Abiotic Stress Tolerance in Plants
Toward the Improvement of Global Environment and Food

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Preface

Stresses in plants caused by salt, drought, temperature, oxygen, and toxic compounds are the principal reason for reduction in crop yield. For example, high salinity in soils accounts for large decline in the yield of a wide variety of crops world over; ~1000 million ha of land is affected by soil salinity. Increased sunlight leads to the generation of reactive oxygen species, which damage the plant cells. The threat of global environment change makes it increasingly demanding to generate crop plants that could withstand such harsh conditions.

Much progress has been made in the identification and characterization of the mechanisms that allow plants to tolerate abiotic stresses. The understanding of metabolic fluxes and the main constraints responsible for the production of compatible solutes and the identification of many transporters, collectively open the possibility of genetic engineering in crop plants with the concomitant improved stress tolerance. Abiotic Stress Tolerance in Plants is a new book with focus on how plants adapt to abiotic stress and how genetic engineering could improve the global environment and food supply. Especially, the application of biotechnology in Asia and Africa would be important. Environmental stress impact is not only on current crop species, but is also the paramount barrier to the introduction of crop plants into areas not currently being used for agriculture. Stresses are likely to enhance the severity of problems to be faced by plants in the near future.

The present book brings together contributions from many laboratories around the world in order to discuss and compare the current knowledge about the role of stress genes in plant stress tolerance. In addition, strategies to introduce these genes into economically important crops and its effects on plant productivity are discussed.

We express our thanks to all the contributors. Our sincere thanks are especially due to Prof. Tetsuko Takabe for her kind help in going through the contents and its arrangement. Finally, it is a profound pleasure to thank Springer for taking up the publication of this book.

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Teruhiro Takabe
SECTION I

SIGNAL TRANSDUCTION
1. INTRODUCTION

Adverse environmental conditions such as drought, high soil salinity, and temperature extremes are found in many agricultural areas. These abiotic stresses can result in severe yield loss to agricultural crops. Plants exhibit various responses to these stresses at the molecular, cellular, and whole plant levels [1-4]. These responses may contribute to increased tolerance to the stresses [5-8]. To breed or genetically engineer plant stress tolerance, it is imperative to identify the genes that control these traits and to understand how these genes and their products are regulated.

With the availability of complete information on a couple of plant genomes and of various genomics and proteomics tools, knowledge on plant abiotic stress responses has been advanced at a great pace in the last few years. In particular, documentation of genes that are regulated by stresses is comprehensive for the model plant Arabidopsis and should be complete for rice soon as well. Nonetheless, abiotic stress signaling mechanisms have been proven to be very complex, and there is still much more to learn. To date, only a handful of genes that play critical roles in plant adaptation to stresses have been identified with confidence. Few pathways that mediate stress responses are revealed in complete. In this chapter, we will give an
overview of the current knowledge on signal transduction mechanisms responsible for signal perception, amplification, transmission, and final activation of stress responses. We will discuss examples of genetic studies or other studies where there is some supporting genetic evidence. This overview will mainly focus on advances made during the past two years since our last review on abiotic stress signal transduction was published [9].

2. ABIOTIC STRESS-REGULATED GENES

One major response of plants upon encountering abiotic stresses is the activation of stress-responsive genes. Genome-wide transcript profiling with Arabidopsis has identified many genes that are regulated by cold, salt, and drought stress (reviewed in [10]). Similar studies were also conducted with crop plants such as rice, barley, maize, and soybean [11-15]. It was suggested that as many as 30% of the genes in the Arabidopsis genome may be affected by abiotic stress at the transcript level [16]. Some of these genes can be activated by multiple stresses and also by the stress hormone abscisic acid (ABA). Generally, more genes are up-regulated than down-regulated [10,17]. This is also true for gene expression in response to ABA. Using the Agilent long-oligo chips, our microarray assay with Arabidopsis seedlings treated with ABA found that over 2000 genes were up regulated by more than 2-fold, whereas about 500 genes were down regulated (unpublished).

The products of some of these stress-inducible genes may play roles in stress signaling and stress tolerance [5,7,18,19]. These include, for example, enzymes that function in the biosynthesis of compatible solutes (osmolytes) or either directly in detoxification of reactive oxidants or in the biosynthesis of antioxidant compounds, ion transporters, ABA biosynthetic enzymes, etc. The products of some other genes may also have protective roles against stress damages yet their modes of action are unclear. These are mainly the late-embryogenesis-abundant protein (LEA)-like proteins [2]. However, some stress-regulated genes may not play a primary role in stress response. Their induction may be merely a consequence of the stress and stress injuries [20]. In some cases, genes physically associated with certain key stress-induced genes in a chromatin region may be regulated by stress, although these genes may not be related otherwise. One example is the UFC (upstream of FLC) gene [21]. FLC is a flowering repressor whose transcript level is down regulated by long-term cold treatment (e.g., vernalization). Interestingly, UFC is similarly regulated by vernalization yet it does not relate to FLC either in sequence or in function. They are merely neighboring genes on the same chromosomal region. This suggests that chromosome location may have a strong influence on the induction of certain genes.

3. AN OVERVIEW ON STRESS SIGNAL TRANSDUCTION

Signal transduction is required for many cellular activities and their coordination. Some signal transduction processes are simple but most others are complex, involving multiple components and occurring in a time and space-dependent
manner. Generally, signal transduction starts with the perception of a stimulus by a specific cellular molecule(s). These sensors or receptors may differ in their molecular identities, modes of signal perception and output, as well as subcellular localizations.

In plant cells, it is also common for receptor activation to result in the generation of second messengers, so called because they represent intracellular signals being translated from the primary external signal. These intracellular messengers are interpreted further by other signaling component(s) and result in the activation of downstream pathways that may have multiple outputs. These pathways usually involve reversible protein phosphorylation. Protein phosphorylation could lead to, among others, the activation of transcription factors that induce the expression of stress-responsive genes.

Signal transduction often requires additional components that recruit and assemble signaling complexes, target signaling molecules, and regulate their lifespan. In many cases, these components themselves are also regulated by the signaling pathways that may have been initiated from the same stress signals. Here we refer to these components collectively as signaling partners.

