al. 2004). Inhibition of TOR by rapamycin also produces ~10 % increase in lifespan (Bjedov et al. 2010). Other components of the TOR pathway, such as S6K (downstream of TORC1), when expressed in dominant negative form enhance oxidative stress resistance in flies, while expression of constitutively active form increases sensitivity to oxidizing agents suggesting a role for TOR in stress phenotypes as well (Patel and Tamanoi 2006). *d4EBP-1* inhibits mRNA translation downstream of TOR and mediates resistance to oxidative stress and starvation (Tettweiler et al. 2005). Some studies in *C. elegans* and yeast have shown transcription factors such as PHA-4, HIF-1, and MSN2/4 to be important in mediating stress resistance and are regulated by TOR (Katewa and Kapahi 2011).
To examine what might be happening at a global translation level downstream of TOR that is inhibited under nutrient-limiting conditions, a genome-wide profiling of translation status of mRNAs was undertaken (Zid et al. 2009). A number of mRNAs encoding mitochondrial electron transport chain (ETC I and IV) and mitochondrial ribosomal proteins were found to be increased suggesting enhanced ribosomal loading of these mRNAs under nutrient-limiting conditions (Zid et al. 2009). However, the exact details of how this occurs and what might be the possible contribution of these genes in TOR and dietary restriction-mediated lifespan remain to be investigated.

13.11.4 MicroRNA-Regulated Aging Processes in Drosophila

Like in *C. elegans*, microRNAs regulate various age-associated processes in flies, besides performing their canonical roles in development (Liu et al. 2007). A conserved microRNA, *miR-34*, was found to be important in regulating longevity as

well as brain integrity in flies. *miR-34* loss caused decrease in survival and late onset of neurodegeneration, while upregulation of *miR-34* increased median lifespan and protected flies from neurodegeneration caused by human polyglutamine disease protein. Some of these aging-associated effects of *miR-34* required repression of adult onset ETS transcription factor Eip74EF involved in steroid synthesis pathway. Taken together, the study demonstrated the role of microRNAs in delaying adult onset of aging by repressing genes involved in development that have harmful effects later in life (Liu et al. 2007).

Another study by Karres et al. (2012) found miR-8 to be directly targeting atrophin levels in flies, preventing a neurodegenerative state DRPLA (*dentatorubral-pallidoluysian* atrophy). Loss of *miR-8* increased atrophin levels that increased apoptosis and behavioral defects. *miR-8*-mediated regulation of atrophin was found to be conserved in mammals as well (Karres et al. 2012).

Additional studies have shown changes in miRNA isoform expression (Abe et al. 2014) with age in *Drosophila*. The authors showed an increase in 2-O′ methylation of some of the miRNA isoforms that increased expression with age. This increased methylation was further found to be associated with increased loading of miRNA transcripts on Ago2 (members of Argonaute family) rather than Ago1 complex with age as revealed by small RNA deep sequencing. Further, *Ago2* mutations that decreased this methylation with age resulted in neurodegenerative phenotypes suggesting that this differential partitioning between Ago1 and Ago2 with age is playing a critical role in preventing decline in brain function occurring with age (Abe et al. 2014).

13.12 Mammalian Aging

13.12.1 Transcriptional Changes with Age in Mice

Age-associated changes in mice have been studied in the heart, muscle, adipose tissue, and macrophages (Zahn and Kim 2007). A study by Edwards et al. (2007) identified 712 transcripts that are age regulated in mice muscle, and this class of genes was enriched for genes involved in p53 apoptotic pathway and those involved in mitochondrial respiration and energy generation, suggesting activation of p53-mediated pathways possibly due to DNA damage response with aging. Decreased expression of p53-regulated genes was found in muscle of calorie-restricted mice hinting toward the existence of p53 as a biomarker for aging.

Additional studies by Bahar et al. (2006) reported an increase in gene expression noise with age in mice cardiomyocytes upon comparison of old mice with young mice showing large variation in transcription with aging. This study suggests a general loss of transcriptional control with age that may ultimately lead to cellular dysfunction and senescence.

Few such studies have also been performed with human samples; however, such studies are difficult to conclude due to large variation in genetic background and environmental parameters that each person is exposed to.

13.12.2 Common Transcriptional Profile in Aging Human Muscle

Transcriptional profiling of muscle from humans of different age has revealed a common gene signature and pathways that are similarly regulated in different human tissues (Zahn et al. 2006). Common genetic signature revealed upregulation of four genetic pathways with age (genes involved in cell growth, extracellular matrix, complement activation, and cytosolic ribosome) while two pathways were downregulated (genes involved in chloride transport and genes involved in mitochondrial ETC). ETC gene reduced expression with age in mouse and flies as well, suggesting that it could be a universal biomarker for aging. Besides muscle, kidney and brain tissue samples were also studied, and genes coding for in cytosolic ribosome (85 genes) were commonly upregulated in all the three tissues with age, suggesting the possibility that old cells upregulate ribosome gene expression as a mechanism to deal with reduced translation efficiency with age. Enhanced expression of extracellular matrix protease genes with age could account for what is observed as fibrosis in the elderly, reflecting tissue proliferation and impairment of function. Taken together, the study demonstrated common aging profile pattern in different human tissues suggesting that core/central signaling pathways could be important in different organs that may help us determine the physiological age of the tissue and in theory; reversing this gene signature could also help in preserving "youthfulness" of the tissue that may also increase longevity. Lastly, except ETC, most of the other pathways appeared to be "primate" or human specific, highlighting the necessity and importance of studying aging pathways in humans as opposed to model organisms that may not completely represent the aging process in mammalian species.

13.12.3 Proteomic Studies in Mammalian Aging

Advancement in large-scale protein analysis and different proteomic approaches has opened new avenues for identification of proteins that alter with age that may provide potential biomarkers and therapeutic targets for aging besides improving our understanding of basic mechanisms underlying the process of aging. Studies employing 2D gel electrophoresis comparing expression of proteins in rat neural retina have identified 4 and 18 proteins that increase and decrease, respectively, with age using MALDI (matrix-assisted laser desorption/ionization) time-of-flight (TOF) MS peptide mapping. Interestingly, as a direct relevance of these proteins in aging, some of them, such as αB-crystallin that increased in retina with age, decreased upon dietary restriction (Li et al. 2004). Mao et al. (2006) found specific changes in expression of ETC components with age in proteome of the mouse brain and liver. Complex IV and Complex I subunits showed decreased expression, while one subunit each of Complex III and Complex V showed increased expression. Besides, a total of 85 proteins, involved in antioxidant pathways, amino acid metabolism, and signal transduction, were also differentially expressed with age in the mouse liver, further highlighting the importance

of 2D gel electrophoresis technique in identifying age-related changes in an organism's proteome. Chen et al. (2003) performed 2D MALDI TOF analysis of human postmortem brain and found five proteins (peroxyredoxin2, two stathmins, and Apo A-1 precursors) to be downregulated when compared to young tissues. A pioneering study comparing human skin tissue from young and old found interferon-γ-induced proteins in primary human keratinocytes from the elderly (Gromov et al. 2003). Among those identified to be dysregulated with age were MnSOD and p85-β subunit of phosphatidylinositol-3-kinase showing the role of interferon γ in aging. Besides changes in absolute protein levels with age, different modifications that occur on the protein with age can also provide important clues to alterations in protein activity and function mediated by such posttranslational modifications (PTM) as organisms/humans grow older. Acetylation, phosphorylation, O- and N-linked glycosylation, methylation, and ubiquitinylation are some of the commonly occurring PTMs on proteins that may be identified using modification-specific antibodies coupled with MS approach (Sharov and Schöneich 2007). One such study (Butterfield and Poon 2005) identified changes in protein oxidation that occur with age in normal vs. DR-treated mouse brain and several models of mice with accelerated aging. Specific carbonyl group levels in proteins such as pyruvate kinase M2, alpha enolase, and F1 ATPase were found to be decreased in DR-treated mice. Also, 3NT (nitrotyrosine) levels of malate dehydrogenase and phosphoglycerate kinase were found to be significantly decreased in CR-treated mice compared to age-matched control. Analysis of brain tissue in CBL57/6 mice strain revealed increased carbonyl levels in beta actin, glutamine synthase, and neurofilament66 in old compared to young mice. Another study by Kim et al. (2006) analyzed 3NT and HNE (4 hydroxynonenal) modifications on histidine of serum proteins in rat and found several proteins such as alphamacroglobulin and serotransferrin to increase in these PTMs with age, reflecting a decline in function of these proteins with age. Future studies employing these sophisticated proteomic techniques may help reveal proteins that actually change in expression or PTM with age in whole organism as well as specific tissues.

13.12.4 TOR in Mammalian Aging

TOR complex as described above performs a critical role in sensing cellular energy and nutrient status and regulates a vast variety of functions/processes including aging and age-associated pathologies such as cancer and neurodegenerative diseases (Johnson et al. 2013). TOR1 and TOR2 were first identified in yeast as genes required for rapamycin-mediated inhibition of growth. Rapamycin, originally identified as an antifungal agent, was used as an immunosuppressive drug, helped uncover the TOR signaling complex (TORC1 and TORC2). mTOR was subsequently purified from mammals and shown to physically interact with rapamycin (Laplante and Sabatini 2012). mTOR has two complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) composed of different constituent proteins and regulate different downstream processes (Johnson et al. 2013). mTORC1, a rapamycin-sensitive complex, inhibited by rapamycin-bound

FK506-binding protein (FKBP12), promotes mRNA translation via two target proteins—S6K (ribosomal protein kinase) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). It also represses autophagy, regulates lipid biogenesis, and controls mitochondrial function and glucose metabolism via HIF-1α (hypoxia inducible transcription factor). Various studies have supported a role for TOR pathway in aging. Mice fed on rapamycin, late in life (600 days old ~60 years of human age), had an extended lifespan, while those fed from 6 months of age produced a small effect in mean lifespan (18 % in females and 10 % in males) (Harrison et al. 2009; Miller et al. 2011). Another study also showed that S6K1 knockout mice have an extended lifespan in females but not in males, suggesting a sex-specific phenotype (Selman et al. 2003). Role of mTORC1 inhibition in longevity also extends to benefits in delaying diseases in different mice models. For example, mice models of neurodegenerative disease such as Alzheimer's and frontotemporal lobar dementia exhibit delayed impairment of brain disease upon mTORC1 inhibition (Spilman et al. 2010; Wang et al. 2012). Some mice studies have also shown reduction in cardiac myopathies and reduced hypertrophy with mTORC1 inhibition (Shioi et al. 2003; Ramos et al. 2012).

A recently published study by Fok et al. (2014) employed microarray to reveal transcriptomic changes in 13 pathways in rapamycin-treated female mice liver showing extended lifespan. Among the downregulated pathways were those involved in cancer such as estrogen receptor signaling pathway and VEGF pathway (involved in angiogenesis during cancer progression). Interestingly, almost half (6 out of 13) of the pathways that appeared to be regulated by rapamycin were aging related such as NRF-2-mediated oxidative stress signaling, protein ubiquitination, mitochondrial function, and IGF-1 signaling (Fok et al. 2014). Further studies in mammals would help to further understand the downstream pathways altered by TOR that mediate major changes in age-related phenotypes and pathologies.

13.12.5 Role of MicroRNAs in Aging in Mammals

Like in *C. elegans* and *Drosophila*, microRNA expression changes that occur with age have been studied to some degree in mammals as well. Inukai et al. (2012) employed deep sequencing of young and old mouse brain to identify 75 known and 18 novel miRNAs that exhibit changes with age. Most of the miRNAs showed a decreased expression in aged mouse brain. As a further validation of miRNAs identified in this study and their relevance to aging, KEGG (Kyoto Encyclopedia of Genes and Genomes) was used to identify potential targets of these miRNAs. Many of the potential targets of these miRNAs were those in the insulin signaling pathway. Specifically, MiR-45 that is known to target *Irs-1* and *Irs-2* (insulin receptor substrate) and MiR-375 that targets *Pdk-1* (kinase in IGF-1 pathway) showed strong downregulation with age validating the importance of the identified microRNAs in aging via modulation of known pathways (Inukai et al. 2012). Some of the other miRNAs identified in the study, miR-101 and miR-433, showing significant decrease with age have been implicated in neurodegenerative diseases such as

spinocerebellar ataxia type 1 (SCA1) and Parkinson's disease, respectively (Lee et al. 2008; Wang et al. 2008).

A study by Zhang et al. (2012) reported changes in miRNA with age in mouse heart, using old and young mice heart tissue, identified miRNAs and miRNA*(passenger strand), clustered into 11 groups with 2–71 miRNAs in each group. Fifty five percent of the miRNAs showed increase in expression, while 45 % showed a decline in expression with age. miR-21 was one of the transcripts whose increased expression was confirmed via in vivo transfection experiments to be mediated by an increase in Ago1 and Ago2 at the protein level (Zhang et al. 2012). Furthermore, some of the miR transcripts identified in the study, miR-494 and miR-106b, have been shown to target IGF2BP1 and IGF-1 signaling to promote senescence and neuron growth, respectively (Ohdaira et al. 2012; Brett et al. 2011) suggesting their relevance in known aging pathways.

13.13 Plant Senescence and Aging

13.13.1 Defining Plant Aging and Senescence

Plant aging though sparsely understood as compared to aging in other organisms is important to understand as broad range of lifespan observed in plant species from few weeks (Yellowwood anemone) to several thousand years in Bristlecone pine is indicative of underlying differences at the molecular level that may yield interesting insights into mechanisms of aging. Aging in plants is a complex phenomenon as different species demonstrate different patterns of life and death cycle (plants referred to as semelparous die after mating and reproduction), and even within plants, different tissues may display large differences in the degree of aging and senescence (e.g., deciduous trees where plant body is made of dead tissues while the canopy is renewed every year, and unlike semelparous plant species, they reproduce repeatedly) (Thomas 2013). Plant longevity is determined by activity of root meristems, terminal shoots, and lateral meristems at each node of the plant axis. Leaf senescence, the most well-studied process in plant aging, is a programmed pathway that is accompanied by various phenotypic changes (chlorophyll loss and yellowing) and alterations in gene expression at the molecular level (Weaver et al. 1998). Many genes known as the senescence-associated genes (SAGs) are known to be induced by darkness, detachment, drought, and hormones such as abscisic acid (ABA) (Weaver et al. 1998). Some degree of overlap exists between stress- and senescence-induced changes in gene expression suggesting commonality between pathways regulating both these processes (summarized in Fig. 13.2). Plant aging could be looked at from different perspectives in terms of vast variation observed at the level of nutrient source, reproduction means, and senescence. Differences in these physiological processes among plant species could also explain and result in vast differences in rate of aging and senescence observed across the plant kingdom, and a few of the cases are described below.

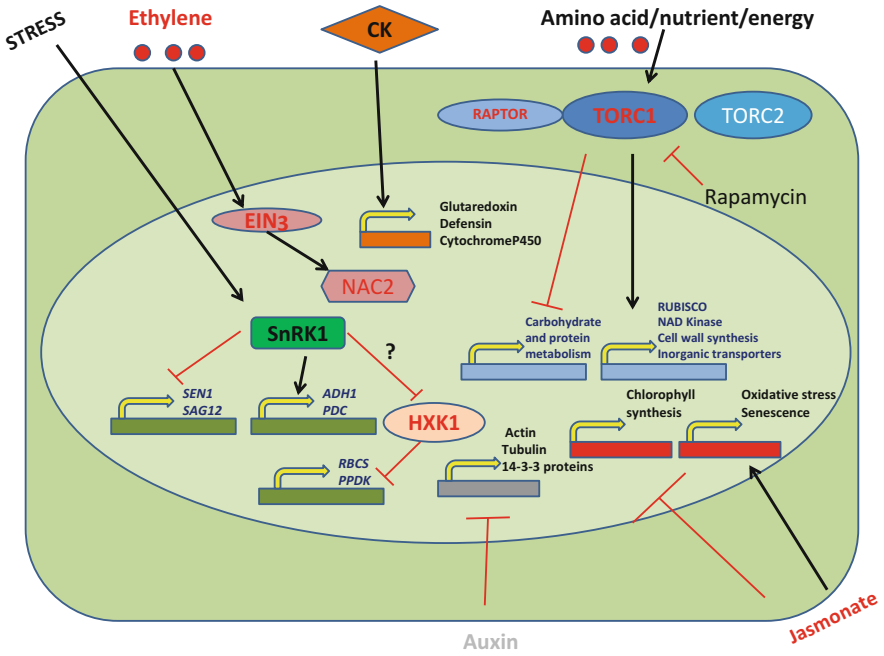

Fig. 13.2 Signaling pathways and downstream genes implicated in plant stress and senescence. Different environmental cues in the form of stress and nutrition are perceived and transduced by plants at the cellular level by a host of different proteins and hormone production. As depicted in this model, SnRK1 (SNF-1-related kinase) integrates flooding and starvation stress to modulate gene expression of senescence- and stress-associated genes. Possible negative regulation of HXK1 (hexokinase) by SnRK1 also contributes to its anti-senescence effects via downstream regulation of genes involved in carbon fixation. TOR signaling branch mediated by TORC1 has been shown to be negatively regulating plant senescence and longevity via possible differential regulation of genes involved in anabolic and catabolic pathways. Hormones act to integrate environmental and developmental signals to either inhibit or promote senescence by causing major changes at the transcriptional level. ABA, ethylene, and jasmonic acid promote senescence, while cytokinin has antiaging role in plants. Auxin besides playing an important role in plant growth and development can either promote or inhibit plant senescence. Genes/proteins that inhibit senescence are shown in *black,* while those promoting senescence are in *red. SEN1* senescence1, *SAG12* senescence-associated gene 12, *ADH1* alcohol dehydrogenase 1, *PDC* pyruvate decarboxylase, *TORC* target of rapamycin complex, *RBCS* ribulose 1,5 bisphosphate carboxylase small subunit, *PPDK* pyruvate phosphate dikinase, *CK* cytokinin

13.13.2 Autotrophy vs. Heterotrophy

Differences in resource allocation within the plant body could have significant consequences on aging and longevity (Thomas 2003). Heterotrophs that depend upon external source of nutrition have constant constraint in terms of balancing their need for repair and maintenance with reproduction, which is different from that of green plants. However, no strict pattern has been reported by Obeso (2002) who found that dioecious woody plants follow this rule, while dioecious herbs do not, which suggest a more complex interaction between growth, reproduction, and aging as a trade-off mechanism.

13.13.3 Starvation as a Cause for Plant Aging

Besides intrinsic parameters such as development, reproduction, and hormones, environmental factors may also trigger senescence (Thomas 2013). One of such factors is starvation due to unavailability of minerals and nutrients that may trigger the senescence-related pathways and processes (Thomas 2003). Nitrogen assimilation and relocalization is one of the well-studied processes that are altered with senescence (Thomas et al. 2002). Nitrogen uptake and localization varies among species depending on N availability in soil. In Arabidopsis, under low nitrogen conditions, most of the mineral is allocated to seeds post flowering (Masclaux-Daubresse and Chardon 2011). However, under high N conditions, part of it was allocated to rosette leaves. Nitrogen mobilization upon starvation involves three crucial processes: activation of senescence-associated proteases, chloroplast dismantling, and autophagy (Desclos et al. 2009; Kato et al. 2004; Guiboileau et al. 2012). Senescence activates proteases within the cell that promote degradation of proteins, resulting in nitrogen release and mobilization. Desclos et al. (2009), Roberts et al. (2012) employed N^{15} labeling/proteomic approach to identify HSP-70 and disulfide isomerase as proteins that were induced under senescence in *B. napus* to possibly prevent protein aggregation. Additional support for nitrogen mobilization under senescence has come from studies showing *Arabidopsis lap2* mutants, lacking aminopeptidase that acts on N-terminal leucine, methionine, and phenylalanine (Waditee-Sirisattha et al. 2011). *lap2* mutants show premature senescence, which could be explained by defective degradation of proteins and reduced levels of nitrogen-rich amino acids (Waditee-Sirisattha et al. 2011; Peoples and Dalling 1988).

Chloroplast disruption, which harbors about 75 % of the total leaf protein, is one of the first events during senescence and starvation. Aspartate protease CND41 present in chloroplast has been implicated in degradation of denatured Rubisco (Kato et al. 2004). Rubisco amount in chloroplasts exceeds that needed for photosynthesis, and thus, a nitrogen storage function has been suggested for it (Feller et al. 2008).

Autophagy also performs a critical role in nitrogen mobilization upon starvation as concluded by hypersensitivity to nitrogen starvation in *atg* mutants defective in autophagy (Guiboileau et al. 2012). N^{15} tracing studies showed reduced nitrogen mobilization in *atg* mutants suggesting a role for autophagic machinery in promoting degradation of toxic proteins within the cell under starvation stress, facilitating nitrogen release (Guiboileau et al. 2012).

A study by Xiao et al. (2010) found a role for *Arabidopsis* acyl-CoA binding protein (*ACBP3*) in starvation- and age-induced leaf senescence. ACBP3 expression was observed to increase with darkness and in senescing rosette leaves. Transgenic *Arabidopsis* lines overexpressing (OE) ACBP3 were found to be showing premature senescence, while RNAi-mediated knockdown of ACBP3 resulted in delayed dark-induced senescence. Measurement of Acyl CoA and lipid profiling in transgenic lines revealed an increase in phosphatidylethanolamine (PE) in ACBP3 overexpression lines, while RNAi loss-of-function lines had decrease in PE content (Xiao et al. 2010). Detailed analysis further showed an increase in phosphatidic acid (PA) and arabidopsides (oxylipin containing galactolipids) in ACBP3 OE lines.

ACBP3 was believed to be promoting senescence by increase in PA and arabidopside molecules, by promoting lipid peroxidation. Further, the authors could also observe disruption of autophagy-related gene ATG8 upon ACBP3 overexpression that disrupted the autophagosome formation by possibly modifying membrane phospholipid metabolism. As discussed by the authors, disruption of nutrient cycling by ATG proteins that conjugate with PE upon alterations in ACBP levels could explain the phenotypes observed in the ACBP3 overexpression and RNAi knockdown lines.

13.14 Microarray Identifies Nutrient Starvation-Induced Expression Changes of Stress-/Senescence-Related Genes

Lack of different mineral nutrients that are required for plant growth and signaling can also trigger major changes in gene expression as found by different groups that performed microarray under phosphate, nitrate, iron, and potassium deficiency (Takehisa et al. 2013). Phosphate deficiency was altered 1,800 genes by more than twofold within 72 h of treatment in *Arabidopsis* (Wu et al. 2003). Phosphate (Pi) starvation was found to produce changes in metabolic genes (carbon/nitrogen metabolism) and genes involved in senescence, stress, photosynthesis, as well as protein degradation suggesting that diverse functions are regulated by Pi. ATP-generating light reaction components PSI, PSII, and chlorophyll a/b binding proteins were found to be downregulated by 2–7-fold. Of the 111 transcription factors that were regulated by phosphate starvation, four AP2 family transcription factors were identified, implicated in stress responses and function (Wu et al. 2003). Sixteenfold upregulation of a CDPK gene was detected that could also be potentially important in stress/senescence effects of phosphate deficiency; CDPKs have been shown to be important in dealing with a variety of stressors such as low/high temperature, wounding, and drought (Chico et al. 2002). *SEN1* (SENESCENCE1) transcript showed 63-fold upregulation at 48 h time point that is also known to be induced by ABA treatment (Oh et al. 1996).

Nitrogen, which is an important macronutrient for plants, also induces major transcriptional changes upon starvation (Krapp et al. 2011). Global spatiotemporal analysis of nitrogen-starved organs in *Arabidopsis* identified 638 and 772 genes expressed differently in root and shoot, respectively. More than hundred genes (142) were differentially expressed in both tissues, and out of these, 20 genes displayed opposite trend. Analysis by the authors revealed significant changes in expression of RNA processing and stress response genes upon long-term nitrogen starvation. Specifically, miRNA169 (a microRNA responsive to N starvation) target genes encoding nuclear factor YA subunits were upregulated that are also involved in drought tolerance (Li et al. 2008). Another study (Cai et al. 2012) performed microarray to identify transcripts that are expressed differentially under N starvation in rice at different time points. At 1 h time point, 48 stress-responsive genes were upregulated and 49 genes were downregulated. At 24 h time point, 26 stress genes

were upregulated while 22 such genes were downregulated, suggesting plausible existence of common transcription factor network acting to bring about these changes upon nitrogen depletion. In addition, miR-350 and miR-399 were also found to be showing altered expression suggesting a conserved role of miRNAs under nutrient stress across species (Cai et al. 2012).

