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Review

Abiotic stress signal transduction in plants: Molecular and genetic perspectives

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Low temperature, drought and salinity are major adverse environmental factors that limit plant productivity. Understanding the mechanisms by which plants perceive and transduce these stress signals to initiate adaptive responses is essential for engineering stress-tolerant crop plants. Molecular and biochemical studies suggest that abiotic stress signaling in plants involves receptor-coupled phosphorelay, phosphoinositol-induced Ca^{2+} changes, mitogen-activated protein kinase cascades and transcriptional activation of stress-responsive genes. In addition, protein posttranslational modifications and adapter or scaffold-mediated protein-protein interactions are also important in abiotic stress signal transduction. Most of these signaling modules, however, have not been genetically established to function in plant abiotic stress signal transduction. To overcome the scarcity of abiotic stress-specific phenotypes for conventional genetic screens, molecular genetic analysis using stress-responsive promoter-driven reporter is suggested as an alternative approach to genetically dissect abiotic stress signaling networks in plants.

Introduction

Environmental stresses such as low temperature, drought and salinity limit crop productivity worldwide. Understanding plant responses to these stresses is essential for rational engineering of hardier crop plants. The process by which plant cells sense the stress signals and transmit them to cellular machinery to activate adaptive responses is referred to as signal transduction. Many signal transduction networks have been established in microbial and animal systems. In plants, the signal transduction pathways for light, several phytohormones and pathogenesis are also being elucidated. Despite decades of physiological and molecular effort, knowledge of how plants sense and transduce low temperature, drought and salinity signals is still very limited. One major constraint hampering our understanding of these signal transduction processes in plants has been the lack or slow pace of application of molecular genetics due to the scarcity of reliable phenotypes specific to the various environmental stresses. These phenotypes are critical for devising genetic screens for mutants, which are the key to dissecting signal transduction pathways in an organism. To circumvent this shortcoming, molecular genetic approaches involving the use of reporter gene expression have been explored and appear very promising for dissecting stress signal transduction pathways in plants. Here, we first survey signaling modules that are implicated in stress signal transduction in plants. References to non-plant systems are made when similar information is not yet available for plants. Even though some of the signaling modules have not been shown to operate in plants, they are important to illustrate the diversity and complexity of signaling networks, and their counterparts probably exist in higher plants due to the conservation of signaling modules across diverse organisms. As low temperature, drought and salinity stresses often lead to the accumulation of ABA and the induction of ABA-induced genes in plants, ABA-related signal transduction is also addressed when applicable. After this survey of common themes in signaling transduction, we discuss the application of *Arabidopsis* molecular genetic approaches in analyzing stress signaling. Topics on general aspects of signal transduction and traditional genetic analysis of stress and ABA signal transduction in plants have been covered extensively in several recent reviews (e.g. Trewavas and Malho 1997, Zhu et al. 1997, Koornneef et al. 1998, Leung and Giraudat 1998, McCourt 1999, Thomashow 1999).

Signaling modules in abiotic stress responses

The relatively independent functional units that make up the signal transduction network are referred to here as modules. Most of the signaling modules were initially described in non-plant systems and many of them have now been found in plants. There are several methodologies currently used to determine whether a signaling module functions in plant stress signal transduction or not. Earlier work on molecular aspects of stress responses is exemplified by expression surveys by differential/subtractive screening (for review see Bray 1993, Shinozaki and Yamaguchi-Shinozaki 1997, Zhu et al. 1997, Thomashow 1999). It is believed that stress-inducible genes may function in the signaling network or may play roles in stress tolerance. A second method is the candidate gene approach. This involves the selection of a candidate gene from a particular known module and examining the expression of that gene or the activity of the gene product under stresses or additionally, by functional complementation in heterologous systems, usually in yeast where stress signal transduction (particularly osmotic stress) is better understood. Additionally, various pharmacological agents have been used as antagonists or agonists to modulate potential signaling pathways. Finally, forward and reverse genetic studies involving the isolation of signal transduction mutants, or mutations in putative regulatory genes can provide compelling evidence for the function of a gene product in particular signaling processes. Currently, the involvement of most of the components outlined in the following modules in stress signaling has not been established by genetic analysis.