Figure 1 depicts a genetic signaling pathway that can serve as a framework for anchoring many of the individual signaling components that are increasingly being reported in the literature.

4. SIGNAL TRANSDUCTION COMPONENTS

4.1. Receptors

4.1.1. Complexity of abiotic stress as signals
Receptors are the molecules that first perceive stress stimulus and then relay the signal to downstream molecules to initiate the signal transduction pathway. However, it is not an easy task to find these receptors. Many of the abiotic stress signals are complex in their nature. They may not simply be physical or chemical signals but rather, a mixture of several favours. For example, low temperature may induce both osmotic stress and mechanistic stress. Drought, conditioned by decreased water potential in the soil, may involve osmotic stress, ionic stress, a mechanistic signal, and heat stress in some cases. Very likely, each of these stress attributes may have different weights with regard to the plant status or the severity of the particular stress in question. Thus, a simple water shortage in the soil may in fact impart very complex and different information to the plants.

One can also expect that there may be multiple cellular sensors to perceive a stress signal or one attribute of the signal. The complex nature of abiotic stress as signals and the redundancy of their perception machinery pose great constraints for the identification of cellular machinery that perceives abiotic stress.
4.1.2. Putative sensors in stress signal perception

Studies in other systems have identified several kinds of receptors that function in stress signal perception. These include receptor-like kinases, two-component receptors, receptor tyrosine kinases, G-protein coupled receptors, ionotropic channel-related receptors, histidine kinases, and nuclear hormone receptors. By sequence homology, most of these receptor families can be found in sequenced plant genomes. However, a few such as receptor tyrosine kinases and nuclear hormone receptors cannot be identified by sequence homology search. Many people thus believe these signaling components nonexistent in higher plants.

Because of their stress inducibility or, in a few cases, phenotypes conferred by their regulated expression, receptor-like kinases, two-component receptors, histidine kinases, ionotropic receptors, and G-protein associated receptors have each been implicated as potential receptors for abiotic stresses or the stress hormone ABA (reviewed in [9,22]). Despite the importance of identifying stress sensors, research effort to find these receptors has been limited. To date, there has been no convincing evidence to support any of the above-mentioned putative receptors as stress sensors.
Thus, it may be helpful to briefly introduce abiotic stress sensors identified in other systems.

In cyanobacteria, histidine kinases were identified as cold-sensors in the activation of selected marker genes [23]. Knockout of these kinases resulted in substantially reduced expression of these genes. In neurons, a TRP \( \text{Ca}^{2+}/\text{cation} \) channel was suggested to be a cold sensor [24]. In fact, similar TRP channels can act as heat sensors as well [25]. However, no TRP channel protein can be found in the sequenced plant genomes using sequence similarity searches. In plant cells, cold-induced \( \text{Ca}^{2+} \) influx has been documented as an early response to cold [26]. Manipulations of \( \text{Ca}^{2+} \) influx can affect the expression of cold-regulated genes (e.g., [27]). Nonetheless, the calcium channels responsible for this \( \text{Ca}^{2+} \) influx have not been identified.

As mentioned above, an abiotic stress may initiate multiple signaling pathways in plants. It may be difficult to directly identify stress sensors through genetic analysis, since knocking out one receptor may not significantly affect the stress signaling outputs. Because ABA is involved in abiotic stress signaling, revealing how ABA is perceived certainly will help reveal how stress signals are sensed. Unfortunately, how ABA is perceived is not known either (reviewed in [22]). Current efforts to uncover ABA perception mechanisms mainly focused on putative receptor-linked components or those putative receptor molecules that are regulated by stress or ABA. For example, Arabidopsis heterotrimeric G-protein \( \alpha \) subunit GPA1 was suggested to be involved in ABA response in guard cells since \( \text{gpa}1 \) mutants were insensitive to ABA inhibition of stomata opening and ABA regulation of inward K\(^+\) channels, yet it does not function in ABA-induced stomata closure. The \( \text{gpa}1 \) mutant seedlings lost water more quickly than the wild type [28]. GPA1 interacts with the G-protein couple receptor-like protein GCR1, yet \( \text{ger}1 \) mutant was more sensitive to ABA than the wild type [29]. The reason for the opposite phenotypes between \( \text{gpa}1 \) and \( \text{ger}1 \) mutants and the modes of action for both proteins are unclear. In addition to the heterotrimeric G proteins, there are different classes of small G proteins [30]. One of the ROP family Rho GTPases, ROP10, was proved to be a negative regulator of ABA responses in Arabidopsis [31]. Since another ROP related to ROP10 was shown to be associated with CLV receptor kinase [32] and ROP10 was localized to plasma membrane, it was hypothesized that ROP10 may be associated with an ABA perception complex on the plasma membrane [31].

Abiotic stresses also generate second signaling molecules (see below). Receptors for these signals should exist in plants, yet none has been identified. In contrast, receptors for inositol trisphosphate, cADPR and sphingosine 1-phosphate are well characterized in animal systems.

4.2. Second intracellular signaling molecules

Several intracellular signaling molecules are involved in stress signal transduction. These include reactive oxygen species, lipid phosphates-derived signals, and cyclic
nucleotides-related signals. In addition, some plant hormones also have the characteristics of secondary signal molecules.

4.2.1. Reactive oxygen species

In addition to the reactive species generated during normal photoreactions and cellular biochemical oxidations, plants also produce reactive oxygen species (ROS) during environmental stresses and in response to pathogen attacks (reviewed in [33]). Although these reactive molecules may have damaging effects on cellular membranes and macromolecules, they play important signaling roles in early stages of stress response. These reactive molecules can activate cellular defense mechanisms to mitigate stress damage. Among others, nitric oxide (NO) and hydrogen peroxide ($\text{H}_2\text{O}_2$) are suggested to play roles in ABA signaling and may function in abiotic stress response as well.