A comprehensive analysis of genes acting under iron-deficient conditions was examined by Higuchi et al. (2011) in barley leaves. The authors found changes at the mRNA level of ribulose 1,5 bisphosphate carboxylase/oxygenase, nitrite reductase, sulfite reductase, and ferredoxin-dependent glutamate synthase that appeared to be decreased. In addition, cell wall, protein degradation, senescence, and ubiquitin genes were upregulated suggesting controlled degradation in leaf cells under Fe-deficient conditions. These observations were consistent with earlier study showing accelerated leaf senescence under low iron conditions in barley (Maruyama et al. 2005).

Shankar et al. (2013) used microarray to identify genes responsive to low potassium conditions in rice seedlings including stress response genes such as GST, heat shock protein, and oxidoreductase, which were found to be altered under low K^+ conditions suggesting activation of oxidative stress response triggered by nutrient deprivation. A total of 55 stress genes showed differential expression including 5 oxidoreductases and 4 GSTs with higher expression under potassium-deficient conditions. Eleven heat shock protein genes and five dehydration-related genes with nine HSPs exhibited reduced expression (Shankar et al. 2013). Other similar studies in *Arabidopsis* and rice have also found altered expression of polyamine synthesis, glutathione reductase, dehydroascorbate reductase, cold responsive, and JA signaling genes under potassium starvation (Armengaud et al. 2004; Ma et al. 2012; Takehisa et al. 2013).

Taken together, transcriptional profiling under nutrient stress examined by different groups revealed changes in metabolism-, stress-, and senescence-related genes. This points toward the possibility that nutrient stress like other abiotic stresses alters plant cell physiology in a manner that evokes cytoprotective mechanisms to deal with the deleterious effects of starvation and senescence.

13.15 Reproductive Development and Aging

A clear role for reproduction and its link to plant longevity has been speculated (Thomas 2013). It is well known that plants exhibit different life forms with annual plants completing their vegetative and reproductive cycle in 1 year, while biennial have vegetative growth in the first year and reproduction in their second year. The perennial plants on the other hand complete their life cycle in many years that extends from less than 10 years in herbaceous species to several thousand years in woody conifers (Thomas 2013). In polycarpic perennial species, reproduction and senescence are not intimately linked, and asexual reproduction produces what are called as clones within an older plant. Plants continue to survive as long as actively

proliferating tissues can keep ahead of old, senescent tissues in different life forms. Senescence processes have mostly been studied in monocarpic species that die after reproduction and seed set. It has been shown that reproducing tissues carries away the nutrients from vegetative tissues and triggers senescence (Davies and Gan 2012). Monocarpic plant species mostly but not always have two phases of aging: the first associated with vegetative growth where certain parts of the plant such as leaves undergo senescence with nutrient mobilization from old to young leaves and the second phase is that of reproduction where there is complete transfer of nutrients to the developing seed and the whole plant ultimately dies (Bosch 2011). Examples of monocarpic species exhibiting this form of life cycle include *Zea mays* and *Glycine max*. Aging and senescence in perennial plants have not been studied in great detail partly due to unavailability of genetic tools for woody plants (Bosch 2011). However, the general consensus for extreme longevity in these plants is that they retain the indeterminacy in some of their vegetative meristems that remain dormant for certain periods of time before resuming growth (Bosch 2011). Meristem in perennial plants retains the ability to initiate new shoot even at an advanced age suggesting existence of far greater regenerative capacity in these plants as compared to monocarpic species (Rohde and Bhalerao 2007).

Studies reporting changes that occur at genetic, biochemical, cellular, and molecular level during leaf senescence suggest similarities in this process in both monocarpic and perennial plants. Different environmental cues are responsible for leaf senescence such as ozone, extreme temperatures, nutritional deficiencies, and pathogen attack. With completion of genome sequencing of *Populus trichocarpa* and development of various genetic tools, it has become a model system for studying different processes including senescence in deciduous species *Populus*. The role of *phytochrome A* (*PHYA*) and *phytochrome B* gene (*PHYB2*) has been found in inducing leaf senescence each season during autumn with shortening of the photoperiod (Olsen and Junttila 2002). Massive changes in gene expression, chlorophyll breakdown, activation of enzymes, and nutrient mobilization have been found to be accompanying leaf senescence in perennial species suggesting strong similarity in leaf senescence with monocarpic species.

In conclusion, the differences in monocarpic and perennial species in terms of aging come from differences in activity of meristem and how its totipotency is maintained at the genetic level that in turn determines the rate of senescence and ultimately death in these different plant forms.

13.16 Functional Genomic and Proteomic Studies Identify Important Regulators of Plant Senescence

Microarray and newer techniques such as RNA-seq have been employed to study developmental and environment-induced changes at transcript level during leaf senescence (Li and Guo 2014). Guo et al. (2013) performed sequencing of senescent leaf cDNA and identified 134 gene encoding transcription factors to be

expressed. A total of 182 gene encoding components of different signaling pathways such as MAPK and receptor-like kinase (RLK) were found. In addition, 116 genes involved in degradation pathways such as ubiquitin proteases and proteinases were found that could be involved in chlorophyll and organelle breakdown that occurs during senescence. Breeze et al. (2011) performed high-resolution microarray analysis of *Arabidopsis* leaves and identified 6,323 differentially expressed target genes that were further clustered into 48 groups based on their expression pattern at 22 time points. Among the identified processes were signaling of senescence-associated hormones, upregulation of senescence-associated transcription factors, and downregulation of chlorophyll synthesis genes as early part of the senescence (Breeze et al. 2011).

RNA-seq methodology employed by Kong et al. (2013) identified differentially regulated genes in age-matched early (K1) and late (K2) senescence cotton line. A total of 1,132 genes were found to be upregulated, and 455 genes were downregulated in K1 compared to K2. Among the upregulated gene category were those involved in catabolism of nucleic acids, lipids, and proteins, while downregulated genes involved those in photosynthesis and anabolism. In addition, several NAC and WRKY transcription factors were found to be upregulated (Kong et al. 2013). The gene expression changes comprise downstream process initiated by transcription factors that bind to their respective promoter to regulate expression. Multiple transcription factor families such as NAC, WRKY, AP2, GRAS, and bZIP have been found to be upregulated with senescence (Guo et al. 2004). Few large-scale studies have identified specific genes regulated by these transcription factors. For example, AtNAC092 (member of NAC family), a positive regulator of senescence, was shown to induce 170 genes and downregulated 48 genes, using microarray studies (Balazadeh et al. 2011). Like NAC, WRKY transcription factor family also acts to regulate leaf senescence (Rushton et al. 2010). WRKY53, a positive regulator of leaf senescence, was shown to bind to promoter region of 63 target genes via genomic pull-down assays (Miao et al. 2004).

Hebeler et al. (2008) used quantitative proteomics to identify proteins that change in expression during early leaf senescence. The authors utilized reciprocal $^{14}N/^{15}N$ labeling of the whole *Arabidopsis thaliana* plants coupled with two-dimensional gel difference electrophoresis followed by MS analysis of nine biological replicates using wild-type plants compared to *old1-1* mutants that display premature leaf senescence. The study identified different subunits of ribulose-1,5-bisphosphate (Rubisco) to be reduced, which reflects breakdown of the photosynthetic machinery with senescence, while increased expression of GST proteins was found that suggested induction of antioxidative stress mechanism in plant cells with age to deal with ROS that are generated upon lipid peroxidation (Hebeler et al. 2008). In addition, serine hydroxymethyltransferase, quinine reductase, and enolase showed a 2–4-fold upregulation in *old1-1* mutants compared to wild type (Hebeler et al. 2008). Increased flavin quinone reductase has been found to be protective against oxidative stress and support the hypothesis that senescing leaves experience elevated levels of free oxygen radicals (Sparla et al. 1999).

13.17 Sugar Sensing by SnRK1 in Plant Senescence

Sugar molecules such as sucrose and hexoses (glucose and fructose) play a crucial role in plant senescence besides acting as energy molecules (Thelander et al. 2004). SnRK1, an SNF (sucrose nonfermenting)-related kinase in plants, performs functions linking sugar signaling to senescence. SnRK1 homologues in yeast (SNF1) and mammals (AMPK) have been linked to important functions in sensing energy status of the organism that is important in maintaining cellular homeostasis and function under nutritional stress (Polge and Thomas 2007). SnRK1 activity has been linked to induction of transcription and posttranslational inhibition (Baena-Gonzalez et al. 2007). Nutrient starvation, high sucrose/low glucose, and other conditions cause senescence and promote SnRK1 activation (Thomas 2013). SnRK1 is believed to suppress senescence possibly via negative regulation of enzymes involved in carbon and nitrogen metabolism by phosphorylation (Thomas 2013). Plants with reduced SnRK1 expression display signs of premature senescence providing evidence for involvement of this sugar sensing kinase in aging-associated processes (Thelander et al. 2004). HXK1 (hexokinase) enzyme catalyzes the first step of glycolysis involving conversion of glucose to glucose-6-phosphate (G-6-P). A possible cross talk exists between HXK1 signaling and SnRK1 in senescence-related process that includes negative regulation of SnRK1 by G-6-P (Thomas 2013). HXK1 complex in the nucleus represses cytokinin signaling that is also a known repressor of senescence (Davies and Gan 2012).

13.18 Interplay Between HXK1 and SnRK1 Signaling in Seedling Growth and Senescence

A possible link between glucose and SnRK1 was uncovered by Cho et al. (2012), who found SnRK1 overexpression lines to be hypersensitive to 6 % glucose while plants expressing kinase inactive SnRK1 were insensitive to glucose. This suggests a plausible function for SnRK1 in sugar signaling. Previous work done by Moore et al. (2003) showed involvement of nuclear HXK1 in sugar sensing that may also have implications for its role in senescence. Catalytically inactive (unable to bind ATP and perform kinase function) HXK1 mutant impaired in glucose phosphorylation still retained transcriptional repression of photosynthesis genes such as *RBCS* (ribulose 1,5 bisphosphate carboxylase small subunit) and *PPDK* (pyruvate orthophosphate dikinase) in response to glucose, suggesting separate roles for HXK1 in glucose metabolism and sugar signaling (Moore et al. 2003). Targeted mutagenesis identified *gin2* mutant that was glucose insensitive as a consequence of a nonsense mutation in HXK1. The *gin2* mutants displayed delayed senescence, which further correlated with increased sensitivity to cytokinin in *gin2* calli but not wild-type callus (Moore et al. 2003). In addition, an impairment in auxin-induced cell proliferation and root growth was observed in *gin2* mutants suggesting involvement of multiple hormonal signaling cascades regulated by HXK1. The slow growth and reduced reproduction of

gin2 mutants is similar to calorie restriction conditions that increase longevity and promote stress resistance in *C. elegans* or *Drosophila* (Mair and Dillin 2008; Katewa and Kapahi 2011). However, how closely this is true in plants with respect to *gin2* and other mutations producing similar defects in nutrient sensing remains to be addressed.

13.18.1 Role of SnRK1 in Stress Responses and Senescence

SnRK1 was characterized in detail with respect to its function in submergence stress and leaf longevity by Cho et al. (2012), who found rice OsSnRK1 to be performing functions similar to its *Arabidopsis* homologue (AtSnRK1). AtSNRK1 as shown by Jossier et al. (2009) integrates nutrient and energy status to regulate plant metabolism. The authors generated transgenic lines overexpressing SnRK1 and found modifications in activity of nitrate reductase and ADP-glucose pyrophosphorylase suggesting important role of AtSNRK1 in metabolism. In addition, lack of induction of osmotic stress-regulated genes (*PR1*, *PR2*, and *PR5*) was found in these lines suggesting misregulated metabolic status of these plants. Transient expression of OsSnRK1 in *Arabidopsis* mesophyll cells with *DIN6* (dark-induced 6) or *GST6* promoter-driven luciferase reporter showed ability of OsSnRK1 in inducing *DIN6* but not *GST6* (a general stress response marker) suggesting specific role of SnRK1 in stress signaling (Cho et al. 2012). The role of protein kinase activity of OsSnRK1 in gene activation was further proven by generating kinase dead catalytically inactive SnRK1 constructs that was found to be inhibiting DIN6-fLUC reporter induction under hypoxia. The authors in the study employed various genetic, biochemical, and molecular biology techniques to show that OsSnRK1 overexpression in *Arabidopsis* increases expression of *ADH1* (alcohol dehydrogenase 1) and *PDC* (pyruvate dehydrogenase carboxylase) that are known gene expression markers under flooding stress conditions. The authors also generated transgenic lines expressing inactive forms of OsSnRK1 deficient in kinase activity that acted as a dominant negative and showed no detectable induction of either *ADH1* or *PDC* that hinted toward the important role of SnRK1 activity upon submergence. Increased binding/recruitment of SnRK1 to the promoter region of target genes was verified using ChIP (chromatin immunoprecipitation) assay, where anti-AMPKα antibody was used to immunoprecipitate SnRK1 bound to DNA under control and submergence conditions (Cho et al. 2012). AMPK (mammalian homologue of SnRK1) has been shown to regulate signaling pathways by phosphorylation of histone H2B in the promoter region of target genes suggesting that SnRK1 could also behave in a similar fashion leading to differential regulation of different target genes under starvation/flood/hypoxic stress (Bungard et al. 2010; Cho et al. 2012).

In addition to stress, SnRK1 function could also be linked to reduced leaf senescence and longevity, demonstrated by a significant delay in onset of leaf yellowing and other detrimental changes in leaf phenotype that start occurring in Columbia *Arabidopsis* plants (Col-0 wild type) at around day 36, but starts around day 40 in OsSnRK1 overexpression lines (Cho et al. 2012). In contrast, transgenic lines expressing kinase dead version (dominant negative) of OsSnRK1 displayed

premature leaf yellowing starting at day 24 among other associated features of senescence. The senescence-associated symptoms were further quantified as chlorophyll content, photosynthetic reaction efficiency, and expression of senescence-associated genes (SAGs). Photochemical efficiency of PSII was found to be intact for a greater length of time in SnRK1 overexpression lines as compared to Col-0 plants, whereas transgenic lines expressing SnRK1 inactive forms had a fast decline in PSII activity. Expression of two senescence-associated genes, *SAG12* (senescence-associated gene 12) and *SEN1* (senescence 1), also showed increased levels in inactive SnRK1 transgenic lines suggesting involvement of SnRK1 in pathways controlling plant aging and longevity (Cho et al. 2012).

13.18.2 miRNA-Mediated Regulation of SnRK1 Signaling

Additional regulation of SnRK1-mediated function in energy deprivation condition was found by Confraria et al. (2013), who performed microarray profiling of wild-type vs. *dcl1-9* mutant plants (deficient in miRNA biogenesis) under starvation conditions and identified 831 genes that were misregulated in *dcl1-9* mutants; 155 of these were predicted/validated miRNA target genes. miRNAs are 20–24 nucleotide long sequences that negatively regulate mRNA expression by targeting them for degradation. Plant miRNAs repress gene expression by translational repression as well as chromatin modification. Major cellular processes and pathways revealed to be coregulated by SnRK1 and miRNAs were those related to translation and organelle function (Confraria et al. 2013). The starvation genes misregulated in *dcl1-9* hypomorphic mutants fell into two categories depending upon whether they were induced or repressed by starvation. Genes that are repressed under energy deprivation and were misregulated included those relating to translation, ribosome, protein folding, redox signaling, and nucleic acid metabolism. Induced genes that were misregulated in *dcl1-9* clustered into amino acid metabolism, protein degradation, chromatin remodeling, autophagy, and fatty acid metabolism-related genes. *TCP* (teosinte-branched 1 cycloidea and proliferating cell antigen) transcription factors were the major class of genes to be repressed by SnRK1/miRNA axis (Confraria et al. 2013). Some unclustered category of induced genes included *SnRK2.10*, *ATG8*, and *AKINβ* involved in salt stress, autophagy, and SnRK1 signaling respectively. *MYB75*, *HSP-70*, and *SnRK3.10* were found in the unclustered repressed gene category involved in anthocyanin synthesis, heat stress, and calcium signaling, respectively (Confraria et al. 2013). To further validate repression of miRNA target genes, *TCP4* and *TCP2* transcripts were assessed by real-time PCR under energy deprivation conditions in plants overexpressing an miR-319 mimetic that acts to quench away miR-139 from other targets. Upon reduced availability of miR-319 and *TCP2* and *TCP4*, repressions under dark conditions were clearly impaired, providing further proof for miRNA-mediated regulation of genes downstream of SnRK1 signaling (Confraria et al. 2013). TCP transcription factors regulate nuclear-encoded mitochondrial gene expression, and this could account for plausible role of starvation-regulated TCP genes in TCA cycle and metabolism. Interestingly, as

noted by the authors, the link between nutrient sensing and miRNA has also been reported in animal species suggesting it to be a conserved mechanism involved in starvation stress (Poy et al. 2007).

13.19 Hormonal Control of Plant Aging

Hormones are small molecules that have profound effects on plant growth and development, integrating different environmental cues to coordinate plant physiological processes including senescence and aging-associated processes. Different hormones participate to control leaf senescence in a programmed manner with the role of cytokinin and ethylene, found to be conserved in senescence across species (Davies and Gan 2012; Li et al. 2013). The role of hormone signaling in plant senescence is further complicated by cross talk between various signaling cascades in influencing the senescence process. Hormone signaling is regulated at multiple levels such as biosynthesis, perception, and the downstream signaling components. Studies of hormone pathway mutants in *Arabidopsis* support the importance of hormones in leaf senescence. Hormone produced at one site may act at distant sites to produce changes in expression of genes as well as protein activities that modulate a variety of processes in the plant system. In this section, we describe plant hormones whose connection to plant longevity and senescence has been found.

13.19.1 Cytokinin in Plant Senescence

Cytokinins (CK) are adenine derivatives with isoprenoid, and aromatic side chains have been known to be a class of senescence delaying hormone for long (Lim et al. 2007). Reduced levels of CKs have been found in leaves undergoing senescence, while exogenous application of CKs delays leaf senescence and promotes longevity (Hwang et al. 2012). Direct evidence for involvement of CKs in plant aging was found when IPT (isopentenyl transferase, a rate-limiting enzyme in CK biosynthesis) was expressed under a senescence-inducible promoter in *Nicotiana* (tobacco), and a significant suppression of senescence was observed in these transgenic plants displaying extended leaf longevity accompanied with reduced senescence phenotypes in adult stages (Hwang et al. 2012). CK signaling is well studied in *Arabidopsis* and is mediated by histidine kinases AHK2, AHK3, and AHK4 that upon binding cytokinin undergo autophosphorylation. This signal is further relayed by phosphotransfer proteins that then activate ARR (*Arabidopsis* response regulators), nuclear-localized proteins, to regulate transcription of CK target genes (Hwang et al. 2012). Interestingly, loss of AHK3 function, a CK receptor, decreases sensitivity to senescence repression with cytokinin treatment, whereas gain-of-function mutation in AHK3 results in delayed senescence suggesting involvement of specific CK signaling receptor in senescence and aging-associated plant functions (Hwang et al. 2012). A study by Buchanan-Wollaston et al. (2005) reported a possibly important role of cytokinin

signaling in inhibiting development and dark-induced senescence as revealed in gene expression analysis showing reduced expression of cytokinin synthase and of several cytokinin response regulator genes. As noted by the authors, type B *ARR* gene, *ARR1*, showed upregulation under both development and dark-induced senescence. Type B ARRs normally activate the expression of type A ARR genes; however, it is possible that under senescence conditions, the N-terminal domain of type B ARRs inhibits this activation in the absence of cytokinin (Hutchison and Kieber 2002).

Recent work done by Bhargava et al. (2013) identified cytokinin target genes using RNA-sequencing. Based on this approach, a host of stress- and senescence-related genes were identified to be acting downstream of cytokinin signaling. A total of 73 genes (60 upregulated and 13 downregulated) were identified to show changes in expression upon cytokinin treatment in the seedlings. A number of glutaredoxin genes (9 out of the 30 known glutaredoxin genes in *Arabidopsis*) were found to be upregulated by cytokinin treatment. Glutaredoxins maintain the redox status of the cell and protect from photooxidative damage and pathogen stress, suggesting that cytokinin might be involved in such functions as well. Other categories of genes identified in this study were bHLH family of transcription factors, three defensin genes, and a cytochrome P450. GO (gene ontology) term analysis also revealed some genes important in pathogen defense, such as defense-associated oxygenase, methyltransferase, and chalcone isomerase, to be under cytokinin regulation again pointing toward the importance of cytokinin signaling in abiotic as well as biotic stresses (Choi et al. 2010; Bhargava et al. 2013).

Černý et al. (2013) performed proteome and metabolome profiling of *Arabidopsis* seedlings expressing cytochrome oxidase and identified 155 proteins that were responsive to *ipt* (cytokinin pathway) activation. Interestingly, functional classification identified stress response genes such as those involved in oxidative stress response and detoxification, defense genes, and cold stress response genes. Potential cross talk with jasmonic acid signaling was also found with increased levels of thylakoid luminal protein and uncharacterized protein and decreased levels of β-glucosidase, ATP sulfurylase, and tryptophan synthase upon cytokinin signaling activation, which is an opposite trend observed with methyl jasmonate treatment (Jung et al. 2007).

Another study done by the same group (Černy et al. 2014) performed proteomic analysis to compare cytokinin-induced changes upon heat shock using two-dimensional gel electrophoresis and identified 148 proteins that were differentially expressed, most of which have no previously reported role in temperature stress. More than 70 % of the proteins that were reported to be modulated by heat were also altered with cytokinin suggesting a potential cross talk between cytokinin and heat stress signaling.

13.19.2 Ethylene as a Promoter of Plant Senescence

Ethylene is a gaseous hormone that has been known to be involved in a variety of plant functions such as seed germination, root development, stress response, and fruit ripening (Merchante et al. 2013). Senescence-promoting roles have been long ascribed to ethylene with the observations showing it as a promoter of ripening and leaf senescence. Ethylene biosynthesis occurs from methionine in a biochemical

pathway, which involves conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) in a rate-limiting step by the enzyme ACC synthase (Alonso and Stepanova 2004). Inhibition of ACC synthase delays senescence in several plant species supporting a pro-senescence function of ethylene (Merchante et al. 2013). Ethylene signaling involves binding of ethylene to receptor such as ETR1 and other homologous proteins that then activates CTR1 (a negative regulator of the pathway). CTR1 further brings about phosphorylation-dependent cleavage of EIN2 (ethylene insensitive 2) that controls activity of downstream transcription factors.

A recent study by Li et al. (2013) found EIN3 (ethylene signaling regulated transcription factor) to promote senescence by inhibition of miR164 repression. Overexpression of EIN3 promoted senescence, while loss-of-function mutations delayed the age-triggered and dark-induced leaf senescence. The authors found EIN3 to be acting downstream of *EIN2* to repress *miR-164* by direct binding to its promoter region. Additionally, ChIP-qPCR assay revealed increased binding of *EIN3* to promoter region of *miR-164* as plant aging progressed. Further, loss of *NAC2* (senescence-associated gene; loss-of-function delays senescence) suppressed enhanced aging observed in *EIN3* overexpression plants suggesting some link between *EIN3*-mediated signaling and *NAC2*. Interestingly, *miR-164*, which has previously been reported to promote mRNA cleavage of NAC class of genes and NAC2, is a positive regulator of this class of genes (Kim et al. 2009). The authors further showed negative regulation of *NAC2* by *miR-164*, elucidating an ethylene-stimulated pathway EIN2-EIN3-miR-164-NAC2, referred to as the NAC2 pathway. Future research into other transcription factors and miRNA-regulated cascades acting downstream of ethylene signaling will help unravel network of genes regulated by ethylene and their functions in leaf senescence.

13.19.3 Dual Role of Auxin in Plant Senescence

Auxin, which is a phytohormone important in a variety of developmental processes such as apical dominance, branching, and differentiation, is synthesized from tryptophan and exists in different forms such as IAA (indole acetic acid) and IBA (indole butyric acid). An aging-promoting role for auxin has been found in studies showing increased levels of IAA in senescing leaves (Lim et al. 2007) and studies reporting auxin-inducible gene *SAUR36* to promote senescence (Hou et al. 2013). Additional studies by Xu et al. (2011) showed senescence receptor-like kinase from soybean (GmSARK) to accelerate senescence when expressed under glucocorticoid-responsive promoter in *Arabidopsis*. Consistent with a pro-aging role for *GmSARK*, inhibition of its expression resulted in delayed senescence. Further, an auxin-dependent role for *GmSARK* was identified when senescence phenotypes observed in *GmSARK*-overexpressing plants were abolished in auxin-deficient mutants (Xu et al. 2011).