Receptors

Environmental signals are thought to be first perceived by specific receptors that, upon activation, will initiate (or suppress) a cascade to transmit the signal intracellularly and in many cases, activate nuclear transcription factors to induce the expression of specific sets of genes. Receptor-coupled protein phosphorylation is a common form of signal initiation. Although none of the receptors for cold, drought, salinity or the stress hormone abscisic acid in plants is determined to certainty, current knowledge indicates that receptor-like protein kinases, two-component histidine kinases, as well as G-protein-associated receptors may represent the potential sensors of these signals.

Receptor-like kinases (RLKs) are found in both animals and plants. Structurally, they consist of an extracellular domain that may function in ligand binding or protein-protein interactions, a transmembrane domain and an intracellular kinase domain. Unlike animal RLKs that usually possess tyrosine signature sequences, plant RLKs have serine/threonine signature sequences. There are a larger number of RLKs in plant genomes, which can be further divided into several subgroups according to the structure characters (e.g. Hardie 1999). Plant RLKs are found to be mainly involved in pathogenesis responses and in plant development where in both cases there seems to exist extracellular ligands for receptor binding. The identification of BRI as a RLK for a potential brassinosteroid receptor (Li and Chory 1997) indicates that there are probably similar RLKs acting as ABA receptors. In *Arabidopsis*, a gene that encodes a receptor like kinase with extracellular leucine-rich repeats, *RPK1*, was found to be induced 1 h after ABA treatment, or dehydration, high-salt and low-temperature treatments (Hong et al. 1997), indicating that this protein kinase may be involved in multiple-stress signal transduction. Considering rapid cellular responses (such as dynamics in inositol phosphates and Ca^{2+} fluctuation) when exposed to stresses, it can be conceived that the activity of a stress receptor should be constitutive, at least at a lower level. Although the relative slow induction of *RPK1* by stress does not exclude the possibility of a constitutive basal level of receptor activity, its role in signal initiation is likely not primary.

The two-component sensor-response regulator systems involving histidine kinases that were initially found in prokaryotes for perception of various environmental signals also exist in eukaryotes, including plants. When the extracellular sensor domain perceives a signal, the cytoplasmic histidine residue is autophosphorylated and the phosphoryl moiety is then passed to an aspartate receiver in a response regulator, which may constitute part of the sensor protein or a separate protein. The 'two-component' sensors may couple with a downstream mitogen-activated protein (MAP) kinase cascade or directly phosphorylate specific targets to initiate cellular responses. Homologous histidine kinases characteristic of this two-component system have been found to function in ethylene (for review, see Chang and Shockey 1999) and probably also in cytokinin signal transduction pathways (Kakimoto 1996, Brandstatter and Kieber 1998). Recently, they have also been implicated to function in the perception of environmental stress signals such as low temperature and osmotic stress in plants, as summarized below.

In cyanobacterium, it was suggested that membrane fluidity might act as a primary sensor for low temperature (Murata and Los 1997). Yet, the change in the physicochemical status of membrane lipids must be further sensed by other molecules closely associated with the membrane. To investigate whether histidine kinases are involved in temperature perception in cyanobacterium *Synechocystis* sp. PCC 6803, all putative histidine kinases in the genome were systematically disrupted and the impact on temperature-induced desaturase gene expression was monitored with the bacterial luciferase reporter. This survey resulted in the identification of two histidine kinases and a response regulator that modulate the induction of some, but not all, low-temperature-induced desaturase genes. Therefore, these histidine kinases probably play roles in part of the low-temperature signal transduction (Suzuki et al. 2000). The failure of complete blocking of all low-temperature-responsive genes also implies that there are multiple temperature sensors in the genome.

The best-characterized two-component histidine kinase is the *Saccharomyces cerevisiae* osmosensor SLN1. Together with the YPD1-SSK1 response regulator, this 'two-component' signal unit regulates the high-osmolarity glycerol (HOG) MAPK cascade, resulting in the production of glycerol to survive osmotic stress. In *Arabidopsis*, a histidine kinase gene, *AtHK1*, was isolated by PCR using degenerate primers. This kinase is structurally related to SLN1. Indeed,

AtHK1 can rescue the salt sensitivity of yeast mutants with deletions of SLN1 and SHO1 (another transmembrane osmosensor), implying that AtHK1 might have a similar function in plants. Expression of *AtHK1* was up-regulated by alterations in osmolarity of external solutions (Urao et al. 1999). Related with this work, it was also found that the expression of two genes that encode 'two-component' response regulator-like proteins in *Arabidopsis* was induced by low temperature, drought and salt stress (Urao et al. 1998). Whether these components play roles in stress signaling awaits functional genetic analysis.