ABA regulation of stomata closure appears to require the generation of $\text{H}_2\text{O}_2$. $\text{H}_2\text{O}_2$ production is a prerequisite for ABA-induced stomatal closure [34-36]. NAPH oxidase may represent the major source for $\text{H}_2\text{O}_2$ production. Mutations in genes that encode catalytic subunits of NADPH oxidase impair ABA-induced ROS production and the activation of guard cell $\text{Ca}^{2+}$ channels and stomata closure [37].

In plants, nitric oxide can be generated by enzymatic reactions as well as non-enzymatic reactions [38]. Both nitrate reductases and NO synthases (NOS) can contribute to NO generation. It appears that these two kinds of enzymes do not function redundantly since mutations in either enzyme could confer specific phenotypes. For example, loss-of-function mutations in Arabidopsis NOS, AtNOS1, impair ABA-induced NO production and stomata closure [39].

ROS may affect stress signal transduction in the activation of stress-responsive genes [40,41], in particular those that encode enzymes in the biosynthesis of antioxidants or enzymes that directly detoxify reactive oxidative radicals. Then, how does ROS affect stress signal transduction? It was demonstrated that ROS is involved in the regeneration of $\text{Ca}^{2+}$ signals through the activation of $\text{Ca}^{2+}$ channels (reviewed in [42]). These secondary $\text{Ca}^{2+}$ signals could initiate additional signal transduction via $\text{Ca}^{2+}$ mediated pathways [9]. Another route is that reactive oxygen species themselves can directly modify signaling molecules through redox regulation. Molecules with cysteine residues as key active sites could be the targets of redox regulation. These molecules could be potential sensors for ROS [20]. Since tyrosine phosphatases in animals are the potential targets of ROS, and these phosphatases could regulate MAPK cascades, it is believed that MAPK pathways are probably the major pathways mediating ROS signal transduction (see Section 4.5.1). An AGC family protein kinase OXI1 appears to be involved in ROS activation of MPK3 and MPK6 since in oxi1 mutant, activation of both MAPK was compromised [43].

Cellular redox environment may also modulate cell signaling by regulating the activity of other signaling components. One example is the regulation of the transcription activator NPR1 by redox status. A reduced milieu in the cytosol facilitates the inactive NPR1 oligomer to change into an active monomer form. The active NPR1 may target the TGA zinc finger transcription factors and activates PR
gene expression [44]. In Arabidopsis genome, there are several NPR1-like genes, but it is not clear whether any of these genes would regulate stress responses and interact with ABF-like zinc finger proteins.

**Lipids-derived messengers**

Membrane lipids may be directly involved in stress response by modulating membrane fluidity or its other physiochemical properties [45]. Yet a more important function of these lipid components is their role in generating intracellular signaling molecules. Lipids and their biogenesis and degradation enzymes play many roles that directly or indirectly regulate or affect plant stress signaling and stress tolerance. For a general discussion of the roles of lipids in cell signaling, readers are referred to a recent review [46]. Some recent advances in lipid signaling of abiotic stress are briefly outlined below.

It is known that phospholipids, the backbone of cellular membranes, can serve as precursors for the generation of second messengers in response to abiotic stresses. While the relevant lipid cleaving enzymes are the phospholipases A2, C, and D, the most studied is the phosphoinositide-specific phospholipase C (PI-PLC). Upon activation, PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce two important molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP₃). DAG and InsP₃ are second messengers that could activate protein kinase C (PKC) and trigger Ca²⁺ release, respectively.

In plants, the role of exogenous InsP₃ in releasing Ca²⁺ from cellular stores has been widely reported [47,48]. Inhibition of PI-PLC activity impairs ABA-induced stomata closure [49] and inhibits osmotic stress-induction of the stress-responsive genes *RD29A* and *COR47* [50]. On the contrary, inhibiting the breakdown of InsP₃ may increase the expression of stress-inducible genes. This was demonstrated with transgenic plants overexpressing Arabidopsis inositol polyphosphate 5-phosphatases [51,52] and with loss-of-function or conditional mutation in an enzyme of inositol polyphosphate 1-phosphatase FIERY1 [53,54].

Accumulating evidence suggests that phosphatidic acid (PA) is also involved in the transduction of stress signals. PA is generated by the Phospholipase D (PLD) hydrolysis of phospholipids. In guard cell protoplasts, PLD activity mediates ABA–induced stomatal closure [55]. PA produced by PLDα1 may interact with and inhibit the activity of ABI1, which is a negative regulator of ABA signaling (see below). Consequently, PLDα1 knockout mutants become less sensitive to ABA [56]. Interestingly, PLDα1 interacts with G-protein α GPA1 [57]. Therefore, PLDα1 and GPA1 may in fact function together in controlling aspects of ABA signaling. Knockout plants in another PLD isoform PLDζ are more susceptible to freezing damage whereas its overexpression enhances freezing tolerance [58].

Several other secondary signaling molecules including InsP₆, sphingosine1-phosphate (S1P), and cADPR were also suggested to regulate ABA responses in guard cells (reviewed in [48]). However, their role in stress signal transduction is unclear.
Phytohormones

Upon encountering abiotic stress, plants may alter their growth and development programs. Cell expansion and division may be halted and thus growth generally slower than under normal growth conditions. Longer-term abiotic stress may also affect plant phase transitions. For example, drought stress can promote flowering. These developmental changes imply that abiotic stress may alter the homeostasis of growth regulators. Although plant hormones are not considered as second messengers, the stress hormone ABA acts like one in many aspects: ABA biosynthesis is activated by abiotic stress [59]; ABA mediates many downstream pathways [22]; and ABA can be subjected to long distance transport and play physiological roles at sites distant from where it is synthesized [60]. In addition to ABA, other plant hormones, in particular ethylene and auxin, are involved in ABA and perhaps abiotic stress responses as well.

The role of ABA in plant stress responses has long been recognized [12,18]. In guard cells ABA regulates ion channels and promotes stomata closure to minimize transpiration water loss [48]. ABA activates the expression of many stress-responsive genes independently or synergistically with stresses. ABA can inhibit the biosynthesis of ethylene and may also potentially reduce the sensitivity of plants to ethylene [61]. The expression of some aquaporin genes or the activity of these water channel proteins may also be regulated by ABA. With the involvement of ABA in these processes, a general consequence is that plants will adapt to the stress with reduced water potential (so that they could lose less and uptake more water) and consequently, a reduced growth rate.