Several transcriptomic studies have been performed to identify auxin-regulated genes (Paponov et al. 2008; Goda et al. 2004; Huang et al. 2008). Huang et al.

(2008) used cDNA AFLP approach to compare wild-type *Arabidopsis* to auxin-insensitive *eta2* mutant and identified 66 differentially expressed transcripts that were involved in RNA metabolism, defense, and transcriptional regulation suggesting a complex network regulated by auxin. Paponov et al. (2008) performed microarray analysis of auxin-treated plants to identify genes involved in biosynthesis, catabolism, and regulation of signaling by other phytohormones. AtNAC2, a transcription factor shown to be involved in salt stress response downstream of auxin signaling, was identified, providing evidence for involvement of auxin in stress-associated functions (He et al. 2005).

The abovementioned transcriptome profiling for auxin-regulated genes did not identify any of the gene encoding SCF/RUB pathways that relay auxin signal to degrade AUX/IAA proteins suggesting posttranscriptional control by auxin signaling to mediate its diverse functions (Dharmasiri and Estelle 2004). Proteomic analysis performed using label-free shotgun approach revealed 69 downregulated and 79 upregulated proteins upon comparison of wild-type plants to *tir1-1* mutants (deficient in auxin receptor) with IAA treatment (Xing and Xue 2012). Among the downregulated proteins, tubulin, actin, and structural proteins were found, which suggests massive change in cytoskeletal organization upon auxin treatment. Other downregulated proteins included GNOM (a membrane guanine exchange nucleotide factor) and 14-3-3 proteins, which act in endocytosis and intracellular signaling, respectively. H2B (histone) protein levels were also reduced, which could be correlated with chromatin decondensation and gene expression that occurs with auxin. In addition, proteins involved in stress response were also altered with auxin treatment, supporting a plausible role for auxin in stress and aging (Xing and Xue 2012).

A paradoxical role for auxin in plant aging was found in studies investigating the role of *YUCCA* gene family in *Arabidopsis* (Kim et al. 2011). YUCCA gene family encodes for flavin-containing monooxygenase enzymes that catalyze the rate-limiting step of auxin biosynthesis (Kim et al. 2011). Increased expression of *YUCCA6* increased IAA levels and downregulated *SAG12* gene expression that was also accompanied with delay in dark-induced leaf senescence. Another study by Lim et al. (2010) found that inhibiting the repressor of auxin signaling *ARF2* (a transcription factor) increased auxin sensitivity together with delayed senescence in two mutant alleles *ore14-1* and *ore14-2* (Lim et al. 2010). In summary, the role of auxin in plant senescence appears to be context dependent and needs future work to elucidate the precise mechanism.

13.19.4 ABA Functions in Plant Stress and Senescence

ABA is an important stress hormone that is derived from isoprene subunits and plays a key role in promoting seed dormancy and inhibition of transition from embryonic to vegetative state as well (Finkelstein et al. 2002). ABA signaling involves PYR/PYL (pyrabactin resistance) receptors that further act with PP2C phosphatase family to repress dephosphorylation of SnRK2 family of protein kinases in order to regulate

downstream transcriptional responses of ABA (Coello et al. 2011). The promotive role of ABA in senescence is supported by the occurrence of leaf senescence by exogenous application of ABA hormone (Lim et al. 2007) and increased expression of ABA biosynthesis genes in senescing leaves (Buchanan-Wollaston et al. 2005). Microarray expression analysis of developmental, cell suspension, and dark-induced senescence in leaves revealed upregulation of 9-cis-epoxycarotenoid dioxygenase (NECD) and two aldehyde oxidases *AAO1* and *AAO3* (enzymes in ABA biosynthesis) suggesting involvement of ABA in all three types of senescence. Protein phosphatases *ABI1* and *ABI2*, implicated in ABA signaling, also showed increased transcript abundance under the different senescence conditions providing further evidence for involvement of ABA pathway in the plant aging process (Buchanan-Wollaston et al. 2005).

An opposing role of ABA in senescence was found by Oka et al. (2012), where ABA reduced senescence in cucumber (*Cucumis sativus*) plants, under low nitrogen conditions. ABA application under low N conditions suppressed chlorophyll loss in these plants, whereas under nitrogen-sufficient conditions, it had no effect. In addition, ABA appeared to decrease transcript levels of chlorophyll degrading enzymes such as chlorophyll b reductase (*CBR*) and pheophytinase (*PPH*) under nitrogen starvation conditions. Thus, while ABA may have a senescence-promoting role in detached leaves, it may actually inhibit senescence in intact plants as shown in this study under a particular nutrient stress (nitrogen starvation in this case).

A similar observation was made by Hermans et al. (2010); short-term magnesium ion deficiency caused changes in ABA-responsive genes at the transcript level, even though the ABA content itself remained unchanged. Magnesium is an important macronutrient for the plants required for enzyme activation and several other physiological processes including photosynthesis in the chloroplast thylakoid membranes to facilitate light reaction (Hermans et al. 2010). Rapid induction of ABA signaling genes such as *ABF2* (abscisic acid responsive factor 2) and *MYB102* was found during magnesium ion deficiency (Hermans et al. 2010). The functional relevance of induction was not studied by the authors, but it would be interesting to know whether ABA in this case too has some protective role under nutrient stress. Future research studies to dissect the downstream signaling components triggered by low nutrients in intact plants promote an anti-senescence role of ABA would yield deeper insights into the biology of such processes.

13.19.5 Jasmonic Acid in Plant Senescence

Jasmonic acid (JA), an oxylipin signaling molecule, performs a wide variety of functions related to development, embryogenesis, fruit ripening, and abiotic and biotic stress response in plants (Kelley and Estelle 2012). Jasmonic acid upon binding its receptor COI (coronatine-insensitive 1) promotes ubiquitin-mediated degradation of JAZ (Jasmonate ZIM domain), which represses jasmonate-responsive genes. External application of methyl jasmonate enhances leaf senescence in a number of species suggesting a conserved role of this hormone in plant senescence (Reinbothe et al. 2009).

A recent study showed dual regulation of *WRKY57* by jasmonic acid and auxin signaling, where senescence-associated genes (*SENESCENCE2* and *SENESCENCE-ASSOCIATED GENE12*) were directly bound by *WRKY57* in their promoter region to repress their transcription (Jiang et al. 2014). In vitro and in vivo assays by the authors showed WRKY57 binds to JAZ4/8 (repressor of JA signaling) to inhibit JA signaling. Also, JA itself was found to be downregulating WRKY57 protein levels. *wrky57* mutants have premature loss of chlorophyll and senescence phenotypes.

A microarray approach was undertaken by Jung et al. (2007) to identify jasmonate-responsive genes in *Arabidopsis thaliana* using 100 μM of methyl jasmonate treatment. The study identified 137 genes upregulated/downregulated by more than twofold under this treatment. A total of 74 genes were found to be upregulated that were involved in functions relating to oxidative stress, cell wall modification, senescence, and defense responses, while those belonging to chlorophyll synthesis were downregulated. Interestingly, significant reduction in expression of ABA-regulated drought/cold stress-responsive genes was noted suggesting an antagonistic relationship between the JA and ABA signaling (Jung et al. 2007).

13.20 TOR Pathway and Nutrient Sensing/Signaling in Regulation of Plant Longevity

TOR (target of rapamycin) signaling plays an important role as an energy sensor that integrates nutrient status of an organism to processes linked with longevity and stress resistance. TOR signaling in plants similar to that in other eukaryotes comprises of two complexes—TORC1 and TORC2—composed of different proteins that regulate different downstream processes. In the presence of abundant nutrients/amino acids, TOR signaling is active with TORC1 (rapamycin-sensitive complex) branch promoting efficient protein translation and inhibiting autophagy, while TORC2 converges on Ras/AKT signaling to regulate actin cytoskeleton assembly (John et al. 2011; Johnson et al. 2013). Inhibition of TOR with drug rapamycin inhibits translation at cellular level that is associated with overall slower growth at physiological level. A recently published study by Ren et al. (2012) has shown TOR to be important in controlling *Arabidopsis* development and lifespan. Loss of TOR results in embryo lethality suggesting an important function performed by this pathway in plants (Menand et al. 2002). TOR inhibition by rapamycin does not produce any visible phenotypes since target proteins of FKBP12 in plants are different from that of animals (Sormani et al. 2007). Transgenic *Arabidopsis* expressing yeast FKBP12 (BP12) is rapamycin sensitive, allowing the identification of role played by TOR in postembryonic growth and plant lifespan. Phenotypic analysis of wild-type plants with rapamycin-treated BP12 lines revealed clear differences in primary root length, lateral root initiation, rosette initiation, and shoot growth with BP12 lines showing overall reduction in all these development aspects (Ren et al. 2012). Closer examination by the authors also revealed reduced hypocotyl seedling elongation and reduced cell size of the root apical meristem suggesting requirement of TOR signaling in cell elongation and expansion (Ren et al. 2012). More importantly, TOR pathway

inhibition by rapamycin in BP12 transgenic lines produced significant extension of lifespan coupled with delayed senescence as determined by reduced loss of chlorophyll, cell death rate, and *SAG12* expression levels in plants with reduced TOR. To gain further insight into broad cellular processes that might be affected by inhibition of TOR, RNA-seq approach was utilized, which showed downregulation of major anabolic pathways such as photosynthesis, cell wall synthesis, and inorganic transporters (Ren et al. 2012). Real-time PCR confirmed downregulation of genes, involved in cell wall restructuring during growth, such as 16 extensin and 7 expansin genes, that could also explain the growth defects in rapamycin-treated BP12 plants. Downregulation of carbon fixation and photosynthesis processes was reflected in reduced expression of genes such as ribulose-1,5-bisphosphate carboxylase small subunit and NAD kinase (Ren et al. 2012). Conversely, upregulation of catabolism-related genes was observed such as genes involved in carbohydrate and protein metabolism. Rate-limiting step of polyamine synthesis catalyzed by *S*-adenosyl methionine (SAM) decarboxylase was observed to be upregulated in BP12 lines with inhibition of TOR pathway (Ren et al. 2012). Consistent with global gene expression analysis, metabolite analysis depicted a similar pattern. Increase in TCA cycle intermediates such as citrate, fumarate, malate, and succinate was observed suggesting reprogramming of the metabolic pathway to compensate for reduced light reaction and provide intermediates for cellular respiration and growth (Ren et al. 2012). Other changes in primary metabolism included increase in polyamines and amino acids that occur as a consequence of increased protein degradation. The authors showed a decrease in arginine BP12 transgenic lines accompanied by an increase in dimethylarginine. Interestingly, dimethylarginine has been previously shown to have antiaging effects correlated with enhanced longevity in *C. elegans* (Takahashi et al. 2011). An increase in anti-ROS molecules such as 5-oxyproline and glutathione (both oxidized and reduced form) was observed suggesting activation of an antioxidative stress pathway downstream of TOR that has also been linked with enhanced oxidative stress resistance and longevity in worms (Robida-Stubbs et al. 2012). The authors could further link TOR inactivation by rapamycin to *rps6* function as *rps6* mutants exhibited growth and development defects similar to BP12 lines having TOR pathway inhibition and also display a smaller but significant lifespan extension compared to rapamycin-treated BP12 lines (Ren et al. 2012). Gain-of-function experiments with both TOR and RPS6 overexpressing lines revealed premature senescence, early ripening, and shorter lifespan (Ren et al. 2012). Taken together, genetic analyses coupled with global transcript profiling established TOR to be a major pathway controlling longevity, metabolism, and stress in plants as well.

13.21 Conclusion

Studies over the past decade have yielded many interesting findings about the aging process, its mechanisms, and potential cross talk with stress signaling cascades. Insulin signaling, dietary restriction pathway mediated by TOR, and sirtuins are some of the major aging pathways conserved across species. Enhanced longevity in

many cases also appears to be accompanied with increased resistance to various forms of external stresses that also support the error theory of aging. Numerous studies taking advantage of various functional genomics approaches have shown multiple pathways that either promote or inhibit aging and senescence. The idea of aging and longevity becomes more complex in plants where definition of aging is still vague. However, some studies have shed light on conserved role of TOR and other nutrient-sensing pathways in aging-related processes in plants suggesting existence of similar pathways (summarized in Fig. 13.3). With fast advancement of functional genomics and proteomics approaches, more findings are expected to be made that will help unravel the as-of-yet unknown genes and pathways controlling the complex aging process in diverse species. Increasing availability of large-scale data at the genomic and proteomic level would provide us with better knowledge of different signaling pathways involved in aging and disease processes. This would provide us with potential biomarkers and therapeutic targets to alleviate morbidity and promote healthy aging in human beings. In plants, increased information and

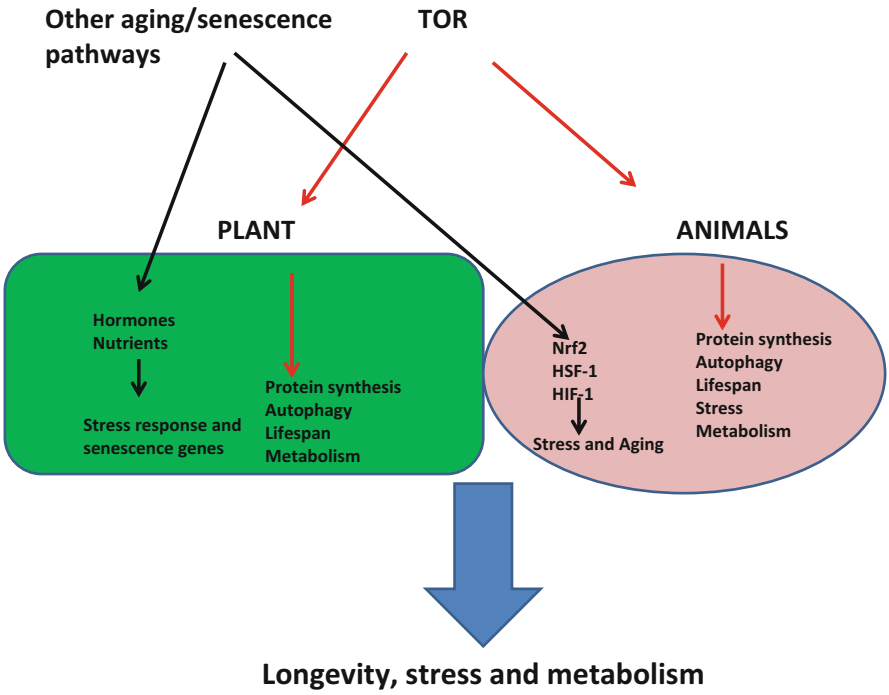

Fig. 13.3 Common pathways/genes important for plant and animal aging. As shown in the figure, TOR pathway, which controls nutrient- and energy-sensing across species, is important for plant and animal lifespan. Inhibition of TOR by rapamycin has beneficial effects on lifespan but also protects against stress- and age-associated diseases in animals. In plants, TOR signaling has been implicated in longevity and metabolism, but its role in stress remains to be addressed. Also, different genetic pathways in both animals and plants impinge on stress (environmental as well as biotic) to regulate aging and senescence processes suggesting cross talk between lifespan and stress pathways. *Nrf2* nuclear (erythroid derived)-like factor, *HSF-1* heat shock transcription factor 1, *HIF-1* hypoxia inducible factor 1

analysis of large-scale data in relation to aging and senescence would provide basic tool in generating more stress-resistant and disease-resistant varieties promoting greater agricultural productivity and facilitating eradication of hunger and malnutrition existent within the human race.

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Part III
Manifestation of Stress Tolerance

Chapter 14
Designing Climate-Smart Future Crops Employing Signal Transduction Components

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Abstract The explosive increase in world population, along with increasing environmental stresses like salinity, drought, and high and low temperatures, has created two major problems: more mouths to feed and less land to farm. Stress perception and thereafter the transduction of the stress signal are the initial steps of a typical stress response of plants. Therefore, understanding the mechanism(s) of plant stress perception and signal transduction is an imperative for designing climate-smart future crops. Recent studies have shown that abiotic stress signaling in plants comprises many components, for instance, receptor-coupled phosphorelay, phosphoinositol-induced Ca^{2+} changes, mitogen-activated protein kinase cascades, and transcriptional activation of stress-responsive genes. In addition, adapter or scaffold-mediated protein–protein interactions and protein post-translational modifications play a major role in abiotic stress signal transduction. An improved understanding of the mechanistic details of abiotic stress-associated signaling in plants combined with functional genomics may aid in pushing the productivity of crop plants closer to the optimum theoretical levels via genetic engineering or breeding approaches. In the present chapter, we discuss the recent progress related to the development of crop plants with enhanced stress tolerance by manipulating various components of the plant signal transduction machinery.

Keywords Signaling • Plant signal transduction • Stress perception • Transgenic plants • Transcription factors • Crop design • Signaling networks • Signaling pathways • Plant cell receptors

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14.1 Introduction

Unlike animals, plants cannot move to escape unfavorable environmental extremes such as low temperature, drought, and salinity. As a consequence of continuous global climate changes, these stresses are bound to further increase in the near future. Therefore, understanding the mechanisms by which plants perceive stress signals and transduce them to the cellular machinery generating a stress response is of fundamental importance to plant biology. Major breakthrough in this field has resulted from the isolation and characterization of functionally diverse stress-inducible genes (Hirayama and Shinozaki 2010, Sahoo et al. 2013).

A typical signal transduction has well-defined stages conserved across various organisms. Signal perception is the first step which triggers the generation of second messengers, among which Ca^{2+} is of exceptional significance in plants. Second messengers further activate different kinases (Ser/Thr/Tyr) via multiple modes, which then lead to phosphorylation of numerous cellular substrates including some kinases and transcription factors as well as other signaling proteins. The final response may vary according to the substrate activated. One of the possibilities is the phosphorylation-dependent activation of transcription factors which can then lead to the upregulation of the stimuli-responsive genes which belong to several categories based on their cellular function. Overview of a typical signal transduction pathway has been shown in Figure 14.1.

Owing to the current explosion of -OMICs datasets and the development of signaling databases, these pathway maps are getting more complex. With the growing knowledge of signal transduction pathways and associated interactions, we discover a higher

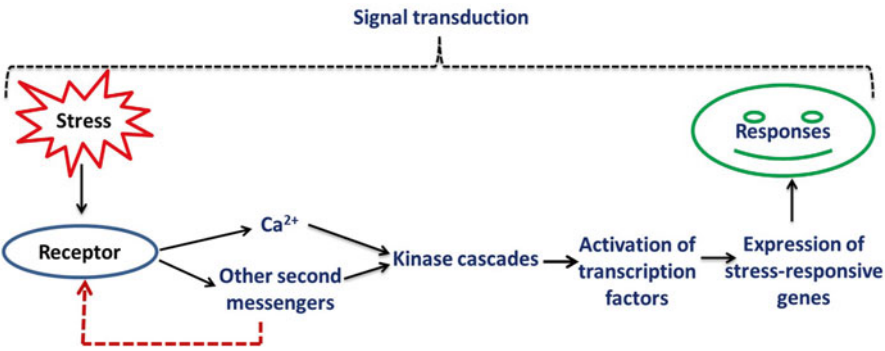

Fig. 14.1 Overview of signal transduction during abiotic stress response in plants. The schematic representation shows a simplified overview of a generic signal transduction pathway operational during plant's response to abiotic stresses. Stress perception is the first step which triggers the generation of second messengers, among which Ca^{2+} is of exceptional significance in plants (see text for details). Second messengers further activate different kinases via multiple modes, which then lead to phosphorylation of numerous cellular substrates including some kinases. The final response may be due to the phosphorylation-dependent activation of transcription factors which can then lead to the upregulation of stress-responsive genes which belong to several categories based on their cellular function (see text for description)

degree of cross talk among them (Kiel and Serrano 2012), and these pathways appear like a tangled web. Untangling this web of pathway interactions is becoming an increasingly daunting task (Eyster 1998; Smékalová et al. 2013). A detailed analysis of these signaling pathways is a prerequisite for utilizing signaling pathways to reengineer plants to cope with environmental stresses. This chapter focuses on recent advances in the basic research of signal transduction pathways and their application in generating climate-resilient crop plants tolerating various abiotic stresses.

14.2 Engineering Crops: Discovering and Tailoring Genes

Plants often come across severe environmental constraints that adversely affect their growth and yield (Umezawa et al. 2006). Therefore, designing novel strategies to engineer for stress tolerance in plants has vast importance. Stresses affect varied responses in plants, such as alterations in gene expression; accumulation of various metabolites such as proline, betaine, and polyamines sugars and sugar alcohols (e.g., mannitol, trehalose, and galactinol); and synthesis of stress-responsive structural and functional proteins like LEA and heat shock proteins (Ramachandra-Reddy et al. 2004). Despite a sufficient understanding of these processes, there are missing links. And hence, functional genomics studies have concentrated much on identifying functional genes that are directly involved in these events. Utilizing the information generated in such functional genomic studies, several successful attempts have been made until now to engineer stress tolerance in plants (Chaves and Oliveira 2004; Vinocur and Altman 2005).

Transcriptome analysis using microarray technology has revealed several transcription factors (TFs) functioning in stress response (Bartels and Sunkar 2005) and hence are potential candidates for genetic engineering. TFs (both activators and repressors) regulate the expression of a wide variety of target genes that have a role in stress response and adaptation. Owing to this, manipulating the expression of both transcriptional activators and repressors is one of the most viable strategies to engineer stress tolerance potential of plants. To date, there have been several efforts in this direction. For instance, STZ—a Cys2/His2-type zinc-finger transcriptional repressor—has been found to get upregulated by various abiotic stresses such as dehydration, salinity, cold, and ABA treatment. Constitutive expression of STZ has been shown to improve tolerance to drought stress (Sakamoto et al. 2004). However, not all transgenic plants constitutively expressing transcription factors have shown beneficial effects, and some plants show growth retardation and alterations in basic metabolism (Vinocur and Altman 2005). Such undesirable traits can be potentially controlled by utilizing stress-inducible promoters to drive the expression of TFs in a stress-dependent manner (Kasuga et al. 1999, 2004).

Several signal transduction components act upstream of TFs, during abiotic stress response, which involves calcium sensing, protein phosphorylation, lipid metabolism, and protein turnover (Vinocur and Altman 2005; Bartels and Sunkar 2005; Boudsocq and Lauriere 2005). Though we have progressed a lot, these complex signaling processes are still not well understood (Zhang et al. 2004; Shinozaki

et al. 2003; Chinnusamy et al. 2004). This also highlights the complexity of the signal transduction network with cross talk, feedback, and interactions delivering proper signals to correct targets at an appropriate time (Zhang et al. 2004; Chinnusamy et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2005). With the advancement of functional genomics approaches, many genes encoding signaling factors have been identified and are available now for engineering stress tolerance. Since signaling proteins control a large array of downstream events, their manipulation through genetic engineering gives an additional advantage resulting into multiple abiotic stress tolerance. For example, a mitogen-activated protein kinase kinase kinase (MAPKKK) when expressed under the control of a constitutive promoter leads to the tolerance against multiple abiotic stresses such as cold, heat, salinity, and drought (Kovtun et al. 2000; Shou et al. 2004). Besides this, signaling factors also function as a molecular switch to control the signal output involved in stress adaptations; they are often found to be activated or inactivated in response to various stress conditions (Mikolajczyk et al. 2000; Li et al. 2000; Monks et al. 2001).

Transcriptomics-, proteomics-, or metabolomics-based studies along with the technical advances in applied research have led to considerable progress in increasing the stress tolerance limits in plants. However, there is a further need to develop suitable transformation technologies systems with controlled expression using tightly regulated stress-responsive promoters (Kasuga et al. 1999; Capell et al. 2004).