In animal systems, there are a large number of hormone and neurotransmitter receptors that are structurally related to rhodopsin, the 7-transmembrane (7TM) domain G-protein-coupled light receptor. They are collectively grouped as G-protein-coupled receptors (GPCRs). Similar receptors probably function in light perception in flagellate green alga (Calenberg et al. 1998). Surprisingly, prototype GPCRs are underrepresented in higher plant genomes. To date, there are only a few sequences that show homology to animal GPCR (Josefsson and Rask 1997, Plakidou-Dymock et al. 1998). On the other hand, over 30 putative 7TM genes exist in *Arabidopsis* genome and the encoded proteins are homologous to the disease-resistant protein Mlo (Devoto et al. 1999). However, they do not show significant homology with animal GPCRs at the amino acid level. It is not yet known whether this group of proteins is the plant version of GPCRs. In any event, it seems that plants do not elect to use these receptors as extensively as do animals. As G-proteins are implicated to play roles in environmental stress signaling, it can be envisaged that there are G-protein-associated receptors in plants that may participate in the perception of environment stresses.

Second messenger/Ca^{2+}-releasing modules

Numerous studies have suggested that Ca^{2+} is involved in various intracellular signaling processes, both in animals and in plants (for review, see Sanders et al. 1999). As such, the concentration of intracellular Ca^{2+} is carefully tuned. Ca^{2+} concentration in the cytosol is low, and upon stimulation, Ca^{2+} is released from intracellular storage or enters the cell via various Ca^{2+} channels. Studies with animal cells have shown that there are several paths that mediate Ca^{2+} transient increase in the cytosol. Ca^{2+} can enter the cells from outside by voltage-gated, receptor operated or store-operated Ca^{2+} channels. Also, Ca^{2+} in intracellular stores can be released through ligand messenger-sensitive channels. These second messengers include inositol polyphosphates, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). Receptors of inositol 1,4,5-trisphosphate (IP_3) have been isolated from animal as well as plant cells. Ryanodine receptors for cADPR were also identified in animals. Interestingly, the activities of these two types of receptors are also stimulated by Ca^{2+}. Furthermore, it was found that the IP_3 receptor is required for coupling the activation of store-operated Ca^{2+} channels in animal cells. These positive feedback regulations underline the phenomenon referred as Ca^{2+}-stimulated Ca^{2+} release. In contrast, the molecular identities of NAADP receptors are unknown and they do not seem to be regulated by Ca^{2+}.

In higher plants and algae, the phosphoinositide module has been implicated to function in the transduction of environmental stimuli such as light, gravity, fungal elicitors, acidity and osmotic stress (for review, see Munnik et al. 1998, Stevenson et al. 2000). The IP_3 precursor, phosphatidylinositol 4,5-bisphosphate (PIP_2) is synthesized via phosphatidylinositol 4-phosphate 5-kinase. An *Arabidopsis* gene encoding this enzyme, *PIP5K*, was induced by water stress and ABA (Mikami et al. 1998). IP_3 is generated by the hydrolysis of PIP_2 by phospholipase C (PLC). In animal cells, there are at least 3 subfamilies of PLC and the regulation of each subfamily uses different signaling pathways, but all PLCs strictly require Ca^{2+} as cofactor for activation. In *Arabidopsis*, there are approximately 10 genes encoding PLCs. An *Arabidopsis* PLC gene, *AtPLC1*, whose product is most similar to members in the animal PLCδ subfamily, was found to be strongly induced by salt and drought stress, and also induced to a lesser extent by low temperature (Hirayama et al. 1995). Exogenous IP_3 was demonstrated to be able to induce Ca^{2+} release from tonoplast vesicles or isolated vacuoles (e.g. Schumaker and Sze 1987). In guard cells, caged IP_3 induced Ca^{2+} increase in cytoplasm and triggered stomata closure (Blatt et al. 1990, Gilroy et al. 1990). After treatment with ABA, there is a transient increase in IP_3 in guard cell protoplasts of *Vicia faba* (Lee et al. 1996). These ABA effects require PLC, as inhibition of PLC blocked ABA-induced Ca^{2+} oscillation and stomatal closure (Staxen et al. 1999). Plant PLCs are similar to members of the animal PLCδ subfamily; they seem to be activated by G-protein as evidenced by pathway activation with G-protein activator, mastoparan (e.g. Quarmby et al. 1992). However, it should be noted that the phosphoinositide module is subjected to multiple levels of regulation via PLC and many other enzymes in the phospholipid signaling pathway. For example, the expression of *AtPLC1* gene was negatively regulated by SOS2 (Zhu et al. 1998), a protein kinase functioning in plant salt tolerance.