In addition to many physiological and biochemical changes that are mediated by ABA under abiotic stress, ABA may also regulate plant development programs and developmental changes such as root patterning. Our current knowledge in this aspect is limited.

4.3. Ca²⁺ as an intermediate signal molecule

The above-mentioned secondary signaling molecules may activate transient increases in cytosolic Ca²⁺ [26]. The sources of cytosolic Ca²⁺ inevitably are either internal or external. Both sources have much higher concentrations of Ca²⁺ relative to the cytosol. Therefore, the gating of Ca²⁺ channels is the major means to control Ca²⁺ transient increase in the cytoplasm. The complex or pumping of Ca²⁺ into vacuoles (or extracellular space) would be the major route for resetting the signals. Currently, a lot is known about both processes in animal cells but related information in plant cells is limited. Most plant Ca²⁺ channels may have diverged significantly in primary sequences from those of animal Ca²⁺ channels. Nonetheless, a putative two-pore Ca²⁺ channel in Arabidopsis was found to share sequence similarity to a voltage-gated Ca²⁺ channel in rats [62]. A recent study reported the identification of an ‘extracellular Ca²⁺ sensor’ [63], yet the biochemical functionality and its mode of action for this protein is unclear.

Internal Ca²⁺ could also contribute to stress-induced Ca²⁺ transients in the cytosol. In animal cells, several Ca²⁺ channels on ER membranes or other
endomembranes are responsible for transient Ca\(^{2+}\) increases in the cytosol. These include the InsP\(_3\) receptors and the cADPR ryanodine receptors. Although InsP\(_3\) and cADPR also exist and function in plant cells, their plant receptors have not been identified. Since many cell signaling events use Ca\(^{2+}\) as an intermediate signaling molecule, it is surprising that no Ca\(^{2+}\) channel has been identified in genetic screens that aim to elucidate these signal transduction mechanisms. Given that many putative ion channel genes exist in the Arabidopsis genome, future reverse genetic studies may help to identify potential Ca\(^{2+}\) channels.

Cytosolic or organelle Ca\(^{2+}\) concentrations are tightly controlled by various Ca\(^{2+}\) pumps and transporters. These Ca\(^{2+}\) transporters restore cytosolic Ca\(^{2+}\) homeostasis after various stimulus disturbances. One Ca\(^{2+}\) transporter is the tonoplast Ca\(^{2+}/H^+\) exchanger (CAX). These exchangers transport Ca\(^{2+}\) from the cytosol into vacuoles, a major storage of Ca\(^{2+}\) within plant cells. Overexpression of CAX1 resulted in increased freezing sensitivity [64] whereas its knockout mutants exhibited increased efficiency of cold acclimation in that the transcript levels for CBF/DREB1 transcription factor genes (see Section 3.7) and their downstream stress responsive genes were higher in the mutant than in the wild type. These cax1 knockout plants are thus more tolerant to freezing stress [65].

4.4. Ca\(^{2+}\)-binding proteins

In contrast to the lack of information on Ca\(^{2+}\) channels in plants, many Ca\(^{2+}\)-binding proteins have been identified in plants [66,67]. These Ca\(^{2+}\)-binding proteins possess Ca\(^{2+}\)-binding motifs that are homologous to those in animal Ca\(^{2+}\) binding proteins. One major Ca\(^{2+}\) binding motif is the so-called EF hand motif, which is conserved across organisms. Major plant Ca\(^{2+}\) binding proteins include calmodulins, SCaBP (SOS3-like Ca-binding proteins)/Calcineurin B-like (CBL) proteins, and Ca-dependent protein kinase (CDPK).

Arabidopsis SOS3 (Salt-Overly-Sensitive 3) was identified because of the salt hypersensitive phenotypes of the sos3 mutant. SOS3 shares sequence similarity with the calcineurin B subunit (CNB) and animal Ca\(^{2+}\) sensor, although SOS3 does not function as CNB in the activation of calcineurin. Rather, it acts as a Ca\(^{2+}\)-binding protein to interact with and activate the AMPK/SNF-like serine/threonine protein kinase SOS2, whose mutation also confers salt sensitivity. The activated SOS2 phosphorylates and regulates ion transporters such as the plasma membrane-localized Na\(^+\)/H\(^+\) antiporter SOS1. This eventually leads to the restoration of ion homeostasis in the cytoplasm under salt stress conditions [68].

In the Arabidopsis genome, there are 9 SOS3 homologs (SCaBP/CBL) and 22 SOS2 homologs (SOS2-like protein kinases -PKS/CBL-interacting protein kinases-CIPK). Individual SCaBP/CBL interacts with PKS/CIPK with different specificities (Gong et al., 2004; Luan et al., 2002). It appears that the various interaction pairs between these two groups of proteins may mediate responses to different abiotic stresses [69,70]. An example is the SCaBP5 and PKS3 interaction that may interpret Ca\(^{2+}\) signatures resulting from ABA or drought stress signals.
Mutations in SCaBP5 or PKS3 confer similar ABA hypersensitive phenotypes in the mutants. In addition, it was found that PKS3 interacts with the ABI2, a type 2C protein phosphatase (see below). ABI2 may negatively regulate the signals perceived by the SCaBP5-PKS3, thus potentially preventing over activation of the downstream signaling pathways. The interaction between PKS3 and ABI2 in this case did not result in detected dephosphorylation or phosphorylation of either partner. It is possible that some other component associated with this complex is the target of ABI2 [71]. More recently, Ohta et al. [72] confirmed this interaction and mapped the protein domain of SOS2 that interacts with ABI2. They found that this interaction is sensitive to the abi2 dominant mutation because the mutated form no longer interacted with PKS3, suggesting that the interaction between PKS kinases and ABI phosphatases may be physiologically significant.