14.3 Signal Perception and Transduction: Untangling the Web of Intracellular Messengers

Cells react to changes in their environment by sensing and producing signals. In multicellular organisms, the individual cells receive external physical or chemical signals and communicate with each other through complex signal sensing and transducing modes to coordinate various functions at tissue and organ level. These signals can be classified into two groups: membrane-permeable and membrane-impermeable. The membrane-permeable signals pass through the cell membrane to bind the specific receptor molecules such as nitric oxide. The membrane-impermeable signal molecules are usually recognized by cell membrane-localized receptors. These include G protein-coupled receptors, growth factors, extracellular matrix components, and cyclic AMP. Eyster (1998) defined signal transduction as "the mechanism by which binding of a molecule to its receptor at the target cell elicits a change in biological activity of the target cell is called signal transduction; or, signal transduction is the transmission of an extracellular signal into an intracellular biological effect." In the past few years, this field has moved so rapidly with the growing addition of new signaling cascades and cross talk among known pathways that the signaling pathways appear as a tangled web with increasing complexity. Described below are the two types of membrane receptors functioning during signal transduction.

14.3.1 Enzyme-Linked Receptors

In this type, the receptor and the enzyme (effector) are distinct domains of the same protein. When a ligand binds to the receptor, the effector enzyme is activated. Ligand binding may also result into dimerization of the receptor or, in some cases, separation of the subunits of an existing dimer (Van der Geer et al. 1994). But in both the situations, ligand binding activates the effector enzyme. These enzyme-linked receptors can be further classified as:

14.3.1.1 Receptor Tyrosine Kinases

Receptor tyrosine kinase (RTK) pathway is one of the most studied signaling pathways that use the enzyme-linked receptor (Eyster 1998). Ligand binding results in autophosphorylation of the RTK as well as subsequent phosphorylation of substrate proteins. The resulting phosphotyrosine (PY) residues interact with SH2 domain (Src homology domain 2) containing proteins, which phosphorylate other proteins and thus change their enzymatic activity (Heldin 1991). Ras-GTPase-activating protein (GAP) and phospholipase C-γ are two such SH2-containing proteins. Phospholipase C-γ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 aids the release of Ca^{2+} from intracellular stores mainly smooth endoplasmic reticulum, whereas DAG activates protein kinase C (PKC) (Schenk and Snaar-Jagalska 1994). The SH2-containing adapter molecules, Shc (Src homology and collagen) and Grb2 (growth factor receptor-bound protein 2), also bind to phosphorylated RTKs. They help in recruiting Sos (son of sevenless) protein, a GEF (G-nucleotide exchange factor), to the plasma membrane (Bonfini et al. 1996). Sos further activates the small G protein Ras by stimulating the exchange of GDP for GTP. Activated Ras, then, activates mitogen-activated protein kinase (MAPK) (Guan 1994). MAPK further conveys messages from the membrane to the nucleus (Van der Geer et al. 1994; Campbell et al. 1995).

Receptor tyrosine kinases (RTKs) are known to regulate several cellular functions, such as proliferation, differentiation, cell survival, and metabolism (Lemmon and Schlessinger 2010; Lim and Pawson 2010). While RTK-mediated tyrosine (Tyr) phosphorylation is well studied in mammals, not much is known about its role in signal transduction in plants (Macho et al. 2014). RTKs were considered to be absent in plants until very recently (Bentem and Hirt 2009). However, studies by Oh et al. (2009; 2010) and Jaillais et al. (2011a, b) have shown that a receptor for brassinosteroids—BRI1 in *Arabidopsis*—possesses both tyrosine and Ser/Thr kinase activity. Nevertheless, there appears to be fundamental differences between RTKs from mammals and similar receptors from plants. More detailed analyses are needed to reveal the exact mode of action of putative RTKs in plants.

14.3.1.2 Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) possess an extracellular domain, a transmembrane domain, and one (or two) cytoplasmic tyrosine phosphatase domain(s). The extracellular domain shows immunoglobulin- and/or fibronectin-like regions and other sequence motifs known to function in cell–cell adhesion (Fischer et al. 1991; Zondag and Moolenaar 1997). The subtype IV PTP tightly binds to Grb2, which belongs to the class of proteins with SH2 and binding of Grb2 inhibit PTP activity (Den Hertog and Hunter 1996).

Tyr phosphatases were first reported in pea proteins in 1986 (Torruella et al. 1986). Since then, several putative members of protein Tyr phosphatase (PTP) family from *Arabidopsis* have been characterized (Gupta and Luan 2003; Bentem and Hirt 2009). Biochemical and genetic analysis confirmed that Tyr phosphorylation is involved in the modulation of MAPK activities in plants and has an important role in plant signaling (Underwood et al. 2007; Ghelis et al. 2008). In animals, PTPs have been classified into three groups, i.e., receptor-like PTP, intracellular PTP, and dual-specificity PTP (DsPTP) (Stone and Dixon 1994). While the receptor-like PTPs have been found only in animals, the other two classes have been shown to exist in all eukaryotes, including plants (Bentem and Hirt 2009).

14.3.1.3 Receptor Serine/Threonine Kinases

Receptor Ser/Thr kinases use Smad3 (Sma/Mad homolog 3, formerly called hMAD-3) proteins to convey signals to the nucleus (Schenk and Snaar-Jagalska 1994; Eyster 1998). In plants, the best examples of receptor Ser/Thr kinases are LRR-RLKs (leucine-rich receptors) which harbor a cytosolic Ser/Thr kinase domain. LRR-RLK functions in plant development and biotic stress defense (Diévart and Clark 2004). Though there are no conclusive reports which show the role of this class of RLKs in abiotic stress response, there are scattered reports which show their abiotic stress-responsive nature (Vaid et al. 2012).

14.3.2 Two-Component System Pathways

In prokaryotes, two-component regulatory system is a major signaling pathway involved in diverse responses, viz., osmosensing, chemotaxis and nutrient sensing. The two-component signaling system is constituted by two proteins: a sensory kinase (protein histidine kinase or HK) and a response regulator (RR). Besides, a histidine phosphotransfer protein (HpT) is often present with these two proteins. The N-terminal portion of the histidine kinase (HK) functions as an input domain as it detects extracellular signals. The C-terminal region possesses the transmitter module (Swanson et al. 1994). In eukaryotes including *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Oryza sativa*, homologous pathways have been identified, which contain an extended C-terminal domain possessing a conserved aspartate residue and called as hybrid histidine kinase (Pareek et al. 2006; Nongpiur et al.

2012). In plants, HKs function via the His–Asp phosphorelay and play a major role in several physiological and developmental processes. Further, recent studies have suggested that some of the sensory HKs may function as in hormone (mainly cytokinin and ethylene) signaling as well as osmosensors. This suggests a possible cross talk among various hormone and stress response pathways (Nongpiur et al. 2012).

14.3.2.1 Guanylyl Cyclases

Guanylylcyclases (GCs) are enzymes which produce cGMP (cyclic GMP) using GTP as a substrate. The cGMP then activates other enzymes such as cGMP-dependent protein kinase (cGK or PKG) or cGMP-dependent phosphodiesterase (Lohmann et al. 1997). The PKG then reduces cytosolic Ca^{2+} levels in cells (Lohmann et al. 1997). The structure of the enzyme-linked receptor form (plasma membrane form) of GCs typically comprises a transmembrane domain, an extracellular domain, an intracellular protein kinase homology domain (KHD), and an intracellular catalytic domain with cyclase activity. In addition to its enzyme-linked receptor form, GCs also exist as a soluble cytoplasmic form and transduce the message of the gaseous signaling molecules, such as NO (Murad 1994) and CO (Verma et al. 1993).

14.3.3 Receptors Without Enzymatic Activity

14.3.3.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are the third largest group of receptors without intrinsic catalytic activity. These receptors possess both extracellular and intracellular loops and structurally pass through the membrane seven times and are therefore referred to as seven-transmembrane pass or serpentine receptors. GPCRs have a well-defined orientation with the N-terminus being extracellular and the C-terminus cytosolic. The extracellular loops of the receptor form the ligand-binding domain. Ligand binding triggers a conformational change in GPCR allowing interaction of the intracellular loops with a linking protein called G protein (Bourne 1997). The G protein is heterotrimeric and comprises three subunits: α, β, and γ. In its inactive state, the "α" subunit has GDP bound to it. The interaction of ligand–receptor complex with Gα causes exchange of GDP with GTP. Then, Gα-GTP binds with an effector enzyme. The association of Gα with the effector enzyme leads to its activation. The signal vanishes when Gα, owing to its intrinsic GTPase activity, cleaves its bound GTP to GDP. Gα-GDP then reassociates with Gβγ and reforms the inactive heterotrimeric G protein complex (Palczewski and Benovic 1991).

Bioinformatics analysis has suggested the presence of about 50 GPCRs (Taddese et al. 2014). Due to its presence in fungi, plants, and unicellular eukaryotes, it is clear that GPCR must have evolved about 1.5 billion years ago, before the plant–fungi–animal separation (Stäubert et al. 2014). Plants possess most of the core elements found in G protein signaling in animals. However, there are differences in the network connections and other properties of plant G protein elements. In plants,

G proteins have been found to be self-activating. Therefore, in plants, the regulatory step of G protein activation is the deactivation step (Urano and Jones 2014).

Earlier it has been shown that G protein-coupled receptor1 (GCR1) interacts with G protein guanine nucleotide-binding protein alpha-1 subunit (GPA1) and functions in drought stress response in plants (Hooley 1999; Pandey and Assmann 2004). Later, other plant GPCRs were also identified by different groups (Moriyama et al. 2006; Liu et al. 2007; Gookin et al. 2008; Pandey et al. 2009). Further, it has been reported that increasing the amount of GTP-bound *Arabidopsis* Gα subunit (AtGPA1)—which was later found to be the fastest known nucleotide-exchanging G protein (Johnston et al. 2007; Jones et al. 2011a, b, 2012)—confers "active" phenotypes (Ullah et al. 2001) confirming that the active Gα form is indeed the GTP-bound form (Chen et al. 2003; Urano and Jones 2013). While we do have basic understanding of G protein and GPCR functions in plants, their role in abiotic stress adaptation remains to be worked out in a greater detail.

14.3.3.2 Cytokine Receptors

Plant cytokines or phytocytokines are peptide hormones, which play an important role in growth and development (Luo 2012). Cytokine receptors comprise an extracellular domain, a transmembrane region, and an intracellular domain (Lee et al. 2011). Several cytokine receptors lack intrinsic catalytic domains. Cytokine receptors associate non-covalently with protein tyrosine kinases. When a ligand binds to the receptor, it triggers Tyr phosphorylation. Some of the recently identified signaling proteins include CLAVATA3p (CLV3p) and phytosulfokine (PSK). Both of them harbor posttranslational modifications and function as signals of cell-to-cell communication. They are sensed by LRR receptor kinases, and the response is typically altered expression of numerous target genes (Lee et al. 2011; Igarashi et al. 2012).

14.3.3.3 Integrins

Integrins are transmembrane glycoproteins functioning as major cell surface receptors for ligands present on adjacent cells or in the extracellular matrix. The functional unit of integrins is heterodimers comprising an extracellular domain, a transmembrane domain, and a shorter cytosolic domain. After ligand binding, integrins form a cluster followed by the activation of focal adhesion kinase (FAK) and phosphorylation of intracellular tyrosine kinases (Clark and Brugge 1995). This also facilitates Grb2 binding thus activating pro-proliferative Ras–MAPK cascade. The downstream signaling post-activation of integrins is mediated by MAP kinases, G proteins, and Ca^{2+}-binding proteins. Besides, there may be changes in the cytosolic [Ca^{2+}] especially during the transduction of mechanical signals as found in onion epidermal cells (Clark et al. 2001a). The first evidence of plant integrins came from a study in soybean root cell suspension in which it was found that addition of GRGDSP peptides significantly increases growth rate (Schindler et al. 1989). Similar reports were observed later in NaCl-adapted tobacco protoplasts (Zhu et al. 1993), defense response in peas (Kiba et al. 1998), and embryo formation in sunflower protoplasts (Barthou et al. 1999). These receptors were also hypothesized to participate in gravity perception.

14.4 Signaling Networks and Signaling Specificity

In nature, plants encounter different environmental stresses in combinations concurrently at various stages of plant development (Shinozaki and Yamaguchi-Shinozaki 2000). Indeed, stress response is a complex interplay of a multitude of signal transduction pathways which often cross talk at various steps, and the output is a converging one (Park et al. 2003). The steps at which cross talk takes place are usually referred to as nodes (Knight and Knight 2001). What we have been studying till now—the linear pathways—are just partial representations of a much complex web of interacting signaling networks.

In some instances, there might be some specific and exclusive responses that lead to a specific change. In other cases, however, multiple stresses often lead to common signaling intermediates. In case of abiotic stresses, very little information is available about the cross talk between different stress signal transduction pathways in plants, as all the stress sensors and most signaling intermediates have not been characterized. Here, we discuss some common signaling mechanisms in plants known to be associated with stress response.

14.4.1 Calcium as Messenger

In plant cells, calcium (Ca^{2+}) is the most important second messenger and is a major component of the signaling network associated with a diverse range of developmental, hormonal, and abiotic stress signals (McAinsh et al. 2013). Spatial and temporal changes in cellular Ca^{2+} concentrations, often referred to as "Ca^{2+} signatures," take place in response to various stress stimuli such as osmotic stress, salinity, drought, oxidative stress, cold, gaseous pollutants, light, plant hormones, and pathogens (elicitors) to present defined stimulus-specific information (Batistič and Kudla 2012). A network of Ca^{2+} binding proteins act as Ca^{2+} sensors as they detect and transduce the signals generated via Ca^{2+} signatures. Ca^{2+} channels are known to play a major role in changes in intracellular Ca^{2+}. Ca^{2+} signaling is ubiquitous in abiotic stress signaling in plants; hence, it may act as an important node at which cross talk can occur. However, specificity and/or cross talk in Ca^{2+} signaling depends upon the magnitude, duration, and intracellular localization of Ca^{2+} "oscillation" signature (Chinnusamy et al. 2004).

Transient increase in cytosolic Ca^{2+} and subsequent activation of Ca^{2+} sensor proteins are two of the responses of plants toward salinity. The discovery of Salt Overly Sensitive (SOS) pathway and the following studies have shed light on a major mechanism activated during ionic stress response in plants. Detailed studies have helped in identifying the components of this pathway. The SOS pathway typically comprises three proteins, viz., SOS1, SOS2, and SOS3 (Zhu 2002). SOS3 is a myristoylated calcium-binding protein and functions as a primary calcium sensor, perceiving rise in cytosolic Ca^{2+} affected by excess cytoplasmic Na^{+}. Upon binding with Ca^{2+}, SOS3 activates a serine/threonine protein kinase SOS2 (Ji et al. 2013; Soni et al. 2013).

SOS3–SOS2 kinase complex then recruits SOS2 to the cell membrane activating SOS1, a plasma membrane-localized sodium–proton antiporter (Na^+/H^+). This causes a subsequent extrusion of excessive Na^+ (Quintero et al. 2011; Ji et al. 2013).

14.4.2 Calcium-Dependent Protein Kinases

Ca^{2+}-dependent protein kinases (CDPKs) are unimolecular calcium sensors encoded by a large multigene family that is found ubiquitously in plants. CDPKs are also present in protists, oomycetes, fungi, and green algae but are absent in animals (Valmonte et al. 2014). CDPKs directly bind Ca^{2+} ions. After binding Ca^{2+} ions, they phosphorylate their substrates which function in a wide variety of cellular and physiological processes such as osmosis, phytohormone response, and stress response. The structure of CDPKs comprises an N-terminal domain which is fused to a kinase (Ser/Thr) domain and a CDPK activation domain (CAD). The CAD has two distinct regions—a pseudo-substrate region and a Ca^{2+}-binding domain (Boudsocq and Sheen 2013; Liese and Romeis 2013). Recent studies indicate that CDPKs perceive specific stress-induced Ca^{2+} signals and translate them into phosphorylation events. These events further trigger downstream signaling processes. The resolution of CDPK-initiated phosphorylation patterns has provided deeper insights into CDPK-mediated signaling indicating a "signaling hub"-like role for CDPKs during plant stress signaling and development (Schulz et al. 2013).

14.4.3 Annexins

The annexin family is another important target for changes in cytosolic [Ca^{2+}] in plant cells. Annexins are Ca^{2+}-dependent membrane-binding proteins constituting multimembered family in plants. They have been shown to take part in Golgi-mediated secretion pathway (Clark et al. 1992, 1999; Blackbourn and Battey 1993; Clark and Roux 1999) and exocytosis of polysaccharides (Carroll et al. 1998). Further, it has been found that GTP inhibits annexin/Ca^{2+}-dependent exocytosis (Carroll et al. 1998). Because of their structural similarity to animal counterparts, it has been hypothesized that plant annexins may also exhibit Ca^{2+} channel activity (Clark et al. 2001b).

14.4.4 MAPK Cascades

Mitogen-activated protein kinases (MAPKs) represent the strongest link for cross talk during abiotic stress signaling in plants. MAPKs have been shown to be involved in developmental, hormonal, and stress (both biotic and abiotic) signaling (Danquah

et al. 2014). This suggests that MAPKs may act as nodes of convergence during response to multiple stresses. It is well known that MAP kinase kinase kinase (MAPKKK) phosphorylates MAP kinase kinase (MAPKK). MAPKK further phosphorylates MAPK. In *Arabidopsis*, three stress-responsive MAPKKKs have been identified, viz., AtMEKK, ANP1-3, and CTR1. AtMEKK1 has been found to be differentially regulated in response to multiple abiotic stresses (Teige et al. 2004; Xing et al. 2008). Further, various MAPKs such as MPK3, MPK4, MPK6, MPK7, and MPK11 have been shown to be involved in plant innate immunity (Droillard et al. 2004; Bethke et al. 2012; Eschen-Lippold et al. 2012), cytokinesis and spindle fiber assembly (Beck et al. 2011; Kosetsu et al. 2010), epidermal patterning (Wang et al. 2007), ovule development (Wang et al. 2008), ABA, and abiotic stress response (Ahlfors et al. 2004; Droillard et al. 2004; Gudesblat et al. 2007b). It was shown that overexpression of DSM1 (drought hypersensitive mutant1), which belongs to plant Raf-like MAPKKK family, improved adaptation to dehydration and oxidative stress in rice (Ning et al. 2010). Similarly, ectopic expression of a MAPK gene from maize, ZmMPK7, in tobacco improved protection from oxidative damage (Zong et al. 2009). These studies suggest a pivotal role for MAPKs in abiotic stress response and adaptation in plants.

14.4.5 ABA-Dependent and ABA-Independent Regulation

The plant hormone abscisic acid (ABA) regulates various aspects of seed development like seed desiccation tolerance and dormancy. Besides, it also plays a pivotal role in both biotic and abiotic stress responses in plants. ABA regulates several aspects of seed development including seed desiccation tolerance and dormancy and also plays a crucial role in both biotic and abiotic stress responses in plants. Although perturbations in ABA biosynthetic pathway are well studied during osmotic stress, still the signaling pathway through which ABA biosynthetic genes are upregulated is unknown. Drought response in plants is characterized either by ABA-dependent or ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2002). ABA-independent stress response has been shown to be dependent on DRE *cis*-elements, whereas ABA-dependent pathways promote gene expression via ABRE *cis*-regulatory elements. NAC {for *N*AM (no apical meristem), *A*TAF1-2 (*Arabidopsis* transcription activation factor), and CUC2 (cup-shaped cotyledon)} and ERFs (ethylene response factors) are prominent examples of transcription factors activated via the ABA-dependent pathway (Sahoo et al. 2013). A typical example of gene participating in ABA-independent pathway is *RD29A*(LTI78 or COR78). The promoter region of *RD29A* possesses DRE or CRT (drought-responsive or C-repeat element). *RD29A* is also considered a stress-marker gene. It is regulated by both cold and drought (Narusaka et al. 2003; Chinnusamy et al. 2004).

14.5 Classification and Interactions of Stress-Associated Signaling Pathways

When plants are confronted to different environmental stresses, there occurs an the initiation of several signal transduction processes. However, there has been no unanimous way to classify these diverse signaling events. Nevertheless, a simple classification may be (Fig. 14.2):

(a) Oxidative/osmotic stress signaling that brings into play MAPK modules
(b) Ca^{2+}-dependent signals resulting in the activation of *LEA*-type genes
(c) Ca^{2+}-dependent SOS signaling pathway regulating Na^{+} homeostasis

Oxidative/osmotic stress (Type I) signaling may contribute to the accumulation and production of compatible solutes and antioxidants. Two such genes shown to take part in this pathway were found through the identification of mutants, viz., *eskimo1* (*esk1*; tolerant to freezing) and *pst1* (salt-tolerant mutant). Xin and Browse

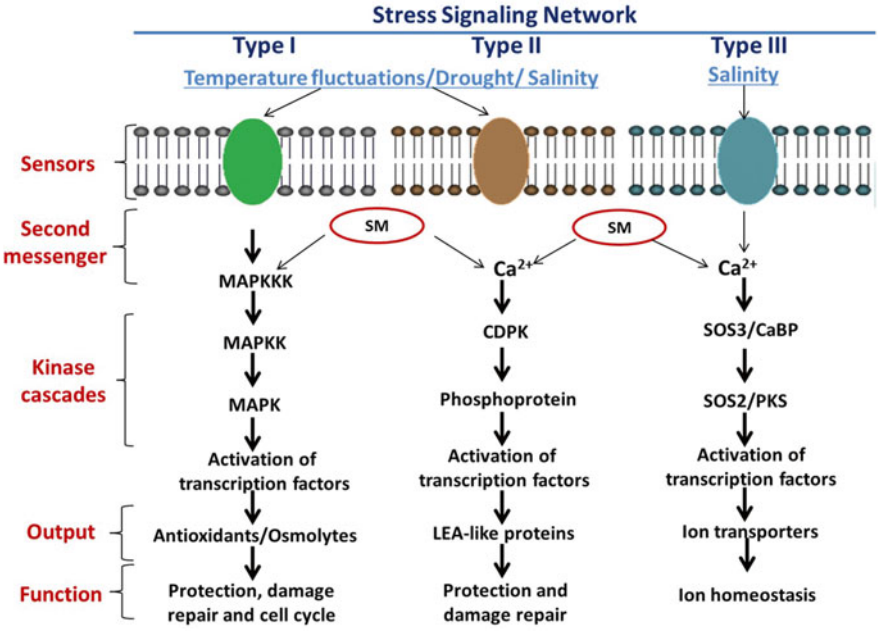

Fig. 14.2 Major types of signaling pathways operational during response to various abiotic stresses. Signaling networks operational in response to various abiotic stresses can be classified into three types. Type I network is functional in heat, osmotic, and oxidative stresses and involves the MAPK cascade resulting in the activation of transcription factors (TFs), eventually leading to the production of compatible solutes and antioxidants. The other two types function in a Ca^{2+}-dependent manner. The type II network is activated in response to the same stresses, which also activate type I network. However, in contrast to type I, the type II network functions via Ca^{2+}-dependent protein kinases leading to the increased synthesis of LEA-like proteins. The other Ca^{2+}-dependent network, type III, is specific to salinity stress and involves the SOS3/SOS2 components, which function in the maintenance of ion homeostasis. See text for details

(1998) have shown that *esk1* accumulates and maintains high level of proline and soluble sugars. Similarly, *pst1* mutant showed high ROS scavenging capacity but shows no effect in the sodium (Na^+) accumulation (Tsugane et al. 1999). Ca^{2+}-dependent signaling (Type II) is the most extensively studied signaling network which leads to the activation of LEA-like genes and also other members of the DRE/CRT classes. Ishitani et al. (1997) have shown that mutants of Ca^{2+}-dependent signaling type include *los*, *cos*, and *hos* mutants that were found in an *RD29A-LUC* reporter-facilitated genetic screen. Ca^{2+}-dependent SOS signaling (Type III) seems to be comparatively specific for the ionic part of salinity stress (Fig. 14.2). Ion transporters are the targets of this type of signaling which regulate Na^+ homeostasis under salinity stress conditions. Mutants of the SOS genes (*sos1*, *sos2*, and *sos3*) are hypersensitive to salt stress, but they show no effect on the activation of the DRE/CRT class of genes (Zhu et al. 1998). Additionally, these mutants accumulate compatible osmolytes like proline under salt stress conditions (Liu and Zhu 1997).