Phospholipids are also hydrolyzed by phospholipase D (PLD) to produce phosphatidic acid (PtdOH), which is a second messenger in animal cells that may activate phosphatidylinositol 5-kinase, PLC and protein kinase C (PKC). In guard cells, it was found that PLD activity increased shortly after ABA treatment and that application of PtdOH had the same effect as ABA to induce stomatal closure (Jacob et al. 1999). Similarly, PLD activity was activated within minutes after dehydration or salt stress treatment (Frank et al. 2000, Munnik et al. 2000). Additionally, PLD was activated by mastoparan, a G-protein agonist, suggesting the involvement of G-protein in early signal transduction (Frank et al. 2000). Two cDNAs encoding PLDs were isolated from the resurrection plant *Craterostigma plantagineum*. One gene, *CpPLD-1*, was constitutively expressed and the second, *CpPLD-2*, was induced transcriptionally by dehydration and ABA treatment (Frank et al. 2000). The de novo synthesis of inositol requires the inositol 1-phosphate synthase (INPS) to convert glucose 6-phosphate into INP. The transcription of *INPS* was enhanced by osmotic stress

in the shoot, but was repressed in the root in common ice plant (Nelson et al. 1998), whereas its expression in *Arabidopsis* was not enhanced by salt stress (Ishitani et al. 1996). The enhanced synthesis of inositol in ice plant is probably required for the synthesis of the osmolyte, D-ononitol. It is unclear if this altered inositol synthesis would affect phosphoinositide signaling or not.

As in animal cells, cADPR is probably also a second messenger in plants that can trigger the release of Ca^{2+} from internal stores and initiates stress-induced gene expression. In animal cells, cADPR is produced by NAD^+ with the ADP-ribosyl cyclase. In plants, cADPR was demonstrated to release Ca^{2+} from vacuoles (Allen et al. 1995). Using the stress-responsive promoter of *RD29A* and *KIN1* fused with *GUS* reporter, Wu et al. (1997) studied the transient expression of the reporter gene in tomato hypocotyl cells. They found that cADPR could induce the expression of the transgenes and this process involved Ca^{2+} and protein phosphorylation/dephosphorylation (Wu et al. 1997). In the guard cells of *Commelina communis*, it was found that ABA-induced stomatal closure is mediated by cADPR (Leckie et al. 1998). The IP_3-induced Ca^{2+} release and cADPR-induced Ca^{2+} each operated in the ABA regulated gene expression (Wu et al. 1997). These two pathways may not be simply redundant. A recent study with human T lymphocyte cells suggested that the two pathways are temporally separated, with IP_3 initiating the signaling while cADPR sustains the signaling (Guse et al. 1999).

Intracellular phosphoprotein modules

Upon receiving a signal from membrane receptors, cells often utilize multiple phosphoprotein cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are perhaps the most common intracellular signaling modes. They regulate a wide range of cellular processes such as enzyme activation, assembly of macromolecules, protein localization and degradation. In plants, many protein kinases (for review, see Hardie 1999) and phosphatases are thought to be involved in environmental stress responses on the basis of pharmacological studies.