Calmodulins have been implicated in several cellular processes through interaction with CaM-binding proteins [67]. The expression of several plant CaM genes is regulated by various environmental stresses such as mechanical stress/touch, cold, salt, or drought stress. Presumably, these CaMs may participate in the transduction of these external stimuli. One of the CaMs, CaM3, was suggested as a negative regulator of COR gene expression, since overexpression of this CaM led to reduced transcript levels of stress-responsive genes RD29A and KIN1 [73]. Consistent with this notion, experimental evidence indicates that a CaM binding protein, AtCaMBP25, may act as a negative regulator of osmotic stress tolerance. Transgenic Arabidopsis plants overexpressing AtCaMBP25 are more sensitive to osmotic stress whereas the antisense plants are more tolerant to salt stress [74].

The involvement of CDPK in stress signal transduction has also been implicated. In addition to stress-inducibility for some of the CDPKs, constitutively active CDPK was demonstrated to regulate stress-responsive reporter gene expression under ABA or stress treatments in protoplasts (Sheen, 1996). Overexpression of a CDPK in rice conferred increased tolerance to cold and salt stress [76]. However, there has been no report showing that loss-of-function CDPK may affect stress signal transduction or stress responses.

Other Ca\(^{2+}\)-binding proteins or Ca\(^{2+}\)-dependent proteins include annexins [77], calnexin and calreticulin. Calnexin and calreticulin may serve as endoplasmic reticulum (ER) chaperones and ER Ca\(^{2+}\) reservoirs. The role of these proteins in stress signaling is unclear. However, plants may have similar ER stress responses as do other eukaryotes [78].

4.5. Phosphoproteins at the core of stress signal transduction

In many signal transduction pathways, protein reversible phosphorylation is the major form of signal relay. The enzymes that catalyze these reversible phosphorylation processes are protein kinases and protein phosphatases. In the Arabidopsis genome, there are over 1,085 protein kinases (cited in [79]) and 112 protein phosphatases [80]. Protein kinases and phosphatases can be divided into several categories based on substrate specificity or on the structure or functional
characteristics. In this section, we present several recent examples on genetic studies of the role of phosphoproteins in stress signaling.

4.5.1. MAPK
Aside from its mysterious position in stress signaling, the mitogen activated protein kinase (MAPK) cascades are known to be involved in plant abiotic stress responses. The cascade is characterized by the sequential phosphorylation of a kinase by its upstream kinase in the order of MAPKKK-MAPKK-MAPK. Early studies found that the transcript levels of certain MAPK genes were enhanced by cold and salt stress. Some late studies followed the kinase activities for these proteins and found the activation of MAPK by stress treatments [81]. In several cases, regulated expression of MAPK components was shown to affect stress sensitivity. For example, expression of an active form of a tobacco MPKKK, NPK1, increases freezing tolerance of transgenic tobacco or maize plants [82,83].

Recently, a MAPK cascade in Arabidopsis was suggested to be involved in cold and osmotic stress signal transduction. This cascade consists of the MAPKKK MEKK1, the MAPKK MKK2, and two MAPKs, MPK4 and MPK6 [84]. Salt and cold stresses activate MKK2, MPK4 and MPK6, whereas in mkk2 mutant plants, MPK4 and PMK6 were no longer activated by cold. The mkk2 mutant plants were also sensitive to freezing and salt stress. Transcript profiling revealed that 152 genes were affected by over-expression of MKK2. These include genes for several transcription factors (such as RAV1, STZ, ZAT10, ERF6, WRKY, and CBF2), disease resistance proteins, cell wall related proteins, enzymes involved in some secondary metabolisms and an ACC (1-aminocyclopropane-1-carboxylate) synthase. Interestingly, several auxin-responsive genes were down regulated. Although this MAPK cascade apparently is involved in stress responses and the CBF2 transcript level was higher in the overexpressing plants, none of the CRT/DREB class of stress-responsive genes was significantly affected by this cascade. This is consistent with our previous prediction that MAPK pathways appear to be independent of the pathways that up regulate the expression of the CBF/DREB class of stress responsive genes [9]. In addition, since genes involved in other hormone biosynthesis (ethylene) and responses (auxin) were altered, it will be important to distinguish the direct targets of this MAPK cascade from those that are regulated by altered hormonal and oxidative stress responses. It is known that the ‘cross-talk’ between various MAPK cascades is intensive [81] and that various feedback regulations are also common within some pathways. For instance, MAPK6 had previously been shown to affect auxin signaling and stress tolerance [82], disease resistance [43,85], and ethylene biosynthesis [86]. Similarly, MPK3 was also suggested to affect ABA inhibition of seed germination [87] and pathogenesis signaling [43,85]. Identifying the targets of MAPK cascades may prove to be challenging.

4.5.2. Other protein kinases
Certain protein kinases are induced by various abiotic stresses either at the transcript level or at the activity level, implying that they may be involved in transduction of
these stress signals. There have been several reports presenting evidence that suppression or overexpression of some of these kinases resulted in altered stress responses in transgenic plants. However, genetic studies regarding the in vivo functionality for these kinases have been lacking. An exception is the SOS2 group AMPK/SNF like kinase (see Section 3.4), which was grouped into the SNF1-related kinase subfamily 3 (SnRK3) [79]. Some other members in the SnRK family were also shown to affect stress signaling and stress responses [79].

The protein kinase OST1 functions in the ABA signaling pathway upstream ABA-induced ROS production [88]. OST1 is related to the ABA-activated protein kinase AAPK in *Vicia faba* [89] and also related to SNF1 protein kinase [90] and was grouped into the SnRK2 subfamily [79]. The *ost1* mutants showed reduced response to ABA in stomata closure yet did not change in ABA responsiveness during seed germination. Ost1 activity is activated by ABA but its gene expression is not. Proteins similar to Ost1 also exist in several other plants and were reported to have similar roles in regulating osmotic stress and ABA signal transduction [91,92]. Further genetics and biochemistry studies are expected to reveal the roles of these kinases in stress signaling by defining their targets and modes of action in stress signaling.