Various stress signal transduction pathways are very specific regarding the input stimuli. The interaction and specificity among these pathways have been dealt with at length by Knight and Knight (2001). Abiotic stresses such as salinity, drought, and cold comprise more than one trait and are multigenic in nature. If common attributes are shared between two stress conditions (such as hyperosmotic stress for salinity and drought), then the signaling during response from the same attribute might be common. Interaction between these signaling pathway types (Fig. 14.2) is not wide, as evidenced by the lack of mutants specific to these pathways and as found via the extensive study of transgenics (Kovtun et al. 2000). For example, in tobacco activation of the HOSAK and MAPKs, SA-induced protein kinase and HOSAK by osmotic stress are not only independent of ABA but also unaffected by the *sos3* mutation (Hoyos and Zhang 2000). Similarly, even though both salinity and drought stresses result in a transient rise in cytosolic Ca^{2+}, the SOS pathway is not activated by drought stress. These different stresses have precise Ca^{2+} signatures that could be deciphered by their particular Ca^{2+} sensors. Allen et al. (2001) have reported that specific Ca^{2+} fluctuations in stomatal guard cells are regulated by stomatal movements.

Under definite conditions, e.g., when components of signaling pathways are ectopically expressed or overexpressed, unnatural interactions among them may occur. The basis of the observed "gain-of-function" effect is the alteration of either the dosage of the signaling molecules or original subcellular localization.

14.6 Utilizing Functional Genomics Approach to Engineer Signal Transduction Components

Functional genomics is a very powerful and complex method to determine the role of individual genes, networks, pathways, and entire genomes. With rapid advances in high-throughput sequencing, proteomics, and metabolomics tools in the past decades, functional genomics has become a vital platform in plant sciences (Moreno-Risueno et al. 2010; Mittler and Shulaev 2013). The numerous plant genomes sequenced have produced a large dataset on the structure, composition, and

organization of different plant genomes, which has paved the way for finding several abiotic stress-responsive genes related to signal transduction pathway (Edwards et al. 2013; Higashi and Saito 2013). Signal transduction pathways are activated in the first few minutes, and the major basis of their contribution toward stress response lies in the modulation of transcription factor activity. Therefore, a comprehensive way to identify genes functioning in these pathways is via concurrent search against transcriptomics- and proteomics-based data of early stress response.

In the signaling related to most of the biological processes, phosphorylation events are a common feature which has a high potential for crop improvement. Without a doubt, a number of members of MAPK modules function in responses to various abiotic stress conditions and have been engineered for conferring tolerance against multiple abiotic stresses such as salt, drought, or low temperature. For instance, it has been shown in *Arabidopsis* that overexpression of MKK2 which is involved in phosphorylation of MPK6 and MPK4 results in constitutive upregulation of many stress-inducible genes and the plants conferring increased salt and freezing tolerance to the transgenic plants (Teige et al. 2004, Samajova et al. 2013). Furthermore, MPK6 and MPK3 play a crucial role in the control of stomatal opening/closure. Additionally, MKK4 and MKK5, which are upstream activators of MPK6 and MPK3, are key regulators of stomatal patterning and development (Wang et al. 2007). Finally, they control stomatal movements by operating in close cooperation with hydrogen peroxide (H_2O_2) and abscisic acid (ABA) (Gudesblat et al. 2007a, b).

Majority of such functional genomics studies have been done in *Arabidopsis*. Today's major aim in agriculture is to translate all the generated knowledge from model plants (like *Arabidopsis*) to different crop plants. To achieve this goal, the first step should be the identification of orthologs in crop plants. Bioinformatics is an important tool aiding this venture (Mochida and Shinozaki 2010). After successful identification of a gene, its overexpression is the straightest way to attain the preferred phenotype. In addition, there are several other methods such as oligonucleotide-directed mutagenesis or TILLING (Targeting Induced Local Lesions in Genomes) to obtain transgenic crops containing modified levels of proteins. At last, artificial or hybrid kinases can be formed that alter proteins apart from their true targets.

Though many signaling factors that play an essential role in abiotic stress response have already been identified, still the complex signaling processes are not fully understood (Chinnusamy et al. 2004). Studying the regulation of signal transduction modules would provide deeper knowledge to develop novel strategies to generate stress-tolerant transgenics plants against multiple abiotic stresses through integrated omics approaches such as proteomics, transcriptomics, phosphoproteomics, metabolomics, and cellomics.

14.7 Conclusion and Future Directions

In an era of accelerated global climate change, developing crop varieties resistant to various abiotic and biotic stresses has become the need of the hour (Battisti and Naylor 2009). Developing stress-tolerant crop varieties requires meticulous efforts

beginning from identifying and characterizing genes to translating the proof of concept to farmer fields. Although, as described in the previous sections, several genes have been found to confer tolerance to one or more abiotic stresses, none of them have been successfully translated to the field-stage. This owes largely to the complexity of the stresses and also to the simultaneous presence of multiple abiotic stresses in the farmers' fields.

Of late, the approach that is gaining widespread acceptability is either the simultaneous manipulation of diverse cellular genes via gene pyramiding tools or altering the expression of "master regulators," which in turn might regulate the function of diverse downstream targets. Components of the signal transduction pathways, due to their capacity to modulate the function of several proteins, constitute an important class of such master regulators. Therefore, they hold great promise in the endeavor to generate "climate-smart" crops tolerating a multitude of diverse stresses.

Findings of numerous studies related to stress response and associated signaling pathways in plants have supported the existence of cross talk among various stress-responsive signaling pathways (Chinnusamy et al. 2004). It has often been found that the initial perception may be specific to a particular stress, but the response pathways may converge at points known as nodes. For instance, many stresses lead to an oxidative damage which triggers the activation of the antioxidant/ROS scavenging pathway (Sahoo et al. 2013). Thus, it is now an accepted belief that plants do respond with both stress-specific adaptive responses and responses which can protect the plants from multiple stresses. Consistently, it has been found that engineering plants for salinity stress tolerance, for example, have also conferred tolerance to other abiotic and biotic stresses as well (Sahoo et al. 2013). This aspect of signaling pathways is an added advantage favoring their utility in studies aiming the development of climate-resilient crops that are well adapted to tolerate multiple stresses.

To date, there has been a little progress in understanding how the interaction of biotic and abiotic stresses is brought about in plants. It is also not known if a particular abiotic stress will accentuate the effect of another abiotic stress or vice versa. There is a need to understand interaction of these stresses in pairs or in combinations with each other and further translate this understanding to design superior crop varieties. Considering the fact that signaling proteins and associated pathways are involved in both biotic and abiotic responses, the challenge lies in the identification of these converging nodules in the large signaling webs. Further, the ultimate desirable outcome of biotechnology in agriculture is ensuring higher yield and nutritional security. The development of such "all-inclusive" crop varieties, however, is a daunting task and requires painstaking efforts which the future studies should aim to employ.

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Chapter 15
Abiotic Stress in Crops: Candidate Genes, Osmolytes, Polyamines, and Biotechnological Intervention

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Abstract Agricultural production and quality are adversely affected by various abiotic stresses including water deficit conditions (drought), salinity, extreme temperatures (heat, cold), light intensities beyond those saturating for photosynthesis, and radiation ($UV_{B,C}$). This is exacerbated when such exposure occurs during seed germination and reproductive phases of development. Estimates of crop losses can amount to billions of US dollars worldwide. To prevent such losses, it is necessary to develop stress-tolerant crops. One approach is to identify resistant germplasm using breeding strategies assisted by molecular markers and transfer those attributes to sensitive varieties, but this approach is a timely process. Introduction of genes that can improve stress tolerance in crops against heat, drought, and salinity is relatively a more effective technology. In this regard, the scientific community is well placed since a number of critical genes, particularly transcription factors that regulate gene expression in response to environmental stresses, have been identified and the proof-of-the-concept validated. Translation of the technology into major crops (rice, wheat, sorghum, and maize) and vegetable/fruit crops is the need of the times.

Keywords Agriculture • Climate change • Environmental stress • Transcription factors • Transgenics

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Abbreviations

ABRE	Abscisic acid-responsive elements
AP2	Apetala 2
CO_2	Carbon dioxide
DREB	Dehydration response element-binding factors
ERF	Ethylene response elements
PA	Polyamines
Put	Putrescine
SAMDC	S-adenosylmethionine decarboxylase
SPD	Spermidine
SPDS	Spermidine synthase
Spm	Spermine
SPMS	Spermine synthase
T-Spm	Thermospermine

15.1 Introduction

Plants being sessile are in direct contact with daily fluctuations in the environment. The environmental stresses that plants face include water deficit (drought), temperature extremes (heat, cold), salinity, flooding, and high light intensities (also ultraviolet radiation). These abiotic stresses adversely affect the growth and productivity of crops. The development of stress-tolerant crops is necessary to sustain agricultural productivity. It is estimated that human population will reach close to ten billion by 2050 (Bengtsson et al. 2006; United Nations World Population Prospects 2010), which will require almost doubling the current agricultural productivity. Moreover, such a humongous increase in crop productivity must be achieved with no increase in arable land and in the face of changing global climate, diminishing natural resources and environmental stresses. Therefore, yield increase of major (e.g., rice, wheat, and maize) and minor (horticultural commodities such as fruits and vegetables) crops has to cope up with and sustain the tremendous world population growth (Godfray et al. 2010; United Nations World Population Prospects 2010). Breeding strategies employing marker-assisted selection for high yielding varieties as well as for identifying germplasm resistant to abiotic and biotic stresses are already in vogue. Another approach is to introduce agronomically important genes as well as those that can improve tolerance to environmental extremes using genetic engineering technology. A number of critical genes that have the potential to improve productivity and impart tolerance/resistance to crops against abiotic and biotic stresses have been identified and validated. Some of them are transcription factors that interact directly or indirectly with the genes associated with the abiotic stress signaling. This unique functional feature makes them a tool of choice to alter genetic traits in any crop plant and allows fine-tuning a plant function rather than making them on/off switches. Many transcription factors have been functionally

characterized in model plants such as *Arabidopsis*, rice, and tomato. Here, we review aspects of candidate genes with a focus on transcription factors and linkage of small molecules (osmolytes, polyamines) to plant abiotic stress tolerance.

15.2 Abiotic Stresses and Agriculture

One of the concerns about sustainability of agriculture has arisen because of global climate change that anticipates global warming and consequently increased carbon dioxide (CO_2) levels and other greenhouse gases in the atmosphere. Thus, crops that can adapt to warmer temperatures can survive. There are benefits of higher CO_2 concentration, which has been rising on an average of 2 μl L^{-1} per year in the last decade (IPCC 2007; Peters et al. 2011)—it increases carbon assimilation and rate of photosynthesis. In fact, it has been estimated that crop production in future may increase by 1.8 % at elevated CO_2 but the production will be reduced by 1.5 % per decade due to global warming (Lobell and Gourdji 2012). Elevated CO_2 can help maintain low stomatal conductance rate that will then reduce water loss and be beneficial during water deficit conditions (Leakey et al. 2009). In C3 plants, the elevated CO_2 can contribute to better photosynthetic ability by keeping the CO_2-fixing enzyme Rubisco saturated and inhibiting oxygenic reactions and photorespiration that cause a drag in yield. In contrast, the elevated CO_2 level in bundle sheath may reduce photosynthetic rate in C4 plants (Long et al. 2006; Leakey et al. 2009).

Irrespective of a positive effect of increased atmospheric CO_2 levels on crop photosynthesis and transpiration, the higher temperatures and drought conditions jointly could cause more damage than good (Shukla and Mattoo 2013). Such a combination could negatively impact growth, plant development, crop yield, and increased soil salinity (Iannacone et al. 2012). Elevated temperatures also reduce the grain filling period in wheat, oat, and field corn (Mitchell et al. 1993; Wheeler et al. 1996; Rosenzwig and Hillel 1998; Ghaffari et al. 2002). In the future, the earth surface temperature is expected to rise by 3–5 °C (IPCC 2007) and this will impact crop productivity due to higher frequency of drought, heat, flood, and heat waves (IPCC 2008; Mittler and Blumwald 2010). Specifically, much warmer and drier summers and variable rainfall will impact the growing season of different crops in Central Europe and Central Africa, causing salinization in the arable land (Porter and Semenov 2005; IPCC 2008; Morison et al. 2008).

Growth of crop plants in suboptimal environmental conditions prevents plants from using their full genetic potential for growth and reproduction (Bray et al. 2000), causing yield losses due to such environmental perturbations (Shao et al. 2008). Abiotic stressors such as heat, cold, drought, and salinity hugely impact agriculture, at times lowering >50 % the average yields of crops (Wang et al. 2003). When all stresses combine, it can have a devastating consequence on plant productivity. Enhanced pressure on global food productivity, due to increasing population, accompanied by changing climatic conditions, will result in a demand for stress-tolerant crops for better crop yield (Newton et al. 2011).

15.3 Gene Expression and Regulation: Transcription Factors as Candidate Genes in Relation to Abiotic Stressors

Plants have evolved with various mechanisms at multiple levels to respond to unfavorable environment including abiotic stresses. It is now known that in response to an abiotic stress, a medley of genes impacting various pathways get induced as also the gene products—proteins, which act as transcription factors. Gene products' functionality involves signal transduction pathways that are tightly regulated and help plants to combat the stress conditions. Gene regulation occurs, at least, at three levels: transcriptional, posttranscriptional, and posttranslational. At each level, various molecular elements and networks are involved. It is at the transcriptional level, however, where the intricacy is of essence, involving the interplay of mainly three important components: chromatin modification and remodeling, *cis*-regulatory elements such as enhancers and promoters that are located upstream and downstream the coding region, and *trans*-regulatory transcription factors (Luo et al. 2012).

Plants respond to abiotic stresses by activating or repressing select genes that fine-tune the complex genetic regulation to synthesize specialized proteins, enzymes, and metabolites. Stress responses are mostly regulated at transcriptional and posttranslational levels via transcription factors (Chen and Zhu 2004). The last few years have seen the use of microarray approaches and next-generation sequencing (NGS) methodologies in unraveling the complex regulatory mechanisms that regulate abiotic tolerance in plants. Thus, elegant and critical studies of genomics in model species including *Arabidopsis*, tomato, and rice have shed light on the regulatory networks involved in abiotic stress tolerance. The new knowledge should help modulate these networks to improve plant growth and crop yields in general and under abiotic stresses, in particular.

A large number (≈1,300) of transcription factor genes are estimated to be functional in *Arabidopsis* (Riechmann and Ratcliffe 2000). Many of them are stress responsive and belong to a family of genes. Each of the stress-responsive transcription factor family has characteristic features such as specific and distinct DNA-binding domains: NAC, ERF/AP2, Zn-finger, DOF, Myb, WRKY, bZIP, and HD-ZIP (Riechmann and Ratcliffe 2000). Molecular studies involving comparative transcriptomic analyses in response to stresses have shown that the expression of specific transcription factors is modulated by multiple stresses (Mittler 2006; Weiste et al. 2007). It is recognized that to conclude that a particular transcription factor is linked to a defined abiotic stress, one needs to take into consideration and evaluate (a) which member of a transcription factor family is involved, (b) whether the plant response occurs without conferring stress tolerance, and (c) the function of the transcription factor is validated *in planta* through transgenic approaches, while transgenic plants must be evaluated for their stress-tolerance level under each stress condition. Such a requirement can ensure that the validated transcription factor is useful for biotechnological tools to improve abiotic stress tolerance and thereby improve crop productivity. We will now evaluate the information available on a few candidate transcription factor genes in relation to abiotic stressors.

15.3.1 bZIP

Basic leucine zipper (bZIP) transcription factors possess 40–80-amino-acid-long bZIP DNA-binding domain. In addition, they contain a highly basic region at the N-terminus and a leucine-rich motif at the C-terminus, which help in dimerization (Jacoby et al. 2002). They regulate DNA activation via dimerization, homo- or heterodimerization. Molecular and biochemical analyses have implicated bZIPs in important plant processes. Examples of these are organ and tissue differentiation (Chuang et al. 1999; Abe et al. 2005; Shen et al. 2007), cell elongation (Fukazawa et al. 2000), nitrogen/carbon balance control (Weltmeier et al. 2006), pathogen defense (Zhang et al. 1993; Despres et al. 2000; Thurow et al. 2005; Kaminaka et al. 2006), unfolded protein response (Iwata and Koizumi 2005), hormone and sugar signaling (Finkelstein and Lynch 2000; Niggeweg et al. 2000; Uno et al. 2000; Nieva et al. 2005), light response (Wellmer et al. 1999; Osterlund et al. 2000), osmotic control (Satoh et al. 2004; Weltmeier et al. 2006), and seed storage protein gene regulation (Lara et al. 2003).

These transcription factors are induced by ABA, which is a known plant stress hormone. ABA levels in plants increase in response to drought, high temperature, and upon induced stomata closer (Davies and Zhang 1991; Saradhi et al. 2000). Interestingly, the induced stress-related genes harbor an ABRE *cis*-element in their promoter region. It is these elements that form the binding sites for bZIP transcription factors.

The members of bZIP family vary from plant to plant, about 75 recognized in *Arabidopsis*, 89 in rice, and 131 in soybean (Kim 2006; Liao et al. 2008; Nijhwan et al. 2008; Wang et al. 2011; Wei et al. 2012). *In planta*, characterization of various bZIPs indicated that they provide abiotic stress tolerance to plants (Kang et al. 2002; Kim et al. 2004; Zou et al. 2007). Overexpression of *OsABI5* resulted in ABA hypersensitivity in rice plants, yet its suppression provided salt tolerance to rice plants (Zou et al. 2008). Also, rice *bZIP72* overexpression led to drought tolerance in transgenic rice plants. *OsbZIP23* overexpression in rice enhanced tolerance to both drought and salinity without compromising the growth and development of the transformed plant (Xiang et al. 2008; Lu et al. 2009).

15.3.2 AP2/ERF

AP2/ERF transcription factors are characterized by the presence of 60–70-amino-acid-long DNA-binding domain. This domain directly interacts with the *cis*-elements such as the GCC box and/or a dehydration-responsive element (DRE)/C-repeat element (CRT), which are present in the promoters of the downstream target genes. This group of transcription factors is classified into five subfamilies: AP2 (Apetala 2), RAV (related to ABI3/VP1), DREB (dehydration-responsive element-binding protein), ERF (ethylene-responsive factor), and others (Sakuma et al. 2002). To date, AP2/ERF transcription factors have been identified in several plants, namely, 163 in rice, 145 in *Arabidopsis*, 132 in grapevine, 200 in poplar, and 85 in tomato (Riechmann and Meyerowitz 1998; Jaillon et al. 2007; Zhuang et al. 2008; Sharma et al. 2010; Sharoni et al. 2011).

Two of the better-studied families are ERF and DREB with broad spectrum implications in plant stresses because many of them have been implicated in abiotic stress. For example, rice *OsDERF1* negatively regulates drought tolerance, demonstrated by showing that knockout rice plants for *OsDERF1* were drought tolerant (Wan et al. 2011). In another report, overexpression of two ERF genes from rice, *AP37* and *AP39*, protected plants against drought and salinity (Oh et al. 2009), while overexpression of *Arabidopsis* ERF, *HARDY*, in rice led to drought tolerance, which was associated with reduced transpiration and enhanced photosynthesis rate (Karaba et al. 2007). Tomato ERF, *TSRF1*, when expressed in rice regulated osmotic and drought responses in the transgenic plants. The improved osmotic and drought tolerance was achieved without growth retardation. This transcription factor specifically activated the rice abscisic acid (ABA) synthesis gene, *SDR*, causing enhanced ABA sensitivity of the transgenic rice plants (Quan et al. 2010). Under flooding stress in rice, two ERF proteins, SNORKEL1 and SNORKEL2, were involved in GA accumulation and caused fast stem elongation in deep water rice (Hattori et al. 2009). Another protein is SUB1A, which inhibited shoot elongation and enhanced survival of submerged transgenic rice (Xu et al. 2006). Similarly, *Arabidopsis RAP2.2* is an ethylene-dependent, hypoxia-induced transcription factor that led to survival of transgenic *Arabidopsis* plants under hypoxia stress (Hinz et al. 2010). Transgenic *Arabidopsis* plants transformed with wheat *TaERF1* showed improved tolerance to salt and freezing stress (Xu et al. 2007), while expression of a modified *Brassica napus* AP2/ERF transcription factor in *Arabidopsis* enhanced cold tolerance (Xiong et al. 2013).

DREB family transcription factors, a subfamily of AP2/ERF, have been tested in a variety of plants—*Arabidopsis*, wheat, tomato, soybean, rice, maize, and barley—for their ability to enhance abiotic stress tolerance (Agarwal et al. 2006b; Lata and Prasad 2011; Mizoi et al. 2012). On the basis of their biological function in divergent species, DREBs are further classified into two subcategories—DREB1 and DREB2—each involved in separate signal transduction pathway under abiotic stresses (Dubouzet et al. 2003). DREB1-type transcription factors are induced by low temperature stress, whereas DREB2 types are induced by dehydration and high salt stress (Liu et al. 1998; Sakuma et al. 2002; Dubouzet et al. 2003; Lucas et al. 2011). Transgenic wheat plants containing *DREB1A* gene, under the control of rd29A promoter, showed enhanced drought tolerance (Pellegrineschi et al. 2004). Similarly, transgenic pea plants expressing rd29A:DREB1A accumulated considerably higher levels of antioxidant enzymes and proline content under drought stress (Bhatnagar-Mathur et al. 2007; Yang et al. 2012); overexpression of *DREB1A* in rice plants improved their drought and salinity tolerance (Oh et al. 2005). Cotton *GhDREB* introduced in wheat led to improved tolerance to drought, high salt, and freezing stresses. These transgenic wheat plants accumulated higher levels of soluble sugar and maintained higher chlorophyll levels in response to added stress (Gao et al. 2009).

In contrast, DREB2-type genes have been studied in a limited number of plant species (reviewed by Agarwal et al. 2006b). However, *DREB2*-type genes also improved abiotic tolerance in tested plants. Maize *ZmDREB2A* under the control of constitutive or stress-inducible promoter enhanced drought tolerance in transgenic *Arabidopsis* plants (Qin et al. 2007). Therefore, additional DREB2-type genes need to be studied to gain more knowledge about their role in plant responses to stresses.

15.3.3 MYB

Like the aforementioned transcription factors, MYB transcription factors are also a family of genes, widely distributed in plants, and characteristically harbor a unique ~50-amino-acid-long DNA-binding domain (MYB domain). They are primarily classified on the presence of the number of MYB DNA-binding domain repeats, generally present from 1 to 4 in tandem in MYB genes (Feller et al. 2011; Katiyar et al. 2012). Based on the number of domain repeats, they are divided into four groups—1R-MYB, 2R-MYB, 3R-MYB, and 4R-MYB. Among these, the R2R3-type MYB domain proteins are more prevalent in plants (Martin and Paz-Ares 1997; Dubos et al. 2010). MYB transcription factors range from 339 in *Arabidopsis*, 230 in rice, to 60 in wheat (Dubos et al. 2010; Feller et al. 2011; Zhang et al. 2012). They regulate a wide range of functions in plants, including plant development, secondary metabolism, hormone signal transduction, disease resistance, and abiotic stress tolerance (Allan et al. 2008; Cominelli and Tonelli 2009).

Comprehensive functional studies on these transcription factors *in planta* showed that they play an important role in abiotic stress tolerance in plants. Examples include the following:

OsMYB3R-2 transgenic rice plants had enhanced cold tolerance (Ma et al. 2009); *Arabidopsis* transgenic plants expressing *OsMYB4* were tolerant to freezing stress (Vannini et al. 2004; Pasquali et al. 2008); *AtMYB96*, R2R3-type MYB transcription factor, integrates auxin and ABA pathway to regulate drought stress (Seo et al. 2009);

Arabidopsis expressing *AtMYB15* plants were ABA hypersensitive with improved drought and cold tolerance; *MYBS3* expression imparted cold tolerance to transgenic rice plants (Su et al. 2010);

Constitutively expressed *OsMYB2* imparted drought and salt tolerance to transgenic rice plants (Yang et al. 2012). Interestingly, *MYB15* was found to negatively regulate the expression of CBF genes (*C*-repeat/DRE-*B*inding *F*actor genes) during cold tolerance. Overexpression of *MYB15* resulted in reduced expression of CBF genes, whereas its loss of function leads to increased expression of CBF genes in cold (Agarwal et al. 2006a).

15.3.4 NAC

NAC transcription factors contain a conserved N-terminal region (NAC domain) that can oligomerize and bind DNA and a C-terminal region that functions as a regulatory domain. NAC factors are found in different plants: 117 in *Arabidopsis*, 151 in rice, 79 in grape, 163 in poplar, and 152 in soybean (Puranik et al. 2012 and references therein). NAC genes affect development (Olsen et al. 2005), senescence (Kjaersgaard et al. 2011; Yang et al. 2011), biotic responses (Olsen et al. 2005; Christianson et al. 2010), and abiotic stress response (Tran et al. 2010).