Most plant protein kinases are serine/threonine kinases that play major roles in protein phosphorelay. A serine/threonine protein kinase from wheat, PKABA1, was among the first plant protein kinase genes to be found that is up-regulated by drought, low temperature and NaCl, as well as by ABA (Holappa and Walker-Simmons 1995). The accumulation of *PKABA1* transcripts in embryos correlates with ABA level as the seeds mature, implying a role of PKABA in the control of dehydration tolerance or seed dormancy. Using a transient coexpression assay, Gomez-Cadenas et al. (1999) found that PKABA1 indeed inhibited ABA-suppressed and GA-stimulated α-amylase gene expression in barley aleurone layers, but did not directly regulate ABA-responsive late embryogenesis abundant (*LEA*) gene expression. The fact that *PKABA1* is induced by ABA and stress in seedlings implies possible functions of its product in vegetative tissues as well. Recently, an ABA-activated protein kinase (AAPK) gene from *Vicia faba* that is homologous to *PKABA1* was found to be specifically expressed in guard cells. Interestingly, introducing a mutated version of AAPK blocked ABA-induced stomatal closure by eliminating ABA activation of plasma membrane anion channels (Li et al. 2000). Since the mutated AAPK showed reduced activity, this inhibitory effect seems to result from a dominant interference of ABA signaling. It is not known whether *AAPK* is also regulated by drought stress. As roots are the first plant organ that senses drought stress in the soil, not surprisingly, roots may have a specific signaling mechanism. Expression of a root-specific protein kinase gene (*ARSK1*) from *Arabidopsis* was strongly induced by drought and salt, as well as ABA treatments (Hwang and Goodman 1995).

A family of protein kinases unique to plants, the calcium-dependent protein kinases (CDPKs), are also involved in stress responses. CDPKs consist of a serine/threonine kinase domain and a C-terminal calmodulin-like domain with up to 4 EF hand motifs. Most CDPKs have a N-terminal motif for myristoylation that potentially facilitates membrane association. The expression of *Arabidopsis ATCDPK1* and *ATCDPK2* was rapidly induced by drought and salt treatments; yet, their expression was not induced by cold, heat shock, or ABA, suggesting that they may participate in an ABA-independent pathway (Urao et al. 1994). By transiently expressing a chimeric gene consisting of GFP reporter driven by a cold-, salt-, dark- and ABA-responsive promoter (*HVA1*) in maize leaf protoplasts, Sheen (1996) showed that coexpression of a constitutively active ATCDPK1 activates the reporter gene, while a non-active CDPK1 mutant cannot. These results suggest an involvement of CDPK in stress signal transduction in plant cells.

In a stress signaling cascade, inactivation of phosphoproteins is usually accomplished by dephosphorylation. There are 4 major subgroups of protein phosphatases: PP1, PP2A, PP2B (calcineurin) and PP2C. Among them, PP2B and PP2C are Ca^{2+}-dependent while PP1 and PP2A by themselves do not need Ca^{2+} to function. As with kinases, studies using phosphatase inhibitors have indicated a role for phosphatases in stress signaling. An often-observed phenomenon in many organisms is that the inhibition of protein phosphatases leads to similar outcomes as the stimulation of protein kinases. For example, in human T cells, inhibition of PP2A induced the phosphorylation of the signal transducers and activators of transcription (STAT)3 and resulted in the translocation of this transcriptional factor to the cytosol (Woetmann et al. 1999). In alfalfa plants, a phosphatase 2A inhibitor, okadaic acid, induced the expression of a cold-induced gene, *cas15*, at 25°C. It was found that cold acclimation strongly inhibited the activity of protein phosphatase 2A and this process was mediated by Ca^{2+}, as treating cells with calcium ionophere A23187 or with calcium channel agonist had similar effects (Monroy et al. 1998). Because PP2A does not need Ca^{2+} for activation, it is possible that PP2A may form a complex with Ca^{2+}/CaM-dependent protein kinase to regulate downstream gene transcription, as was demonstrated in human T lymphocyte cells (Westphal et al. 1998). The physical association of a protein phosphatase with protein kinases may be a common phenomenon. A good example in plants is the association of

kinase-associated phosphatase (KAPP) with RLKs (Stone et al. 1994). KAPP may be a common regulator of a subset of RLKs (Braun et al. 1997) and has been shown to participate in the signaling of shoot apical meristem development (Williams et al. 1997, Stone et al. 1998).