4.6. Protein phosphatases

Protein phosphatases dephosphorylate phosphoproteins and thereby attenuate the function of protein kinases. Protein phosphatases can be classified by their substrate specificity as serine/threonine phosphatases, tyrosine phosphatases, and dual specificity phosphatases. Among them, serine/threonine phosphatases are the largest group of phosphatases in plants. According to their sequence (structure) characteristics and cation requirements, serine/threonine phosphatases can be classified further into PP1, PP2A, PP2B, and PP2C. Among these phosphatases, some PP2C, PP2A, PTP, dsPPase have been implicated in ABA or stress signal transduction [93,94]. The best-known example is the PP2C involvement in ABA signal transduction.

Early genetic studies using the inhibitory effect of ABA on seed germination identified the ABA-insensitive 1 (ABI1) and ABI2, two homologous 2C type phosphatases (reviewed in [22]). However, due to the dominant nature of both mutations, their roles in ABA signaling were not clear. Mutation analysis and reporter-gene assays in protoplast systems suggested that these ABI may function as negative regulators of ABA signaling [95]. Consistent with this notion, recessive intragenic revertants of *abi1* exhibited hypersensitivity to ABA in seed germination and vegetative growth [96]. Although ABA hypersensitive phenotypes for loss-of-function *abi1* and *abi2* mutants have not been reported, the discovery of other PP2C as negative regulators of ABA signaling [97,98] supports the idea that some PP2C may function as negative regulators of ABA signaling.

Following the isolation of the dominant *abi1-1* and *abi2-1* mutants, many researchers used these mutants in their studies of abiotic stress signaling and plant stress tolerance. It is clear that ABA or stress induction of many ABA and
stress-regulated genes are impaired in abi1 [99] or abi2 mutants (reviewed in [22]). Further studies using both mutants have found that abi1-1 and abi2-1 have defects in reactive oxygen species generation or their regulation on ion channels. The abi1-1 mutants are impaired in ABA-induced ROS production whereas abi2-1 guard cells are defective in H2O2-activated Ca2+ channel regulation [35].

To reveal the functionality of ABI1 and ABI2, it is essential to identify their targets. In yeast two-hybrid assays ABI1 interacts with the homeodomain transcription factor AtHB6 [100]. ABI1 and ABI2 also interact in vitro and in vivo with the SOS2 class of protein kinases [71,72] (see above sections). Some signal molecules such H2O2 and fatty acids were shown to bind to ABI1 or other PP2C (reviewed in [93]). ABI1 may also be regulated by PA derived from PLDα hydrolysis of phospholipids. PA binding of ABI1 inhibits ABI1 phosphatase activity and therefore will activate ABA signaling in response to ABA [56]. Interestingly, Zhang et al. [56] reported that ABI1 was predominately localized in the cytoplasm but tended to be relocated to plasma membrane in response to ABA treatment. It should be noted that PA is generated upon ABA treatment and is also membrane-localized. Subcellular localization of ABI1 and ABI2 were not reported before. If ABI1 does not localize in the nucleus where AtHB6 is found [100], the interaction between AtHB6 and ABI1 may not occur in vivo.

Previous pharmacological studies suggested that PP2A might be involved in cold stress signaling [101]. Recently, PP2A was shown to play roles in ABA activation of slow anion channels in guard cells because the rcn1 mutant exhibited ABA-insensitivity to ABA in stomatal closure and was impaired in slow anion channels regulation by ABA [102]. RCN1 encodes the regulatory subunit of PP2A. Because RCN1 is involved in response to ethylene and auxin [103,104], it is not clear whether the role of RCN1 in ABA signaling is the consequence of the regulation of an ABA signaling component(s) by PP2A or a result of the complex interaction between different plant hormones.

4.7. Transcription factors in stress signaling

Presumably, the targets of some protein kinases will be transcription factors that upon activation will bind to cis-elements in the promoters of stress-responsive genes and thus activate their transcription. Transcription factors may themselves be regulated at the transcription level by other upstream transcription factors. These further upstream transcription factors are often in a constitutively active state but are contained by repressors or held physically separate from their target genes (e.g., in cytoplasm or inaccessible to the target regions within the nucleus). Regulation of these transcription factors is therefore an important way to control gene expression. Common means for the release of repression include conformation changes by protein phosphorylation, degradation by ubiquitination, and trafficking between subcellular localizations. Protein kinases could play roles in all these processes, yet examples in plants are still very rare. Nonetheless, a lot has been learned regarding gene activation by transcription factors during abiotic stress signal transduction.
Several classes of transcription factors are involved in the activation of stress-response genes in plants. These include the AP2/ERF (ethylene responsive element binding factor), Zn finger, basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), MYB, and NAC transcription factors. The CBF/DREB transcription factors belong to the AP2/ERF class and have been studied in detail. These transcription factors bind to the C-repeat element (CRT)/dehydration-responsive element (DRE) in the promoters of many stress-responsive genes [3,19]. Nonetheless, CBF/DREB may not be the sole transcription factors in the regulation of CRT/DRE genes. A homeodomain transcription factor, HOS9, regulates cold signal transduction and cold tolerance through a pathway independent of the CBF/DREB transcription factors [105]. Because of their functional redundancy, null mutation in a single CBF/DREB transcription factor may not necessarily give rise to a visible phenotype. On the other hand, several experiments demonstrated that overexpression of CBF/DREB transcription factors could lead to an enhanced expression of stress-responsive genes and increased tolerance to various abiotic stresses [3,19].

Because CBF/DREB transcription factor genes are also induced by stress, upstream transcriptional activators must exist. Recently, it was suggested that CBF2 probably is a negative regulator of other CBF genes, since in cbf2 knockout mutant, the transcript levels of CBF1 and CBF3 were slightly higher than in the wild type, and the cbf2 mutant seedlings were more resistant to freezing stress [106]. Another putative transcription factor for CBF/DREB1 genes is ICE1. A dominant mutation in ICE1 resulted in impaired cold-stress regulation of CBF genes, whereas overexpression of ICE1 increases cold-induced CBF and the downstream gene expression. These transgenic plants are also more tolerant to chilling and freezing stress [107].