Transgenic rice plants expressing *OsNAC1* exhibited enhanced drought tolerance, which was associated with induced stomatal closure and reduced transpiration in the

transgenic plants (Hu et al. 2006). Similarly, stress-responsive NAC gene (*SNAC2*) expression imparted cold and salinity tolerance to transgenic rice plants (Hu et al. 2008). *OsNAC6* overexpression in rice conferred drought and salt tolerance to transgenic rice plants (Nakashima et al. 2007). *OsNAC10*, when expressed under a root-specific promoter, imparted drought tolerance to transgenic rice plants (Jeong et al. 2010). Enhanced tolerance to drought and salt stresses by the expression of some NAC genes led to induction of other genes related to stress pathways. Examples include the following: *OsNAC045*-enhanced drought and salt tolerance was associated with the induction of stress-responsive genes, *OsLEA3-1* and *OsPM1* (Zheng et al. 2009); *OsNAC5* expression in rice plants activated *OsLEA3* gene along with conferring salinity tolerance to transgenic rice plants (Takasaki et al. 2010). Transcription factors also control each other's function; for example, expression of *ANAC096* showed an involvement with ABRE *cis*-element-binding factors for the transcriptional activation of ABA-inducible genes when subjected to dehydration and osmotic stresses (Xu et al. 2013).

15.3.5 WRKY

WRKY transcription factors contain a DNA-binding domain of 60 amino acids and a zinc finger motif at their C-terminus end (Rushton et al. 1995) and bind to W box (C/TTGACT/C) *cis*-elements, which are present in the promoters of several stress-responsive genes (Pandey and Somssich 2009).

WRKY family transcription factors are also abundant: 74 in *Arabidopsis*, 100 in rice, 197 in soybean, 66 in papaya, 104 in poplar, 68 in sorghum, 38 in *Physcomitrella patens*, 35 in *Selaginella moellendorffii*, 80 in pinus, and 45 in barley (Chen et al. 2012 and references therein). They participate in various processes in plants, including plant growth, seed development, leaf senescence, defense, and responses to biotic and abiotic stresses (Chen et al. 2012).

Like the other transcription factors mentioned above, functional *in planta* studies on various WRKY transcription factors showed their involvement in multiple abiotic stress responses. Examples include the following: expression of *OsWRKY89*, induced by UV-B radiation, enhanced the tolerance of transgenic rice plants to UV-B irradiation through increasing the wax deposition on leaf surfaces (Wang et al. 2007a); expression of *OsWRKY11* under a heat-inducible promoter imparted drought and heat tolerance to transgenic rice plants (Wu et al. 2008); *OsWRKY13* interacts with the *OsNAC1* in the abiotic stress response pathway in plants (Qiu et al. 2009); *AtWRKY34* participates in the pollen-specific cold stress response in mature *Arabidopsis* plants via interacting with the CBF proteins (Zou et al. 2010).

15.4 Heat Shock Proteins in Abiotic Stress

Protein denaturation and aggregation impair protein and cellular function. Temperatures higher than those optimal for normal growth impact many plant cellular processes and negatively affect grain filling and yield (Craufurd et al. 1993;

Morita et al. 2005). A group of proteins was selectively found to accumulate in diverse living cells in response to a short exposure to higher temperatures. This group of proteins became to be known as heat shock proteins (HSPs). It is understood that these proteins are normally synthesized to maintain homeostasis and are involved in a number of plant processes. The fact that HSPs have an important role in maintaining functional conformation of other proteins by assisting their folding and stabilizing them, and as chaperones in the assembly and transport of nascent proteins, has catalyzed research on their role in plant life and in response to abiotic stresses (Vierling 1991; Wang et al. 2003, 2004; Kazuko and Shinozaki 2006; Mu et al. 2013). HSPs are a large family of proteins classified into chaperonins such as GroEL/Hsp60, Hsp70 family, Hsp90 family, Hsp100 family, and small HSP family (12–40 kD). HSPs/chaperonins that function in abiotic stresses include the late embryogenesis abundant (LEA) proteins (Kazuko and Shinozaki 2006). The small HSP (sHSP) family is made of six classes, thought of as the most abundant stress-induced proteins, and have a conserved α-crystalline domain (ACD) at their C-terminus (Goyal et al. 2012). sHSPs are distributed throughout a cell in different subcellular organelles such as chloroplasts, mitochondria, nucleus, cytosol, and endoplasmic reticulum (Vierling 1991; Wang et al. 2004).

Most abiotic stresses cause protein misfolding, affecting the protein structure. The ability of a plant to quickly respond and synthesize in abundance HSPs may impart a defense against drastic effects of the extreme stress. Thus, the misfolding of susceptible proteins is preventable if sufficient molecules of HSPs are around to help the proteins to properly refold. For instance, gene shuffling was used to improve the function of Rubisco activase, a critical protein in carbon fixation, which led to better performance of transgenic *Arabidopsis* at moderately high temperatures (Kurek et al. 2007). The association of the chloroplast chaperone, a HSP cpn60b, with Rubisco activase was found to acclimatize photosynthesis to higher temperatures (Salvucci 2008). Studies on how plant HSPs respond to extreme environments can provide insights into the role(s) of HSPs in plant tolerance to abiotic stresses (Wang et al. 2014). Notably, overexpression of *TaHsfA2d*, similar to rice transcription factor *OsHsfA2d*, in *Arabidopsis* led to tolerance of the transgenic plant to elevated temperatures as well as to salinity and drought (Chauhan et al. 2013). The model plant showed higher yield variance under constant heat conditions. Thus, manipulation of HSP transcription factors such as TaHsfA2d in crop plants may impart tolerance to a number of abiotic stresses and sustain crop yield. *Arabidopsis* has 21 classes of A1 heat shock factor homologues, which play an essential role in the transcription activation of HSPs (Liu and Charang 2013). Heat stress response genes were chaperoned by *HSFA1* and *HSFA2*, suggesting that these HSP factors could be used to engineer stress tolerance in crop plants.

HSPs have been suggested to catalyze a better flow of water and nutrients in plants so that cellular metabolism becomes robust (Camejo et al. 2005). HSPs also mitigate reactive oxygen species effects on plants and can thereby protect plants from damage and help plant tolerance to different stressors (Lipiec et al. 2013).

15.5 Small Molecules and Stress Tolerance

15.5.1 Osmoprotectants (Osmolytes)

Osmoprotectants, or osmolytes, by definition help plants to survive in extreme osmotic stress. Osmolytes include N-methylated amino acids and amines (glycine betaine, sarcosine, and trimethylamine-N-oxide), amino acids (glycine, proline, and glutamate), and polyols (mannitol and trehalose). These small molecules often accumulate in plants that face an osmotic stress and repair the cytosolic imbalance caused by stress exposure (Hsiao et al. 1976). Osmolytes such as proline stabilize proteins and cell membranes from the damaging effects of desiccation as well as scavenge reactive oxygen species generated due to abiotic stresses such as drought, low temperature, and salinity (Hayat et al. 2012 and references therein). For example, roots of salt-tolerant alfalfa plants accumulate twice as much proline as the salt-sensitive plant roots (Fougere et al. 1991; Petrusa and Winicov 1997).

Genetic approaches have validated the fact that plants use small molecules to adjust to harsh environmental conditions. Overaccumulation of proline and soluble sugars provided tolerance against abiotic stresses in transgenic rice plants (Ito et al. 2006). Overexpression of tomato ERF transcription factor, *JERF3*, protected transgenic rice plants against drought and osmotic stress, which correlated with elevated proline and soluble sugar levels in transgenic rice plants (Zhang et al. 2010). In yet another study, expression of rice *MYB2* transcription factor was found to protect the transgenic rice plants from salt-mediated oxidative stress, which correlated with higher accumulation of proline and soluble sugars, lesser H_2O_2 production, and a low level of lipid peroxidation in the transgenics (Yang et al. 2012).

Glycine betaine (GB) is a quaternary amine whose accumulation correlates with plant tolerance to abiotic stresses. Genetic manipulation of GB biosynthesis led to protection of modified plants against various abiotic stresses (Chen and Murata 2011 and references therein). Table 15.1 lists examples of genes in GB biosynthesis pathway tested for their ability to provide abiotic stress tolerance. Two key enzymes are involved in GB biosynthesis: one, choline oxidase, which oxidizes betaine to betaine aldehyde and the second betaine aldehyde dehydrogenase, which converts betaine aldehyde to GB (Weretilnyk et al. 1989). Choline oxidase gene (*codA*) from *A. globiformis* has been used for GB production in transgenic plants. Transgenic rice plants that expressed heterologous bacterial *codA* gene overaccumulated GB and were more tolerant to salt and cold stress (Sakamoto and Murata 2002). Transgenic tomato plants expressing the *codA* gene were also tolerant against cold, salt, and oxidative stress. This tolerance was attributed to the protection of photosynthesis and reproductive organs and increased ROS detoxification (Park et al. 2007a, b). The authors hypothesized that GB may have a role in the protection of quaternary structure and enzyme activity of proteins from damaging effects of environmental stresses (Sakamoto and Murata 2002). Others have also reported that GB inhibits ROS accumulation, protects photosynthetic machinery, activates some stress-related genes, and protects membranes (Chen and Murata 2008).

Table 15.1 GB pathway genes and abiotic stresses

Gene	Gene source	Plant species transformed	Tolerance tested	Reference
codA	*Arthrobacter globiformis*	*Arabidopsis thaliana*	Tolerance to multiple stresses	Hayashi et al. (1997), Alia et al. (1998), Sakamoto et al. (2000), Sulpice et al. (2003)
codA	*Arthrobacter globiformis*	*Oryza sativa*	Tolerance to salt, cold, and drought stress	Sakamoto and Alia (1998), Kathuria et al. (2009)
codA	*Arthrobacter globiformis*	*Solanum lycopersicum*	Cold, salt, and oxidative stress tolerance	Park et al. (2007a, b)
codA	*Arthrobacter globiformis*	*Solanum tuberosum*	Tolerance to salt, drought, and oxidative stress	Ahmad et al. (2008)
betA	*Escherichia coli*	*Zea mays*	Cold and drought tolerance	Quan et al. (2004)
betA	*Escherichia coli*	*Gossypium hirsutum*	Drought tolerance	Lv et al. (2007)
BADH	*Spinacia oleracea*	*Nicotiana tabacum*	Heat stress	Yang et al. (2005)
BADH	*Atriplex hortensis*	*Triticum aestivum*	Heat and drought stress	Wang et al. (2010a, b)

Further, rice transformed with bacterial choline dehydrogenase gene accumulated higher levels of GB and the transgenic rice plants had improved tolerance to drought and chilling conditions (Quan et al. 2004). Similarly, wheat plants expressing the *Atriplex hortensis betaine aldehyde dehydrogenase* (*BADH*) gene were found more tolerant to drought and heat. These transgenic wheat plants had efficient photosynthesis rate, along with higher accumulation of GB, proline, and soluble sugar (Wang et al. 2010a, b).

Trehalose is a nonreducing sugar that also acts as an osmolyte in plants (Wingler 2002). Its functions in bacteria and yeast include sugar storage, metabolic regulation, and abiotic stress protection. Trehalose biosynthesis pathway genes from bacteria, trehalose-6-phosphate synthase (TPS) and/or terhalose-6-phosphate phosphatase (TPP), when expressed in plants enhanced the protection of transgenic plants against drought, salt, and cold abiotic stresses (Avonce et al. 2006 and references therein). Rice *TPP1* constitutively overexpressed in rice plants also developed improved salt and cold stress tolerance (Ge et al. 2008).

15.5.2 Polyamines

Polyamines are organic nitrogenous biogenic amines present ubiquitously in living cells. In plants, the main polyamines are the diamine putrescine (Put) and polyamines spermidine (Spd), spermine (Spm), and thermospermine (T-Spm) (Handa and Mattoo 2010). Their implication in abiotic stress responses dates back at least

Table 15.2 Polyamine biosynthesis pathway genes and abiotic stresses

Gene	Gene source	Plant species transformed	Tolerance tested	Reference
ADC	Oat	Rice	Salt	Roy and Wu (2001)
ADC	*Datura stramonium*	Rice	Drought	Capell et al. (2004)
ADC1	*Arabidopsis*	*Arabidopsis*	Freezing	Altabella et al. (2009)
ADC2	*Arabidopsis*	*Arabidopsis*	Drought	Alcázar et al. (2010a)
ADC	*Arabidopsis*	Eggplant	Multiple	Prabhavathi and Rajam (2007)
ODC	Mouse	Tobacco	Salt	Kumria and Rajam (2002)
SAMDC	Tritordeum	Rice	Salt	Roy and Wu (2002)
SAMDC	Human	Tobacco	Salt, osmotic	Waie and Rajam (2003)
SAMDC	Carnation	Tobacco	Multiple	Wi et al. (2006)
SAMDC1	*Arabidopsis*	Arabidopsis	Multiple	Alcázar et al. (2006)
SAMDC	Yeast	Tomato	Heat	Cheng et al. (2009)
SPDS	*Cucurbita ficifolia*	*Arabidopsis*	Multiple	Kasukabe et al. (2004)
SPDS	*Cucurbita ficifolia*	Sweet potato	Drought and salt	Kasukabe et al. (2006)
SPDS	Apple	Pear	Multiple	Wen et al. (2008)
			Oxidative	He et al. (2008)

six decades. An early report showed a relationship between potassium deficiencies and increased Put levels in barley plants (Richards and Coleman 1952). Studies employing polyamine-deficient mutants, application of exogenous PAs, and inhibitors of PA brought to the fore the association of polyamines with stress responses (Navakouidis et al. 2003; Alcázar et al. 2006; Liu et al. 2007; Wang et al. 2007b). Polyamine biosynthesis in plants derives from arginine (Arg) and/or ornithine (Orn) (Mattoo et al. 2014). A major focus in relation to stresses such as drought, chilling, heat, salinity, and hypoxia has been on the following genes/enzymes—Arg decarboxylase (ADC), Orn decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), Spd synthase, Spm synthase, and thermospermine (T-Spm) synthase (Flores and Galston 1982; Galston 1983; Bouchereau et al. 1999; Alcázar et al. 2006; Groppa and Benavides 2008; Alcázar et al. 2010b; Gill and Tuteja 2010; Bitrián et al. 2012). Transgenic approaches involving the study of genes or mutants have shown that PAs have a protective role against abiotic stresses (Alcázar et al. 2006; Kusano et al. 2008; Gill and Tuteja 2010; Gupta et al. 2013; Shukla and Mattoo 2013). Table 15.2 lists genes encoding polyamine biosynthesis pathway proteins tested for abiotic stress tolerance.

Exogenous polyamines affect plant cell membranes, minimize stress-induced growth inhibition, activate osmotic responsive genes, and elevate level of antioxidant enzymes (Ben-Arie et al. 1982; Ali 2000; Iqbal and Ashraf 2005; Tang and Newton 2005; Ndayiragiji and Lutts 2006; Afzal et al. 2009; Yiu et al. 2009; Zhang et al. 2009). Polyamine metabolism-deficient mutants, *Arabidopsis spe-1* and *spe-2*, were less tolerant to salt (Kasinathan and Wingler 2004; Watson et al. 1998). An ADC2 knockout mutant (*adc2-1*) was more sensitive to salt stress, which was reversed by exogenous Put (Urano et al. 2004). In the same way, *Arabidopsis*

acl5/*spms* mutant, defective for Spm production, is more sensitive to drought and salt stress, and Put application reverses the sensitivity to the defined abiotic stresses (Yamaguchi et al. 2006, 2007).

Heterologous overexpression of PA genes, ADC/ODC/SAMDC/SPDS, which leads to accumulation of higher PA levels in transgenic plants, was found to make the transformed plants—*Arabidopsis*, rice, tobacco, and tomato—tolerant to abiotic stresses (Minocha and Sun 1997; Thu-Hang et al. 2002; Kasukabe et al. 2004; Wen et al. 2008). Overexpression of oat, *Datura stramonium*, and *Arabidopsis ADC* in rice and *Arabidopsis* resulted in stress tolerance to salt, drought, and freezing abiotic stresses in transgenic rice and *Arabidopsis* plants (Capell et al. 1998, 2004; Roy and Wu 2001; Alcázar et al. 2010a). Likewise, *ODC* overexpression in transgenic tobacco plants showed them to have greater tolerance to salt stress than the control plants (Kumria and Rajam 2002). Similarly, heterologous *SAMDC* gene overexpressed in tobacco, rice, and tomato also led to higher polyamine accumulation, and the transgenic tobacco, rice, and tomato plants had tolerance against salt, osmotic, and heat stresses (Roy and Wu 2002; Waie and Rajam 2003; Alcázar et al. 2006; Wi et al. 2006; Cheng et al. 2009), while overexpression of *Spermidine Synthase* (*SPDS*) gene led to transgenic *Arabidopsis*, pear, and potato plants to be tolerant against drought, salt, and oxidative stresses due to the higher polyamine level in transgenic plants (Kasukabe et al. 2004, 2006; He et al. 2008; Wen et al. 2008).

15.6 Conclusions

Abiotic stresses are a major detriment to crop productivity and quality. Plants respond to environmental stresses in many different ways. Several genetic players in such responses have been identified and characterized. Among these are marker genes, transcription factors, and effective small molecules including osmolytes and polyamines. Together they provide candidacy and valuable indicators to be explored in building endogenous tolerance/resistance to individual or groups of different stresses that plants encounter in agricultural production systems on a daily basis. The path to creating new germplasm that can withstand various environmental stresses is before us. Efforts to critically mine gene function should be expanded to define which approach would enhance which crop and crop management practice. A systems biology approach, data mining, and clustering patterns of transcriptomic, proteomic, and metabolomic data addressing abiotic stress responses are needed to translate basic research into field application. Critical, controlled, and highly monitored field trials are required to enlist agriculturists to bring this bounty to the farmer.

Verily, although a good number of potential candidate genes that respond to different stresses are known, in a majority of the cases, validation has been restricted to the use of only the model plants. The success of some of the field-tested, novel germplasm/genotypes provides a positive spin and is encouraging for yet more field studies to be conducted with engineered crop plants. Only then, we will be able to produce super new high yielding crops that can withstand harsh environmental stresses including the global climate change and feed the world.

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Chapter 16
Abiotic Stress Tolerance and Sustainable Agriculture: A Functional Genomics Perspective

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Abstract Crop growth and productivity is being seriously constrained by a range of abiotic stress factors all over the globe. Literature revealed that abiotic stress factors [temperature extremes (heat and cold), water extremes (drought and flooding), salinity, sodicity, wounding, metal/metalloid toxicity, excess light, radiations, high speed wind, nutrient loss, and anaerobic conditions] are the key reason for declining the usual yield of major crop plants by more than 50 %, which causes significant economic losses every year. A number of genes and their products respond to abiotic stress factors at transcriptional and translational level; therefore, genetic engineering for abiotic stress resistance is an important goal for protecting/improving agricultural crop productivity. Adaptation of plants to various environmental insults is reliant upon the establishment of cascades of molecular networks involved in stress perception, signal transduction, and the expression of stress-specific genes and metabolites. Thus, engineering stress-responsive genes which can protect and/or preserve the function may be a potential target to enhance stress tolerance in plants. Genetic engineering and DNA markers have now emerged as important gear in crop improvement.

Keywords Abiotic stress tolerance • Functional genomics • Stress signaling • Genetic engineering • Crop plants

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16.1 Introduction

The sessile nature of plants presses them to recognize, distinguish, and respond to their immediate environmental conditions with suitable reactions. The major cause of the crop yield loss worldwide and subsequently enormous economic loss as well has been credited to abiotic stress factors. Among the major abiotic stresses, drought, salinity, metal/metalloid toxicity and deficiency, and extreme cold and heat temperatures are severely impacting plant growth, development, and productivity. A major part (>22 %) of agricultural soils on the globe is affected by salinity (FAO 2004). Heavy metals are frequently used as components of fungicides, pesticides, or disinfectants. Phosphate fertilizers, pesticides, and sewage sludge are among the major contributors of anthropogenic toxic metals in agricultural soils (Madyima et al. 2002). Crop plants may take up varied metal/metalloids (such as As, Cd, Cr, and Hg), where these metals/metalloids may accumulate in various concentrations in plant's edible and nonedible parts. Also, the low temperature [chilling (<20 °C) and/or freezing (<0 °C)] is limiting plant growth, development, and productivity (Jeon and Kim 2013). Morphological, physiological, biochemical, and molecular changes may be caused by the previous potentially interrelated and/or in isolation or in combination stress factors that in turn may affect overall plant development and productivity (Shanker and Venkateswarlu 2011). Nevertheless, salinity, drought, flood or waterlogging stress, high temperature, low temperature, metal toxicity, UV-B radiation, O_3, high light, and mechanical damage led to excessive reactive oxygen species (ROS) generation. A serious imbalance between the production of ROS and antioxidant defense may cause oxidative stress that in turn may damage biomolecules and finally arrest cellular metabolism. However, plant cells possess antioxidants like α-tocopherol, glutathione, and ascorbate and antioxidative enzymes such as superoxide dismutase (SOD, EC1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC1.11.1.6), and glutathione reductase (GR, EC1.6.4.2) that participate in ROS-scavenging (Mittler et al. 2004; Gill and Tuteja 2010). Functional genomics approaches such as genomics, transcriptomics, metabolomics, and proteomics can be useful for the evaluation of abiotic stress mechanisms in plants. These approaches can also be useful in identification of genes that are supposed to control the specific traits responsible for abiotic stress tolerance in plants (Cramer et al. 2007). Various functional genomics tools with greater sensitivities are now available for the understanding of complex cell-specific regulation. Nowadays, functional genomics approaches can be utilized for studies of whole organs like leaves, roots, etc. (Widodo et al. 2009). Different tools are available for identifying the function of gene at large genome scale like microarrays, serial analysis of gene expression (SAGE), and gene tagging and via use of physical and chemical mutagens. The transgenic approach is also used to elucidate the function of a gene or its product. Hence, using these technologies, the functional traits in plants contributing towards abiotic stress tolerance can be explored. The present chapter will focus on functional genomics approaches for abiotic stress tolerance in crop plants.

16.2 Abiotic Stress Responses

Plant responses to drought, salinity, metal/metalloid, and other stresses are discussed in detail in the light of recent literature.

16.2.1 Salinity

16.2.1.1 Salinity Stress vs. Plant Growth and Photosynthesis and Its Variables

Literature is full on the plant responses to salinity stress in terms of seedling establishment, growth, development, and yield with particular reference to the characterization of physiological upsets. NaCl stress has been evidenced to negatively impact plant growth in terms of severely decreased biomass (Zhu 2001; Nedjimi and Daoud 2009). Plant's phenological stages are differentially affected by salinity levels (Moradi and Ismail 2007; Manchanda and Garg 2008). Flowering and maturity are delayed due to salinity (Greenway and Munns 1980). Reduced germination, seedling emergence, and establishment can be caused by intolerable salinity (Munns and James 2003; Ashraf and Foolad 2005). Responses of plants at germination or early seedling development stage have been be used to predict mature plant responses to salinity stress (Maiti et al. 1996). In rice, seedling stage with consequent poor crop establishment followed by reproductive stage were reported to be highly sensitive to salinity stress (Moradi and Ismail 2007). Depending on a range of factors such as level of soil salinity, plant phenological stages, and experimental genotypes, plants may exhibit the reduction in photosynthetic leaf area due to increased soil salinity levels (Munns 2002; Jimenez et al. 2003). Soil salinity may also differentially affect photosynthesis and its related physiological variables (Munns et al. 2006; Chaves et al. 2009). Soil salinity may impact CO_2 intake by the photosynthetic organs, modify the structure and function of photosynthetic organelles, alter light reactions, and/or reduce rate of transport of assimilates and intermediary compounds to the metabolic sinks (Lawlor and Cornic 2002; Manchanda and Garg 2008; Chaves et al. 2009). In *Pueraria lobata*, high salinity stress was reported to cause a significant reduction in photosynthesis, stomatal conductance, leaf water potential, and chlorophyll pigments (Al-Hamdani 2004).