The suggestion that serine/threonine phosphatase PP2A regulates cold-responsive genes in plants is intriguing. The common heterotrimeric form of PP2A consists of a structural A subunit, a regulatory B subunit and a catalytic C subunit. Each subunit has different interacting proteins. In yeast and animal cells, PP2A has been shown to affect cell cycling, vertebrate axis determination (Wnt signaling) and apoptosis. To identify PP2A targets or proteins associated with PP2A in plants, Harris et al. (1999a) used an *Arabidopsis* PP2A cDNA catalytic subunit as a bait to conduct a yeast two-hybrid screen and obtained one protein, TAP46, that was demonstrated to associate with PP2A in vivo. This protein shows similarity to yeast TAP42 and mammalian α4 proteins, both of which function in the target of rapamycin (TOR) pathway in response to nutrient availability, and whose activities lead to the activation of ribosomal S6 kinase and inactivation of a translation initiation inhibitor 4E-BP1. This positively regulates the translation initiation factor eIF-4E and results in increased mRNA translation (Gingras et al. 1999). The precise functions of TAP42 and α4 protein are unknown but they seem to act as adapters in the PP2A signaling complex to regulate PP2A activity. Interestingly, the expression of *TAP46* in *Arabidopsis* is induced by low-temperature treatment, but not by heat shock (Harris et al. 1999a). Together with a previous finding that a ribosomal S6 kinase homolog was induced by low temperature and drought stress (Mizoguchi et al. 1996), it seems likely that a signaling branch using part of the TOR module involving MAPK/CDPK-PP2A/TAP42-S6K may be activated by low temperature and other stresses and modulate the transcription/translation of stress-responsive genes in plants.

An additional connection between PP2A and stress signaling is that PP2A may regulate MAPK cascade by dephosphorylation of the core kinases; however, other serine/threonine or dual specificity phosphatases also regulate the MAPK cascade. In *Arabidopsis*, a cDNA encoding a tyrosine-specific protein phosphatase (PTP1) was isolated by using degenerate primers against conserved tyrosine-specific phosphatase sequence. It was found that the expression of *PTP1* was significantly enhanced by NaCl but was down-regulated by short-term (6 h or shorter) cold treatment (Xu et al. 1998). The significance of this differential regulation by two unrelated stresses is not clear. In a study of the interaction between low temperature and salt stress in the regulation of the stress-responsive gene *RD29A*, Xiong et al. (1999c) observed that when *Arabidopsis* seedlings were treated for 4.5 h in the cold, the induction of *RD29A* by NaCl or ABA (in the cold) was completely blocked, while longer cold treatment (2 days) did not block ABA induction. It is possible that this interaction between low temperature and salt stress occurs at the level of PTP1, as evidenced by similar regulation of *PTP1* expression by cold and salt stresses (Xu et al. 1998). By analogy with other systems and also implicated in plants (e.g. Knetsch et al. 1996), the role of this PTP1 in stress signaling may be the dephosphorylation of a MAP kinase on a tyrosine residue.

The characterization of a yeast mutant defective in calcineurin B (CNB) (PP2B) underscores the crucial role of this protein phosphatase in salt tolerance (Mendoza et al. 1994). By functional complementation, plant transformation or expression studies, several homologous plant proteins are implicated to function in salt tolerance in a similar way to the yeast CNB (Pardo et al. 1998, Kudla et al. 1999, Piao et al. 1999). Identification of the *SALT OVERLY SENSITIVE* (*SOS*)*3* gene product as related to CNB provided compelling evidence to support the role of calcineurin-like protein in plant salt-tolerance (Liu and Zhu 1998). However, SOS3 does not seem to function through a protein phosphatase. Rather, SOS3 interacts with and activates a protein kinase encoded by *SOS2* (Liu et al. 2000, Halfter et al. 2000). The SOS3-SOS2 protein kinase complex is becoming a well-studied module that functions in plant salt tolerance (for review see Zhu 2000).