Several other putative signaling components that regulate the CBF/DREB class of transcription factors were identified in genetic screens for altered stress-inducible RD29A::LUC (luciferase) reporter gene expression. Mutation in the HOS1 gene resulted in increased stress-responsive gene induction by cold. HOS1 is a novel protein containing a RING finger domain that potentially participates in protein degradation. Since hos1 mutant seedlings had higher expression of CBF transcription factor genes, it is hypothesized that HOS1 may target positive regulators CBF transcription factors for proteolysis. Other potential regulators include FRY1/HOS2 [54], FRY2/CPL1 [109], and LOS4 [110]. FRY2 encodes a novel RNA polymerase II C-terminal domain (CTD) phosphatase [109,111,112] and may function in the regulation of transcript elongation. FRY1 encodes a bifunctional enzyme with both inositol polyphosphate 1-phosphatase and nucleotidase activities [53]. Both fry1/hos2 and fry2 mutants had higher transcript levels of several CBF transcription factor genes and higher level induction of stress-responsive genes [53,54,109], whereas los4 had a lower transcript level of CBF genes [110]. Because of the nature of these proteins, fry1/hos2, fry2/cpl and los4 may indirectly regulate the transcription of CBF either through upstream signaling pathway regulation or through regulation of the transcription machinery under abiotic stress.
4.8. Chromatin remodeling factors

Because genes are packed in chromatin, remodeling of chromatin structure to allow positive transcriptional regulators access to the genes is thus a critical step toward gene activation. It is conceivable that stress signal transduction involves components in chromatin remodeling. An example in this regard is the activation of the yeast High Osmolarity Glycerol 1 (Hog1) pathway. Upon osmotic stress, a MAPK pathway is activated and leads to the phosphorylation of the MAPK Hog1. Activated Hog1 is recruited to specific promoter regions by transcription factors. Hog1, once bound to the promoter complex, then recruits histone deacetylase Rpd3 to deacetylate histone and activate osmoreponsive genes [113]. Currently, little is known about the regulation of chromatin remodeling by most abiotic stresses except for low temperature.

Many plants in the temperate region require an exposure to prolonged low temperature (winter) to promote flowering in the spring, a process referred to as vernalization. In Arabidopsis, vernalization involves the down regulation of the flowering suppressor Flowering Locus C (FLC). FLC has a dosage repressing effect on flowering time. Vernalization modifies the FLC locus into a repressed state by histone methylation and therefore promotes flowering transition [114]. It should be noted, however, that vernalization and cold acclimation are two different processes [115]. Plants respond to them differently both in terms of gene expression and physiological consequences.

4.9. Posttranscriptional regulation in stress signaling

Gene regulation could occur at the level of transcription, posttranscription, translation, and posttranslation. Current studies of stress gene regulation are mainly focused on the transcription level. Although other processes of gene regulation are also important, it is only until recently that the importance of posttranscriptional regulation of stress responsive genes has become evident. Particularly, genetic studies of ABA and stress signal transduction have demonstrated that aspects of mRNA processing are critical for stress and ABA signal transduction.

In screens for components that affect the activation of the RD29A::LUC reporter gene, several mRNA processing factors or RNA-binding proteins were isolated. The SAD1 (Supersensitive to ABA and Salt 1) encodes a Sm-like U6 small ribonucleoprotein (snRNP) that is required for mRNA splicing and export. The sad1 mutant plants are hypersensitive to ABA and osmotic stress in gene expression, seed germination, and vegetative growth. The mutant plants are also defective in ABA biosynthesis because drought regulation and self-regulation of ABA biosynthetic genes are impaired in the mutant [59,116]. A second component is the FRY2/CPL1 RNA Pol II CTD phosphatases [109,111,112]. FRY2/CPL1 contains two dsRNA binding domains, suggesting that structured RNA may regulate the FRY2/CPL1 activities [109]. In the same screen, a RNA helicase, LOS4, was found to be required for cold acclimation and cold-regulated gene expression [110] (see Section 3.7). All these studies indicate a potential role of RNA processing in stress and ABA signal transduction.
Using different approaches, several other groups have discovered similar components functioning in ABA signal transduction. ABH1 (CBP80) is an mRNA cap binding protein. The \textit{abh1} mutant was isolated by its hypersensitivity to ABA during seed germination. The \textit{abh1} guard cell ion channels are also hypersensitive to ABA [117]. Because of its enhanced sensitivity to ABA in guard cells, \textit{abh1} plants can withstand water shortage for a longer time than the wild type plants. Mutation in another cap binding protein, CBP20, which is in complex with ABH1, confers phenotypes similar to ABH1 [118]. CBP80 and CBP20 are single copy genes and therefore, their mutations confer pleiotropic phenotypes such as small statue and serrated leaves. The \textit{abh1} mutation also suppresses the late-flowering phenotype conditioned by mutation in \textit{FRIGIDA} [119].

Other RNA-binding proteins that potentially affect ABA signaling include HYL1 and AKIP. The \textit{hyl1} mutant is hypersensitive to ABA during seed germination, and also hypersensitive to cytokinin, auxin, glucose, and salt and osmotic stress [120]. HYL1 is a dsRNA binding protein and appears to affect the levels of several miRNA [121,122]. The ABA-activated protein kinase (AAPK) interacting protein AKIP is similar to heterogeneous nuclear RNA-binding protein A/B in animals [123]. AKIP probably functions in the targeting or trafficking of certain mRNA [123] and may also regulate the stability of mRNA species that encode ABA signaling components.

Another group of molecules that can potentially regulate stress signaling and also plant developmental adaptation to stress is small RNA. Small endogenous RNA such as micro RNA (miRNA) and short interference RNA (siRNA) may regulate the transcript stability of some stress signaling components. The biogenesis of some of these small RNA may also be regulated by stress or ABA [124].

4.10. Regulation of stress signaling components by protein modifiers

The above-mentioned components are directly involved in stress signaling. In many cases, however, their roles in stress signaling may be regulated by other components that are not directly involved in the signal relay. Protein modifiers that are responsible for protein lipidation, glycoslation, methylation, sulfation, and ubiquitination regulate protein targeting, activity and longevity. Some of these processes are known to affect abiotic stress signaling.