16.2.1.2 Salinity Stress vs. Relative Water Content and Ion Accumulation and Toxicity

High soluble salts of soil solutions generate a low osmotic potential that lowers the soil-water potential and cause a range of consequences including disrupted ion imbalance, ion toxicity and osmotic stress, and perturbation in the plant water balance (Panda and Khan 2003; Demiral and Turkan 2005; Mandhania et al. 2006). Increased salinity may tend plant water and osmotic potential towards more negativity (Aziz and Khan 2001; Khan 2001). Reduced root and leaf water potential (Ψw) has extensively

been reported in high salinity-exposed plants (Maggio et al. 2007; Ashraf et al. 2008). Increasing EC of the nutrient solution was reported to cause decrease in total Ψw (Maggio et al. 2007). Salinity reduces water potential in avocado (Chartzoulakis et al. 2002) and sugar apple (Nogueira et al. 2004).

Accumulation of Na^+ and/or Cl^- in leaves and consequent alterations in cellular turgor and various physiological processes in various plant species treated with a range of salt concentrations have been widely investigated and reviewed (Munns 2005; Manchanda and Garg 2008). There exists a differential pattern of accumulation and/or partitioning of Na^+ and Cl^- in plants (Zhao et al. 2002), and their effects on growth and leaf area (Maggio et al. 2007). Nevertheless, the accumulation and partitioning of Na^+ and Cl^- in salinity-treated plants can be genotype dependent (Jungklang et al. 2003; Garg and Singla 2004). Na^+ and/or Cl^- preferentially accumulates in roots over shoots via salt tolerance (salt exclusion) mechanism and thereby maintains a substantial potential for osmotic water uptake into the roots by restricting the spread of Na^+ to shoots (Chartzoulakis et al. 2002; Aydi et al. 2008). However, plant shoot may also exhibit a high Na^+ level when compared to roots. Some halophytes can survive even if they accumulate up to 50 % of their dry weight as Na^+ in shoots (Tester and Davenport 2003). In salt-treated *Lycopersicon esculentum* plants, older leaves were reported to accumulate more Na^+ than younger leaves (Maggio et al. 2007). The extrusion of root cell-harbored Na^+ from the cytoplasm into the apoplastic space is possible (Shi et al. 2003), or Na^+ can also be compartmentalized into the vacuole (Zhang and Blumwald 2001).

16.2.1.3 Salinity Stress vs. Oxidative Stress and Its Metabolism

Increased plant growth medium salinity may cause the production of ROS and subsequently the oxidative stress (Apel and Hirt 2004; El-Tayeb 2005; Sekmen et al. 2007; Azevedo Neto et al. 2008; Khan and Panda 2008; Türkan and Demiral 2008). Non-metabolized ROS-accrued damage in photosynthetic pigments and biomolecules, leakage of electrolytes via lipid peroxidation, and disrupted cellular metabolism are known (Gill and Tuteja 2010). NaCl may result into a significant increase in the levels of H_2O_2 and the lipid peroxidation (measured as MDA, malondialdehyde; product and indication of lipid peroxidation) (Hernandez et al. 2000, 2002; Demiral and Turkan 2005; Mandhania et al. 2006; Sekmen et al. 2007; He and Zhu 2008; Hasanuzzaman et al. 2011). Extensive reports are available on salinity-induced increase in SOD activity (Hernandez et al. 2000; Sreenivasasulu et al. 2000; Manivannan et al. 2008). The CAT activity decreased in *A. doliolum* under salt stress (Srivastava et al. 2005) but increased in *C. arietinum* leaves in the same type of stress (Eyidogan and Oz 2005). However, an elevated CAT activity has been reported to confer salinity stress tolerance by reducing oxidative damages and improving physiological adaptation in different plant species including *Vigna radiata* (Nahar et al. 2012) and *T. aestivum* (Hasanuzzaman et al. 2011). A wild salt-tolerant tomato cultivar was reported to exhibit increased activity of mitochondrial and peroxisomal MDHAR and elevations in AsA content and AsA/DHA ratio, whereas these traits decreased in salt-sensitive cultivar

(Hasanuzzaman et al. 2012a). In mung bean seedlings, enhanced MDHAR activity and higher AsA level together with upregulated other antioxidant components conferred salt tolerance (Nahar et al. 2012).

16.2.2 Drought

16.2.2.1 Drought Stress vs. Plant Water Balance and Photosynthesis and Its Variables

Plant drought tolerance is significantly governed by water retention capacity, relative water content, and membrane stability, where a higher RWC can confer the higher drought tolerance (Ritchie et al. 1990; Martin and Ruiz-Torres 1992). Under moisture stress conditions, tolerant cultivars showed less reduction in RWC as compared with that of susceptible ones (Sairam et al. 1990, 1997). Considering the drought stress impact on photosynthesis and its variables, photosynthesis rate can be decreased/stopped under severe water stress condition (Yordanov et al. 2003; Secenji et al. 2005). In fact, altered cell expansion, cell division, and stomatal movement can be responsible for drought-mediated decreased photosynthesis and the respiration (Morgan 1984; Khanna-Chopra and Sinha 1991). Also, drought stress can significantly modulate stomatal function by affecting the hydraulic conductance of the soil-leaf pathway (Hubbard et al. 2001). Drought-accrued decrease in the contents of photosynthetic pigments has also been widely demonstrated (Khanna-Chopra et al. 1980; Pastori and Trippi 1993; Krause et al. 1995).

16.2.2.2 Drought Stress vs. the Contents of Amino Acid, Proline, and Mineral Nutrients

Drought stress may cause a decline in protein content and an increase in the level of amino acids (Labanauskas et al. 1981; Navari-Izzo et al. 1990; Sudachkova et al. 1996; Hirel and Lea 2001). Low relative water content (RWC) was evidenced to stimulate the transcription and translation of genes of enzymes (e.g., 1-pyrroline-5-carboxylase reductase) involved in the synthesis of glutamate that was argued to cause enhancement in the production of proline. Drought stress may affect plant nutrient status by affecting the nutrient uptake from the soil solution (Levitt 1980; Alam 2001). A general trend of decrease in mineral nutrient (N, P, K, and S) uptake was observed in a number of drought-exposed plant species (Pinkerton and Simpson 1986; Tanguilig et al. 1987; Alam 2001). Since the transpiration significantly controls nutrient transport from roots to the upper shoot parts, any decrease in its rate may cause a decreased uptake of nutrients such as N (Tanguilig et al. 1987). It has been reported that both NH_4^+ and NO_3^- decreased and the decrease was greater for NO_3^- than for NH_4^+ (Tanguilig et al. 1987). Erdei et al. (2002) found a ploidy level-dependent increase or decrease in K^+ concentration; they found a decrease in K^+ concentration in tetraploid lines of wheat, while a decrease in the rest of the lines of wheat.

16.2.2.3 Drought Stress vs. Oxidative Stress and Its Metabolism

After drought stress of 12 days, significant rise in MDA, $O_2^{\bullet-}$, and H_2O_2 was recorded in the leaves of *Malus* spp. Drought also led to considerable damage of the cellular membranes (Wang et al. 2012a, b). More than 70 % of total H_2O_2 was proved to produce due to photorespiration under drought stress (Noctor et al. 2002). *Brassica napus* seedlings exposed to 10 and 20 % PEG-6000 for 48 h showed increased oxidative stress as indicated by increased H_2O_2 levels by 33 and 66 %, respectively, and increased MDA levels by 45 and 99 %, respectively (Hasanuzzaman and Fujita 2011). Enhanced SOD activity has been found in drought-exposed plants including *T. aestivum* (Moran et al. 1994), *V. unguiculata* (Manivannan et al. 2007), *Phaseolus vulgaris* (Zlatev et al. 2006), and *O. sativa* (Sharma and Dubey 2005). Drought stress can modulate CAT activity in plants (Sharma and Dubey 2005; Simova-Stoilova et al. 2010; Alam et al. 2013). Drought stress was reported to increase (Simova-Stoilova et al. 2010) or decrease (Sharma and Dubey 2005) the activity of CAT. Nevertheless, a higher CAT activity was reported to confer drought tolerance in *Brassica* spp. (Alam et al. 2013). Significantly, increased APX activity was reported in drought-exposed poplar species (Yang et al. 2009). Mild drought stress increased MDHAR activity in rice seedlings, whereas a severe drought stress caused a decrease in its activity (Sharma and Dubey 2005). Compared to the parent, drought-exposed hybrid *P. densata* plants showed a higher activity of MDHAR, elevated AsA, and downregulated DHA content (Gao et al. 2009). Drought stress-mediated modulation of GR (Lu et al. 2007) and GPX (Mohammadkhani and Heidari 2007; Shehab et al. 2010) activity has also been reported.

16.2.3 Metal and Metalloids

16.2.3.1 Metal/Metalloid Stress vs. Plant Growth, Development, and Photosynthesis and Its Variables

Owing to high sensitivity of seed germination to metal pollution and a lack of some defense mechanism therein, germination assay is considered as a basic procedure for the determination of metal/metalloid phytotoxicity (An 2004; Xiong and Wang 2005). Inhibited seed germination has been reported in a number of plants including As-exposed *Oryza sativa* (Abedin and Meharg 2002), Cu-exposed *O. sativa* (Ahsan et al. 2007), Cd-exposed *Hordeum vulgare* (Wu et al. 2003), and *Lycopersicon esculentum* (Dong et al. 2005). Cd-exposure has been extensively reported to impact photosynthesis and related variables such as altered photosynthetic apparatus (Tukiendorf and Baszynski 1991; Vassilev et al. 1995; Dahlin et al. 2000; Wojcik and Tukiendorf 2005; Khan et al. 2006, 2007; Mobin and Khan 2007; Anjum et al. 2008a; Singh et al. 2008a), Chl metabolism (Sandalio et al. 2001; Wu et al. 2003; Drazic et al. 2004; Hsu and Kao 2004; Vassilev et al. 2005; Kovacik et al. 2006; Mobin and Khan 2007; Anjum et al. 2008a; Ekmekci et al. 2008; Singh et al. 2008a), functioning of photochemical reactions (Skorzynska-Polit and Baszynski 1995), activities of the Calvin cycle enzymes (Krupa 1999), ribulose-1,5-bisphosphate carboxylase (Rubisco) (Vassilev

et al. 2004; Mobin and Khan 2007), and carbonic anhydrase (CA) (Khan 1994; Mobin and Khan 2007; Singh et al. 2008a). Increased As can cause significant alterations in chloroplast shape (Miteva and Merakchiyska 2002). Excess Cu may primarily target the reaction center of photosystem II (PS II), where both the donor and acceptor sides of PSII can be inhibited (Bernal et al. 2004). Carotenoid (Car) content can also be modulated by Cd (Rai et al. 2005; Lin et al. 2008; Singh et al. 2008a, b).

16.2.3.2 Metal/Metalloid Stress vs. Oxidative Stress and Its Metabolism

Extensive reports are available on Cd-mediated oxidative via elevated ROS generation (Sandalio et al. 2001; Iannelli et al. 2002; Leon et al. 2002; Milone et al. 2003; Wu et al. 2003; Mobin and Khan 2007; Anjum et al. 2008a; Singh et al. 2008a,b). Cd-exposure may enhance the activity of SOD (Cho and Seo 2005; Mishra et al. 2006; Wu et al. 2006; Khan et al. 2007; Mobin and Khan 2007; Anjum et al. 2008a, b; Ekmekci et al. 2008; Hasan et al. 2008; Singh et al. 2008a, b). In contrast, significantly decreased SOD activity can also be evidenced in Cd-exposed plants (Iannelli et al. 2002; Leon et al. 2002; Guo et al. 2007; Filek et al. 2007). Cd-exposure may differentially impact the chloroplastic and cytosolic Cu/Zn SODs, Fe-SOD, and Mn-SOD (Sandalio et al. 2001). As-exposure may result into an increased (*Pteris vittata*) or decreased (*P. ensiformis*) SOD activity (Srivastava et al. 2005). Cu (up to 1.5 mM) was reported to cause increase in SOD activity (Tanyolaç et al. 2007). High Ni concentration-exposed *T. aestivum* plants (Gajewska et al. 2006) and hairy root of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominathan and Doran 2002) exhibited a decrease in SOD activity. Regarding metal-/metalloid-mediated modulation of CAT activity, Cd-exposure may cause both a decrease (Cho and Seo 2005; Balestrasse et al. 2001; Leon et al. 2002) and an increase (Hsu and Kao 2004; Khan et al. 2007; Mobin and Khan 2007) in CAT activity. A higher CAT activity was argued to improve plant Cd tolerance (Hasanuzzaman et al. 2012b; Nahar et al. 2012) and As tolerance (Hasanuzzaman and Fujita 2013). Higher APX activity was supportive to enhance heavy metal tolerance including Ni, Cd, As, and so on (Nahar et al. 2012; Hasanuzzaman and Fujita 2013). In *Oryza sativa* shoots and roots, MDHAR and DHAR activities were increased under Ni stress, which was supposed due to activation of AsA regenerating system to improve AsA level (Maheshwari and Dubey 2009). Al stress can also cause increase in MDHAR activity (Sharma and Dubey 2007). Hasanuzzaman and Fujita (2013) showed that higher GR was involved in reducing H_2O_2 and lipid peroxidation and maintaining GSH/GSSG in *T. aestivum* seedlings under arsenic stress. In scots pine seedlings, upon imposition of Cd stress, GR activity increased; at the same time the GSH pool and GSSG content increased. This result expresses the role of GR in maintaining GSH pool level in plants (Schützendübel et al. 2001). Nahar et al. (2012) also described the similar results regarding GR activity and GSH pool in mung bean seedlings under Cd stress together with significantly reduced oxidative stress. Cd-exposure increased GPX activity in *C. annuum* plants (Leon et al. 2002). Higher GPX activity corroborated with enhanced oxidative stress tolerance as a result of As (Gupta et al. 2009; Hasanuzzaman and Fujita 2013) and Cd (Domínguez et al. 2010).

16.2.4 Other Stresses

Among other stresses, cold and frost stresses impact most crops, which follow high temperature, ozone, and UV-B stresses. Plant tolerance to chilling (0–15 °C) and freezing (<0 °C) varies considerably, where plants belonging to temperate environments may considerably be tolerant and easily acclimatized to cold (nonfreezing temperatures: −5 to −30 °C) (Levitt 1980; Chinnusamy et al. 2010). UV-B radiation-accrued elevation in ROS level and subsequent damages to biomolecules are well reported (Du et al. 2011; Singh et al. 2011). Owing to a strong oxidizing nature of ozone (O_3), it can interact with apoplastic constituents (Yan et al. 2010) and protein carbonylation, increased lipid peroxidation, and changes in cellular permeability (Hasanuzzaman et al. 2012a) mainly through elevating ROS generation. Increase in temperature (such as 22, 30, 35, and 40 °C) may enhance the accumulation of H_2O_2 (Kumar et al. 2012). In fact, a range of factors such as inhibited C3 cycle, increased photosynthetic electron flux to O_2, and over-reduction of respiratory electron transport chain (ETC) can cause an imbalance between light absorption and light use (Hu et al. 2008). Nevertheless, chilling temperature can strongly impact the light-independent photosynthetic reaction and cause therein elevated generation of ROS (Wise 1995). Higher CAT activity was reported to confer high temperature tolerance in *T. aestivum* (Hasanuzzaman et al. 2012c). High temperature stress increased APX activity in *T. aestivum* seedlings, and supplementation of sodium nitroprusside with heat stress enhanced its activity again that eliminated oxidative damage in those seedlings (Hasanuzzaman et al. 2012c). The APX activity increased under different varieties of barley under cold stress; but the extent of increase was higher in tolerant variety compared to sensitive variety, which also reduced oxidative stress (Dai et al. 2009). Chilling tolerant rice cultivar (Xiangnuo-1) had significant higher APX activity. In chilling-sensitive cultivars, its activity reduced that resulted in higher MDA content and electrolyte leakage (Huang and Guo 2005). Light in the range of 1,000–1,200 μmol m^{-2} s^{-1} may increase GR activity (Zhou et al. 2009). Ascorbate deficient *Arabidopsis* mutant vct1 showed increased MDHAR activity and decreased AsA content at UV-B exposure (Gao and Zhang 2008). The MDHAR activity did not change wheat leaves under UV-B stress. On the other hand, subsequent recovery from freezing stress resulted in sharp increase in MDHAR activity (Yang et al. 2007). Wheat seedlings were protected from oxidative damage caused by high temperature stress because of higher DHAR activity and AsA contents where other antioxidants were upregulated at the same time (Hasanuzzaman et al. 2012c). Different tomato cultivars showed differential DHAR activities in response to O_3 stress. In "Valenciano," O_3 stress increased DHAR activity, whereas in "Nikita" and "Alisacraig," the same stress did not increase DHAR activity (Marco et al. 2008). Crofton weed subjected to cold and drought stress showed higher GR activity, and under heat stress, it showed lower GR activity (Lu et al. 2007). Transgenic *Gossypium hirsutum* plant overexpressing GR showed chilling stress tolerance and photoprotection (Kornyeyev et al. 2003). In chilling-sensitive rice cultivars showing lower GR activity, increased MDA content and electrolyte leakage were evident. But chilling-tolerant rice cultivar (Xiangnuo-1) had higher GR activity that reduced the oxidative damage (Huang and Guo 2005). High light intensity markedly

increased GR activity and GSH level in lettuce leaves (Zhou et al. 2009). A higher GST activity in *T. aestivum* seedlings was involved in decreasing oxidative damage induced by high temperature (Hasanuzzaman et al. 2012c).

16.3 Abiotic Stress Signaling in Plants

Abiotic stress like extreme temperature, drought, salt stress, and mineral deficiencies decreases the productivity of crops and affects sustainability. In order to meet the demand of ever-growing population, it becomes necessary to increase the crop productivity. This can be done by understanding the mechanisms behind the tolerance of the crops to different stresses. Functional genomics provides a useful tool to study the traits in plants contributing to abiotic stress. The identification and quantification of transcript/metabolites in specific cell types and/or tissues can be now possible with the advancement of these technologies. Functional genomics allows the wide range of gene function analysis via high-throughput technology like next-generation sequencing. The data coming out of sequencing gives the large information about the genes to be analyzed. The *Arabidopsis* and rice genome sequence are the results of such high-throughput technology that paved the way towards understanding the function of genes at genome-wide scale. The large collection of ESTs and cDNA sequences also help in knowing the structure and function of genes for the plants whose genome is not sequenced yet. The function of a gene can be studied at transcript, protein, and metabolome level or through large-scale mutagenesis. These different fields are termed as transcriptomics, proteomics, metabolomics, and phenomics. Nowadays, different tools are available for identifying the function of gene at large genome scale like microarrays, serial analysis of gene expression (SAGE), and gene tagging and via use of physical and chemical mutagens. The transgenic approach is also used to elucidate the function of a gene or its product. Hence, using these technologies, the functional traits in plants contributing towards abiotic stress tolerance can be explored. The resulted data can be used in generating abiotic stress-tolerant crops. The pictorial diagram depicting the role of functional genomics in generation of stress-tolerant plants has been shown in Fig. 16.1.

16.3.1 Approaches and Tools to Study Abiotic Stress Responses in Plants

Functional genomics approaches such as genomics, transcriptomics, metabolomics, and proteomics have been used for the evaluation of abiotic stress mechanisms in plants. These approaches can identify the genes that control the specific traits responsible for abiotic stress tolerance in plants (Cramer et al. 2007). Nevertheless, various functional genomics tools with greater sensitivities are now available for the understanding of complex cell-specific regulation. Also, the previous genomics approaches can be utilized for studies of whole organs like leaves, roots, etc. (Widodo et al. 2009).

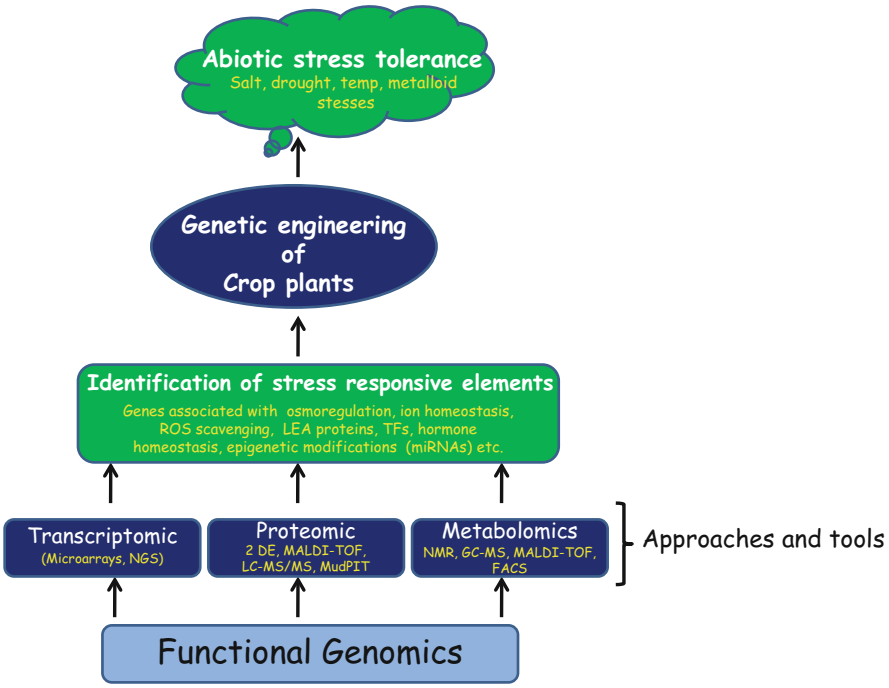

Fig. 16.1 Functional genomics involve the use of transcriptomic, proteomic, and metabolomic approaches for the identification of stress-responsive elements whose genetic engineering led to the generation of abiotic stress-tolerant crop/model plants

16.3.1.1 Transcriptomics

Microarray has been the first tool to be used to study the genome-wide transcript profiling in many abiotic stress-exposed plant species (Ueda et al. 2004, 2006; Walia et al. 2007; Gruber et al. 2009; Kim et al. 2012). With the advancement in the technique, microarrays were further used to study the tissue- and cell-specific transcript profiles in model plant *Arabidopsis* and other crop plant species including maize, rice, barley, and soybean (Brady et al. 2007; Dinneny 2010; Spollen et al. 2008; Pu and Brady 2010; Long 2011; Rogers et al. 2012). However, to date, only few have led to the identification of stress tolerance pathways (Deyholos 2010).

A new strategy used to detect and accurately quantitate changes in the transcriptome is high-throughput, gene expression profiling and deep sequencing technology (RNA-Seq). It is also known as next-generation sequencing (NGS). It has been successfully used for mammalian systems, yeast, and plants (Mortazavi et al. 2008; Nagalakshmi et al. 2008; Lister et al. 2008). Recently, the transcriptome of model plant *Arabidopsis* (Weber et al. 2007), *Glycine max* L. (soybean; Fan et al. 2013), *Lolium perenne* L. (perennial ryegrass; Studer et al. 2012), *Triticum aestivum* (wheat) endosperm (Gillies et al. 2012), *Zea mays* (maize; Li et al. 2010), *Sorghum*

bicolor (sorghum; Dugas et al. 2011), *Panicum virgatum* L. (switchgrass; Wang et al. 2012a, b), horse gram (Bhardwaj et al. 2013), and extremophile *Thellungiella parvula* (Dassanayake et al. 2011) has been studied. Studies have shown that RNA-Seq provides better quantitation and accuracy than microarrays (Jain 2012). Very few studies have reported the results regarding abiotic stress tolerance in plants utilizing NGS (Deyholos 2010). This includes the studies on drought stress (Dong et al. 2005), salt stress (Molina et al. 2008; Fan et al. 2013), and cold stress (Tamura and Yonemaru 2010). NGS technology together with complete genome sequence has successfully identified the transcriptionally regulated candidate genes in response to osmotic stress and abscisic acid (ABA) (Dugas et al. 2011). Hence, utilizing these new technologies in combination with the completed genome sequences will provide a powerful tool to uncovering genetic traits even in more complex species such as wheat.

16.3.1.2 Proteomics

Owing to the direct involvement of protein in plant stress responses, the dissection of proteome composition of stressed plants has become a significant tool for elucidating the possible relationships between protein abundance and plant stress tolerance. Exhaustive exploration of plant stress response-associated proteins is very important because the changes in gene expression at transcript level do not necessarily correspond with the changes at protein level (Bogeat-Triboulot et al. 2007). Herein, the extracted proteins are analyzed via electrophoresed using the isoelectric focusing (IEF)-aided two-dimensional electrophoresis (2-DE) (O'Farrell 1975). Two-dimensional difference gel electrophoresis (2D-DIGE) (Ünlü et al. 1997), the mass spectrometry into protein chemistry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Gevaert and Vandekerckhove 2000; Natarajan et al. 2005), "shotgun proteomics" or "LC-MS/MS-based proteomics" (Swanson and Washburn 2005), and also the multidimensional protein identification technology (MudPIT) (Wolters et al. 2001) have been significant techniques added to the 2-DE-based large-scale proteome analyses. These techniques in isolation or combination have made possible the identification and large-scale functional analyses of stress-responsive genes in different plant species (Komatsu and Ahsan 2009; Nanjo et al. 2010). A large number of proteomic studies have also been done in rice, maize, barley, and wheat in response to abiotic stresses (Rai et al. 2005; Zhang et al. 2011a, b).

16.3.1.3 Metabolomics

Metabolites are required for growth and maintenance of living cells. They are the building blocks for structural and enzymatic molecules. Metabolites are a key link between genetic information and a phenotype and are a measure of the

physiological state of an organism. A range of analytical technologies, including chromatographic separation techniques (such as liquid and gas chromatography, coupled to mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy), have been successfully used for the analyses of metabolites in many different organisms, tissues, and biofluids (Roessner and Beckles 2009). Metabolomic analysis has been done extensively for the analyses of plant responses to different abiotic stresses (Widodo et al. 2009; Urano et al. 2010; Obata and Fernie 2012). The study conducted in two genotypes of barley showed that the tolerant variety accumulates higher level of metabolites involved in cellular protection of leaves compared to less-tolerant variety (Widodo et al. 2009). Most of the studies regarding metabolites have been done in whole tissues (either leaves or roots) because of the impossibilities in isolating tissue and cell types from plant tissues. Single cell metabolomic approach was successful in *Arabidopsis* that allows the detection of about 68 metabolites by gas chromatography–mass spectrometry GC/MS (Keerberg et al. 2011). Today, spatial distribution of a single metabolite in plant tissues is now possible with the help of matrix-assisted laser desorption ionization (MALDI) coupled with analysis of every ionized molecule in a mass spectrometer (Kaspar et al. 2011). MALDI has been optimized for the analyses of lipids (Horn et al. 2012). However, efforts are now being made to its use also in proteins/peptides characterization (Jun et al. 2010; Lee et al. 2012; Peukert et al. 2012). However, there are very little applications of metabolomics to the spatial analysis of salt stress responses in plants. Therefore, this approach provides the potential to monitor the cell- and tissue-specific adaptation mechanisms in cereals and provide novel ideas for the development of more tolerant crop genotypes. Techniques such as laser microdissection (Yi et al. 2012), fluorescence-activated cell sorting (FACS) (Borges et al. 2012; Evrard et al. 2012), or MALDI-based mass spectral imaging (Kaspar et al. 2011) may pave the way for such highly spatially resolved analyses.

16.3.2 Genetic Engineering of Crop Plants for Abiotic Stress Tolerance

Genetic engineering has become one of the important tools employed for enhancing the yield of the crop plants growing under major abiotic stresses such as water deficit, extreme temperatures (high or low), and ion imbalance (toxicity and/or deficiency). Owing to the fact that the introgression of genomic portions (QTLs) often led to undesirable agronomic characteristics from the donor parents and the least information on QTL genes, the introduction and/or overexpression/downregulation of selected genes has become a viable option to develop genetically engineered plants as well as to hasten the breeding of the improved varieties. To this end, model plant systems such as *Arabidopsis*, tobacco, and *Medicago* have provided the fundamental tools for achieving more insights into the genetic and biochemical basis of plant stress adaptation (Bohnert et al. 2006). Despite the recent developments, there are a number of limitations of such genetic engineering

in crop plants (Pardo 2010; Umezawa et al. 2006; Yang et al. 2010; Cominelli and Tonelli 2010).

Genetic engineering of the crops for abiotic stress tolerance can be broadly categorized into the following groups: (a) manipulating the single target gene, (b) manipulating the regulatory genes, (c) manipulating the genes associated with hormone homeostasis, (d) manipulating genes in tandem of metabolic pathway, and (e) regulation of epigenetic modifications. Different ways of genetic engineering (including manipulating the single target and the regulatory genes) done towards increasing the abiotic stress tolerance in crops have been highlighted in Table 16.1, and the details have been discussed in the proceeding text.

16.3.2.1 Manipulating the Single Target Gene

Single target genes potentially associated with specific stress metabolites or proteins have been the main focus of single target gene manipulation technique aimed at improving plant abiotic stress. In particular, information gained through exhaustive molecular studies on the proteins and enzymes involved in ROS scavenging, osmolyte (proline, betaine, and sugars such as trehalose and polyamines)-associated genes, and also molecular chaperones and ion transporters have unveiled genes underlying key physiological and biochemical processes. A brief description on the genes associated with osmoregulation and ROS detoxification, basics of manipulating late embryogenesis abundant proteins, and the genes associated with water and ion homeostasis are given hereunder.

Osmoregulation-Associated Genes

Owing to the role of elevated polyamines, glycine betaine, proline, trehalose, fructan, trehalose, mannitol, and galactinol in the plant stress adaptation, pathways involved in their synthesis and accumulation have been attempted to improve plant abiotic stress tolerance (Rontein et al. 2002; Alcázar et al. 2006; Hussain et al. 2008; Munns and Tester 2008). In particular, improved stress tolerance through elevated levels of polyamines (PAs) has been achieved by overexpressing genes associated with ornithine or arginine decarboxylases (ODC, ADC), *S*-adenosylmethionine (SAM) decarboxylase (SAMDC), and spermidine synthase (SPDS) in a number of plant species including *Arabidopsis* (Alcázar et al. 2006), *Nicotiana tabacum* (Wi et al. 2006), *Oryza sativa* (Capell et al. 2004; Roy and Wu 2002; Prabhavathi and Rajam 2007), *Solanum tuberosum* (Kasukabe et al. 2006), and *Solanum melongena* (Prabhavathi and Rajam 2007). The overexpression of a betaine aldehyde dehydrogenase (BADH) gene in wheat (*Triticum aestivum*) improved tolerance of this plant to drought and heat stress through improving osmotic adjustment and antioxidative defense capacity (Wang et al. 2010). Improved yield has been reported under stressful field conditions through the introduction of betA (encoding choline dehydrogenase) gene in crop plants such as maize (*Zea mays*) (Quan et al. 2004) and *T. aestivum*

Table 16.1 Various genes/TFs/proteins employed in genetic engineering of crop/model plants for abiotic stress tolerance

	Engineered plant species	Gene	Targeted approach	Observations/stress tolerance	References
Manipulating osmolyte accumulation	*Triticum aestivum*	Betaine aldehyde dehydrogenase (BADH) gene	Overexpression	Improved osmotic adjustment and antioxidant capacity leading to increased stress tolerance	Wang et al. (2010)
	Zea mays T. aestivum	betA (encoding choline dehydrogenase) gene	Ectopic	Increased yield under abiotic stress conditions	Quan et al. (2004), He et al. (2010)
	Gossypium hirsutum	Choline monooxygenase (CMO) gene involved in glycine betaine biosynthesis	Ectopic	Increased yield under saline conditions	Chen and Murata (2011)
	O. sativa	Moth bean Δ1-pyrroline-5-carboxylate synthetase (P5CS) gene	Ectopic	Proline accumulation increased tolerance under osmotic stress	Su and Wu (2004); Vendruscolo et al. (2007)
	Cicer arietinum	Constitutive expression of *P5CSF129A*	Ectopic	Proline accumulation increased the transpiration efficiency of plants	Bhatnagar-Mathur et al. (2009)
	Soybean	Δ1-pyrroline-5-carboxylate reductase (P5CR)	Ectopic	Higher proline accumulation led to increased relative water content and glucose and fructose level under drought stress conditions	de Ronde et al. (2004)
	T. aestivum	Mannitol-1-phosphate dehydrogenase (mtlD) gene	Ectopic	Resulted in improved tolerance to drought and salinity stress	Abebe et al. (2003)
	O. sativa Medicago sativa	Two *Escherichia coli* trehalose biosynthetic genes (otsA and otsB)	Overexpression	Improve tolerance to abiotic stresses	Garg et al. (2002), Suarez et al. (2009)
Manipulating antioxidant machinery	*Medicago sativa*, *T. aestivum*, *S. tuberosum*	Mn-superoxide dismutase (Mn-SOD)	Overexpression	Resulted in higher tolerance to abiotic stress and improved yields under field conditions	McKersie et al. (1996), Gusta et al. (2009), Waterer et al. (2010)
	Solanum lycopersicum	Ascorbate peroxidase (APX) gene	Overexpression	Improved tolerance to exposure to direct sunlight, under field conditions	Wang et al. (2006)
	O. sativa	Catalase (Cat) gene	Overexpression	Resulted in improved growth and yield under salt stress	Nagamiya et al. (2007)

	O. sativa	Constitutively co-expressing glutathione-*S*-transferase (GST) and CAT genes	Overexpression	Enhanced tolerance to salinity and oxidative stresses at the vegetative stage	Zhao and Zhang (2006)
	N. tabacum	Co-expression of three antioxidant enzymes, copper zinc superoxide dismutase (CuZnSOD), APX, and dehydroascorbate reductase (DHAR)	Overexpression	Resulted in a higher tolerance to salt stress	Lee et al. (2007)
Manipulating LEA proteins	*O. sativa*	OsLEA3-1 in rice, was shown to improve yields under drought stress in transgenic wheat and rice	Overexpression	Resulted in improved yields under drought stress, without yield penalties under control conditions	Xiao et al. (2007)
	T. aestivum, *O. sativa*	LEA protein HVA1 from *Hordeum vulgare*	Overexpression	Improved yields under drought stress conditions	Bahieldin et al. (2005); Xu et al. (1996)
	Fragaria × *ananassa*	Dehydrin WCOR410 gene from wheat	Overexpression	Improved leaf freezing tolerance	Houde et al. (2004)
	O. sativa, *Z. mays*, *A. thaliana*,	RNA chaperones: CspA of *E. coli* and CspB of *B. subtilis*	Ectopic	Resulted in enhanced tolerance to abiotic stress, by maintaining growth, photosynthesis, and development	Castiglioni et al. (2008)
	O. sativa	Heat-shock protein gene, sHSP17.7	Overexpression	Resulted in improved drought and osmotic stress tolerance	Sato and Yokoya (2008)
	A. thaliana	Rice plasma membrane aquaporins OsPIP-1 and OsPIP2-2. Overexpression of TaNIP in *Arabidopsis* enhanced plant tolerance to abiotic stresses	Overexpression	Resulted in improved salinity and dehydration tolerance	Guo et al. (2006a, b)
	A. thaliana	Wheat nodulin 26-like intrinsic proteins (NIP) gene	Overexpression	Resulted in improved tolerance to abiotic stress	Guo et al. (2006a, b)
	Tomato	Tobacco gene encoding aquaporin (NtAQP1)		Provided protection against salinity stress through increased photosynthetic rate	Sade et al. (2010)
	Tomato	Tonoplast intrinsic protein (TIP) aquaporin gene SlTIP2;2	Overexpression	Increased cell water permeability and whole-plant transpiration, which resulted in improved salt and drought tolerance	Sade et al. (2009)

(continued)

Table 16.1 (continued)

	Engineered plant species	Gene	Targeted approach	Observations/stress tolerance	References
Manipulating regulatory gene	*A. thaliana*	Nuclear factor Y (NF-Y) complex comprised of three subunits: NF-YA (HAP2), NF-YB (HAP3), and NF-YC (HAP5)	Overexpression	Conferred tolerance to abiotic stress	Mantovani (1999), Nelson et al. (2007)
	Z. mays	ZmNF-YB2	Overexpression	Transgenics showed enhanced tolerance to drought stress	Nelson et al. (2007)
	O. sativa	SNAC1 (STRESS-RESPONSIVE NAC 1)	Overexpression	Transgenics showed increased yield under drought stress conditions	Takasaki et al. (2010), Hu et al. (2006), Hu et al. (2008)
	O. sativa	Two NAC genes, OsNAC5 and OsNAC6	Overexpression	Resulted in stress tolerance via upregulation of the expression of OsLEA3	Takasaki et al. (2010)
	O. sativa	OsNAC10 under control of a root-specific promoter (RCc3)	Overexpression	Transgenics yielded more grains under drought stress conditions	Jeong et al. (2010)
	N. tabacum, *A. thaliana*, *O. sativa*, *T. aestivum* canola	Dehydration-responsive element (CBF1/DREB1B) genes	Overexpression	Resulted in improved tolerance to drought, salinity, and temperature stress	Jaglo-Ottosen et al. (1998), Liu et al. (1998), Gilmour et al. (2000), Dubouzet et al. (2003), Jaglo et al. (2001)
	O. sativa	HARDY (HRD), encoding a AP2/ERE-like TF	Overexpression	Resulted in reduced transpiration and increased water use efficiency (WUE) under control and drought conditions	Karaba et al. (2007)

Manipulating the genes related to hormone homeostasis	*O. sativa*	LOS5/ABA3, a key enzyme in the last step of ABA biosynthesis	Overexpression	Trangenics showed improved yield under drought stress conditions	Xiao et al. (2009)
	Tomato	LeNCED1 (a drought-inducible gene)	Overexpression	Resulted in increased ABA accumulation and improved drought tolerance	Thompson et al. (2007)
	Canola	Era1 (encodes the β-subunit of farnesyl transferase)	Downregulation	Displayed enhanced yield under mild drought conditions	Wang et al. (2005)
	O. sativa	Harpin-encoding (hrf1) gene	Overexpression	Improved drought tolerance through increased levels of free proline	Zhang et al. (2011a, b)
	N. tabacum, *O. sativa*	IPT (isopentenyltransferase) expression under the control of SARK (senescence associated receptor kinase), a maturation- and stress-induced promoter	Overexpression	Resulted in increased drought tolerance and superior yields	Peleg et al. (2011a, b)
	Manihot esculenta Crantz	IPT under control of a senescence-induced promoter, SAG12		Displayed higher tolerance to stress	Zhang et al. (2010)
	Tomato	IPT	Overexpression	Resulted in increased yield under salt stress conditions	Ghanem et al. (2011)
Manipulating genes of metabolic pathway	*A. thaliana*, *O. sativa*	Co-expression of *E. coli* P5C biosynthetic enzymes gamma-glutamyl kinase 74 (GK74) and gamma-glutamyl phosphate reductase (GPR) and the antisense transcription of proline dehydrogenase (ProDH)	Overexpression and downregulation	The transgenic plants displayed improved tolerance to heat stress associated with the accumulation of cell wall proline-rich proteins	Stein et al. (2011)
	N. tabacum	Co-expression of DHAR, GR, or GST. Simultaneous overexpression of the CuZnSOD and APX genes in plastids	Overexpression	Resulted in the increased tolerance of the transgenic plants to a variety of abiotic stresses. Led to increased germination rates and longevity of long-term stored seeds	Le Martret et al. (2011), Lee et al. (2010)

(He et al. 2010). Chen and Murata (2011) reported higher yield in salinity-exposed cotton (*Gossypium hirsutum*) through expression therein of gene encoding choline monooxygenase (CMO), involved in GB biosynthesis. Expression of genes associated with enzymes of proline biosynthesis/metabolism enzymes such moth bean Δ1-pyrroline-5-carboxylate synthetase (P5CS) (de Ronde et al. 2004; Su and Wu 2004; Vendruscolo et al. 2007; Verbruggen and Hermans 2008; Bhatnagar-Mathur et al. 2009) has been reported to improve osmotic/salinity adaptive capacity in a different plant species. Constitutive overexpression of the mannitol-1-phosphate dehydrogenase (mtlD) gene was reported to improve plant tolerance to drought and salinity (Abebe et al. 2003). Earlier, trehalose synthesis-related gene expression has been evidenced to improve abiotic stress tolerance in different plan species (Goddijn et al. 1997; Garg et al. 2002; Jang et al. 2003; Suarez et al. 2009).

Detoxifying Reactive Oxygen Species

As mentioned earlier, non-metabolized ROS (such as O_2, H_2O_2, $O_2^{\bullet-}$, and $HO^{\cdot}$) can impact important biomolecules including proteins, DNA, and lipids (Miller et al. 2010). However, plant's capacity to efficiently metabolize has been extensively reported to be significantly improved by overexpressing a range of genes associated with ROS scavenging/detoxifying enzymes including aldehyde dehydrogenases (ALDHs) (Nakazono et al. 2000), Mn-superoxide dismutase (Mn-SOD) (McKersie et al. 1996; Gusta et al. 2009; Waterer et al. 2010), ascorbate peroxidases (APX) (Wang et al. 2006), and catalase (CAT) (Nagamiya et al. 2007). In transgenic rice plants, Zhao and Zhang (2006) constitutively co-expressed glutathione-S-transferase (GST) and CAT genes and achieved improved tolerance to salinity-caused oxidative stresses (Zhao and Zhang 2006). Additionally, co-expression of CuZnSOD, APX, and dehydroascorbate reductase (DHAR) was reported to help tobacco to significantly tolerate salt stress (Lee et al. 2007).

Manipulating Late Embryogenesis Abundant Proteins and the Genes Associated with Water and Ion Homeostasis

Improved stress tolerance can also be achieved through manipulating the genes encoding the late embryogenesis abundant (LEA) proteins, where these proteins play crucial roles in cellular dehydration tolerance (Umezawa et al. 2006). Extensive reports are available on LEA-mediated prevention of protein aggregation and improved antioxidant capacity in drought-exposed plants (Goyal et al. 2005; Hand et al. 2011; Kovacs et al. 2008). Xiao et al. (2007) reported OsLEA3-1 overexpression-mediated improved yields under drought stress in rice, whereas Bahieldin et al. (2005) and Xu et al. (1996) evidenced improved drought tolerance in wheat and rice, respectively, by overexpressing barley (*Hordeum vulgare*) LEA protein HVA1. Freezing tolerance was achieved in strawberry (*Fragaria*×*ananassa*) by overexpressing wheat dehydrin WCOR410 gene (Houde et al. 2004). Sato and Yokoya

(2008) reported improved drought and osmotic stress tolerance in rice by overexpressing their sHSP17.7, small heat-shock protein gene.

Owing to the role of membrane protein, aquaporins in the transport of water, small neutral solutes, and CO_2 (Tyerman et al. 2002; Bienert and Chaumont 2011), their manipulation has been reported to improve plant stress tolerance via enhanced water and ion homeostasis (Li et al. 2004; Guo et al. 2006a, b; Sade et al. 2009, 2010). Guo et al. (2006a, b) and Sade et al. (2010) reported improved abiotic stress tolerance in *Arabidopsis* via heterologous overexpression of rice OsPIP-1 and OsPIP2-2 and that of wheat nodulin 26-like intrinsic protein (NIP) gene (TaNIP), respectively. Constitutive overexpression of TIP aquaporin gene SlTIP2 was evidenced to improve salt and drought tolerance in tomato through increased cell water permeability and whole-plant transpiration (Sade et al. 2009).

16.3.2.2 Manipulating the Regulatory Genes

Because of their involvement in almost all biological processes, and their regulatory role in the expression of many stress-responsive genes, transcription factors (TFs) have been the major target candidates for the generation of stress-tolerant crops (Yamaguchi-Shinozaki and Shinozaki 2006). A large number of plant transcription factors (TFs) have been linked to plant stress responses. These TFs have been highlighted in Table 16.1. Improved abiotic stress tolerance was reported in nuclear factor Y (NF-Y) complex overexpressing *Arabidopsis* (Mantovani 1999; Nelson et al. 2007). Constitutive expression of ZmNF-YB2 showed enhanced drought tolerance in transgenic maize (Nelson et al. 2007). Hu et al. (2006, 2008) and Takasaki et al. (2010) are among the researchers who reported regulated stomata movement and photosynthetic activity-mediated improved yield in drought-exposed transgenic rice overexpressing SNAC1 (STRESS-RESPONSIVE NAC 1). The expression of stress-inducible genes such as OsLEA3 was reported to be upregulated due to overexpression of two NAC genes in rice (OsNAC5 and OsNAC6) (Takasaki et al. 2010). Extensive reports are available in literature on the significance of dehydration-responsive element (DRE)/C-repeat (CRT) proteins in plant tolerance to drought, cold, and salinity (Yamaguchi-Shinozaki and Shinozaki 1994). In particular, improved plant tolerance to drought, salinity, and temperature stresses has been evidenced as a result of CBF1/DREB1B gene overexpression (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Jaglo et al. 2001; Dubouzet et al. 2003). In HARDY (HRD)-overexpressing rice, Karaba et al. (2007) reported increased drought tolerance via reduced transpiration and increased water use efficiency (WUE).

16.3.2.3 Manipulating the Genes Associated with Hormone Homeostasis and the Genes in Tandem of Metabolic Pathway

Considering the significance of phytohormones in plant growth, development, and stress responses (Klingler et al. 2010; Peleg and Blumwald 2011), several phytohormone-associated genes are being manipulated to improve plant stress

tolerance (Schroeder et al. 2001; Wang et al. 2005; Thompson et al. 2007; Xiao et al. 2009; Wilkinson and Davies 2010). Overexpression of ABA biosynthesis gene—LOS5/ABA3—was reported to improve rice yield under drought stress (Xiao et al. 2009). In tomatoes, overexpression of LeNCED improved drought tolerance via increased ABA accumulation (Thompson et al. 2007; Wang et al. 2005). In drought-exposed transgenic canola with ERA1 antisense (driven by the drought-inducible RD29A promoter), Wang et al. (2005) evidenced enhanced yield. Additionally, overexpression of a Harpin-encoding (*HRF1*) gene was found to improve transgenic rice tolerance to drought via increased proline level and regulated stomatal closure (Zhang et al. 2011a, b). Overexpression of many genes (such as IPT, a gene encoding isopentenyltransferase) involved in cytokinin biosynthesis was also reported extensively to improve plant stress tolerance (Kunkel et al. 1999; Rivero et al. 2007, 2010; Ma 2008; Zhang et al. 2010; Peleg et al. 2011a, b).

Reports are also available in context with achieving improved plant stress tolerance through manipulating genes in tandem of metabolic pathway. Increased proline accumulation was reported in *Arabidopsis* and tobacco co-overexpressing simultaneously *E. coli* P5C biosynthetic enzymes gamma-glutamyl kinase 74 (GK74) and gamma-glutamyl phosphate reductase (GPR) and the antisense transcription of proline dehydrogenase (ProDH) (Stein et al. 2011). Improved stress tolerance has also been reported in a number of plants as a result of the co-expression of genes associated with enzymes such as dehydroascorbate reductase (DHAR), glutathione-*S*-transferase (GST), and glutathione reductase (GR) (Le Martret et al. 2011) and CuZnSOD and APX genes (Lee et al. 2010).

16.3.2.4 Regulation of Epigenetic Modifications

Plant gene activity in response to environmental stimuli can also be modulated by a range of epigenetic processes such as DNA methylation, histone modifications, small RNA (sRNA) molecules, and transposable element activity (Henderson and Jacobsen 2007; Feng et al. 2010; Hauser et al. 2011). Considering these facts, the studies aimed at improving tolerance to abiotic stress in crops are on high leap on the control of methylation and histone patterns (Baulcombe 2004; Khraiwesh et al. 2011; Scippa et al. 2004; Dyachenko et al. 2006; Sunkar et al. 2006; Zhong et al. 2010). The role of CpHpG hypermethylation in salt tolerance has been reported (Dyachenko et al. 2006). Zhong et al. (2010) reported the increased salt tolerance in wheat employing the methylation inhibitor 5-azacytidine, whereas decrease levels of histone acetylation were proved by Scippa et al. (2004) to result in higher photosynthetic rates in water-stressed tomato. Important gene expression regulators such as small non-coding RNAs including small RNAs (sRNAs), short interfering RNAs (siRNAs), and microRNAs (miRNA) have been the focus of recent plant genetic research aimed at targeting epigenetic modifications (Baulcombe 2004; Khraiwesh et al. 2011). In *Arabidopsis*, the overexpression of miR398 was reported to increase oxidative stress tolerance (Sunkar et al. 2006), whereas in Sly-miR169c overexpressing tomato, enhanced drought tolerance was argued as a result of reduced

water loss and regulated stomatal opening (Zhang et al. 2011a, b). Different examples of genetic engineering undertaken in several crop plants to enhance the abiotic stress tolerance are listed in Table 16.1.

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