Protein lipidation facilitates membrane localization of the modified proteins. Prenylation (including farnesylation and geranylgeranylation) is particularly required for signal transduction that involves small GTPases. Although the detailed plant pathways that are regulated by prenylation are unclear, it is known that ABA signaling requires that some of its components be modified by prenylation. Mutations in subunits of protein farnesyl transferase or geranylgeranyl transferase made the mutant plants hypersensitive to ABA in seed germination and stomatal regulation [125,126]. Nonetheless, the regulation of the stress-responsive genes \textit{NCED3} and \textit{ABA1} in \textit{era1} mutant does not appear to be hypersensitive to ABA or NaCl [127], suggesting that stress and ABA activation of these genes may not require \textit{ERA1}. 
Myristoylation is another form of protein lipidation. Several proteins in stress responses are known to be modified by myristilation. These include CDPK [128,129] and the Ca$^{2+}$-bindign protein SOS3. Myristoylation is required for SOS3 function in salt tolerance [130]. Other protein modification processes such glucosylation, sulfation, and nitrosylation may also affect stress signal transduction, but currently there is little experimental information to confirm this hypothesis. An Arabidopsis mutant defective in an oligosaccharyltransferase that potentially affects protein glycosylation is more sensitive to salt and osmotic stress [78].

Protein ubiquitation is often used by cells to target signaling proteins for degradation, thereby regulating signal transduction. The role of proteolysis in cell signaling was established for several processes such as light signaling, hormone (auxin, ethylene, GA, and ABA) signaling and signaling for pathogenesis [131,132]. Although information regarding the role of protein ubiquitation in stress signaling is limited, its role in ABA signaling is now well documented. Ubiquitination was found to regulate ABA signaling component ABF5 during seed germination and early seedling development. ABF5 is a bZip transcription factor whose mutation confers insensitivity of seed germination to ABA inhibition. Following seed germination, ABF5 is ubiquitinated and the germinated embryos established as seedlings. ABA can stabilize ABF5 and therefore prevent seed germination and seedling establishment [133]. When the 26S proteasome regulatory particle subunit RPN10 was mutated, ABF5 was stabilized and, therefore, the rpn10 mutant seeds are hypersensitive to ABA inhibition of seed germination [134]. Ubiquitin process involves the ubiquitin activating (E1), conjugating (E2) and ligating enzymes (E3). The SCF (Skp1/Cullin/F-box/Rbx1/2) complexes represent a major type of E3 ubiquitin ligases. F-box proteins in the SCF complexes may define substrate specificity. In Arabidopsis, there are about 700 putative F-box proteins [132]. One F-box protein has been suggested to be involved in ABA signal transduction since the knockout mutant became insensitive to ABA during seed germination [135].

Protein ubiquitination also removes denatured or unfold proteins. These unnatural proteins may become abundant under abiotic stress conditions. Chaperone proteins could restore some of these proteins to their native state [136]. Abiotic stress induces the expression of several heat shock proteins (Hsp). However, the role of these Hsp proteins and other chaperones in stress signaling is not very clear. In animal cells, an important role for some of these chaperones is the assembly of hormone receptor complexes. In plants, all the chaperone proteins such as Hsp90, Hsp70, immunophilins, cyclophilins, TPR (tetratricopeptide repeat) domain adaptor proteins exist, yet no obvious nuclear hormone receptors are found in completely sequenced Arabidopsis and rice genomes. The role of these proteins in stress signaling will await discovery in future studies.

4.11. Role of scaffolds and adaptors and vesicle trafficking in stress signaling

Signal transduction often requires the assembly of protein complexes. This involves recruiting, organizing, and anchoring of individual components. Specific proteins are evolved to play this scaffold and adaptor roles. These proteins usually contain
conserved protein-protein interaction domains. Although the role of scaffold protein in signaling is expected, there has been little information regarding these proteins in plant stress signaling. Other proteins with a physical role in supporting signaling molecules or their trafficking include cytoskeleton and its associated proteins. Cytoskeleton reorganization was suggested to play roles in early steps of cold signal transduction [45,137].

Vesicular trafficking is required for many signal transduction processes. In yeast cells, the barley stress and ABA-induced protein AtHVA22 interacts with vesicle trafficking component, which has high homology with the Arabidopsis RHD3 protein, a protein involved in root hair development [138] and trafficking between ER and Golgi body [139]. Previously, it was shown that drought and ABA affect root hair development in Arabidopsis [140,141]. It is not clear whether this process requires RHD3 or whether RHD3 is defective in drought responses.

Components in other aspects of vesicle trafficking, such as syntaxin-like proteins, are also implicated or demonstrated in ABA and osmotic stress response [142,143]. Rab GTPases that regulate vesicle trafficking [144] may also indirectly affect plant abiotic stress signal transduction and stress tolerance. Indeed, Mazel et al. [145] reported that overexpression of AtRabG3E resulted in increased tolerance to salt stress.

5. SIGNAL TRANSDUCTION PATHWAYS AND NETWORK INTEGRATION

In the previous sections, we presented an overview on individual components involved in stress signal transduction. Needless to say, there are many more components to be discovered, and some of them (such as stress signal receptors) are essential to understanding signal transduction. However, with more and more components being described, an important next task will be to sort out the pathways that integrate these different components. Since individual pathways interact with one another at various levels, the signal pathways actually constitute a signaling network. While integrating these different pathways, one thing often discussed is the interaction (cross-talk) or specificity of various pathways.

Evidently, many genes can be activated by multiple stresses and by the plant hormone ABA (see previous sections). This could result from pathway interactions at any signaling steps (Figure 1). For instance, different stresses many share similar intrinsic attributes (Section 3.11). An obvious example is that drought and salt stress both lead to osmotic stress. In this case, the signaling pathway activated by osmotic stress would likely be shared by both drought stress and salt stress. On the other hand, the ionic stress generated by Na+ would be specific to salt stress. Additional layers of interactions could occur at the generation of second signal molecules (see Section 3.2). Particularly, various stresses may affect the biosynthesis of and response to various plant hormones such as ABA and ethylene. Abiotic stresses as well as biotic stress also generate reactive species. The signal transduction pathways initiated by a common secondary signaling molecule would be similar in terms of the usage of signaling components and the outputs of the signaling pathways, even if their primary signals are quite different.