ABA signaling is central to any discussion of stress responses since osmotic stress leads to ABA accumulation. The *Arabidopsis abi1* and *abi2* are dominant mutants that showed insensitivity to exogenous ABA during germination. These mutants also exhibit deregulation of stomates resulting in a wilty phenotype at reduced humidity conditions. The *ABI1* and *ABI2* genes encode homologous serine/threonine protein phosphatase 2C (Leung et al. 1994, Meyer et al. 1994, Leung et al. 1997) and may have overlapping functions. Previous studies based on these insensitive mutants and ABA deficient mutants led to the consensus that there exist both ABA dependent and ABA-independent stress signaling pathways. Besides their roles in controlling ABA-dependent signal transduction in guard cells, it was found that constitutive ABI1/PP2C abolished ABA-induced *HVA* gene expression (Sheen 1996). However, these two processes may also be related as the *abi1* and *abi2* mutations reduced ABA-induced cytoplasmic Ca^{2+} rise in guard cells (Allen et al. 1999). Thus, one can expect that active PP2C had less effect on constitutive CDPK-induced *HVA* gene expression (Sheen 1996). As the *abi1* mutation was dominant, its direct role in ABA signaling was not clear. Using reporter gene assay with mutated ABI1, Sheen (1998) concluded that the observed *abi1* phenotypes may result from dominant-interfering (Sheen 1998) and the in vivo role of these proteins may be negative regulation of ABA action. This is supported by the isolation of recessive *abi1* alleles. Gosti et al. (1999) found that these recessive mutations in the *ABI1* locus actually confer sensitivity to ABA either for seed germination or for seedling growth, indicating the ABI1 is indeed a negative regulator of ABA signaling (Gosti et al. 1999).

Despite much interest in the ABI1 and ABI2 proteins, the in vivo substrates of these enzymes are still not known. In yeast two-hybrid screens, it was found that *SOS2* interacted with ABI2 (J. K. Zhu, unpublished observation), suggesting there probably exist connections between ABA signaling pathways and the SOS module. Also, an alfalfa PP2C with homology to *Arabidopsis* ABI2 was shown to act as wound-induced MAPK-specific phosphatase that regulates MAPK activity (Meskiene et al. 1998, Baudouin et al. 1999). Thus,

it is also possible that ABI proteins may connect to MAP kinase modules.

MAPK modules

The MAP kinase pathways are intracellular signal modules that mediate signal transduction from the cell surface to the nucleus (for review see Robinson and Cobb 1997). MAPK cascades are likely conserved in all eukaryotes. They seem to be widely used as osmolarity signaling modules. The core MAPK cascades consist of 3 kinases that are activated sequentially by an upstream kinase. The MAP kinase kinase kinase (MAPKKK), upon activation, phosphorylates a MAP kinase kinase (MAPKK) on serine and threonine residues. This dual-specificity MAPKK in turn phosphorylates a MAP kinase (MAPK) on conserved tyrosine and threonine residues. The activated MAPK can then either migrate to the nucleus to activate transcription factor directly, or activate additional signal components to regulate gene expression, cytoskeleton-associated proteins or enzyme activities, or target certain signal proteins for degradation.

The cooperative activation of the 3 kinases results in an ultrasensitivity that makes the cascade operate in an on-or-off switch manner and prevents noise activation of the cascade (Huang and Ferrell 1996). It has been shown that different MAPK pathways may share common components; yet, activation of one pathway may not necessarily affect another pathway. The specificity is realized by scaffold proteins (such as Ste5 in yeast) that hold these kinases or by a specific component in the signaling cascade (O'Rourke and Herskowitz 1998). The yeast HOG1 pathway is the best-studied MAP kinase pathway. It was also found in the fungal pathogen *Magnaporthe grisea* (Dixon et al. 1999). In plants, MAPK cascades have been shown to participate in auxin and cytokinin signal transduction and cell-cycle regulation and are implicated in wound and pathogenesis responses as well as in environmental stress signal transduction (for review, see Jonak et al. 1999).

In alfalfa plants, a MAPK was activated within 10 min of cold treatment. It was also activated by drought stress as well as mechanical stress, but not by heat, salt stress or exogenous ABA (Jonak et al. 1996), suggesting that this MAPK mediates drought and cold signaling via an ABA-independent pathway. A salicylic acid-induced protein kinase (SIPK) belonging to the MAP kinase family that is activated by salicylic acid, pathogen attack and wounding was also found to be activated within 5–10 min after osmotic stress (Mikolajczyk et al. 2000). Similarly, in tobacco cells, the SIPK (a MAPK) and another protein kinase, HOSAK, were activated by osmotic stress and this activation is independent of Ca^{2+} or ABA (Hoyos and Zhang 2000). On the other hand, in barley aleurone protoplasts, it was found that a MAP kinase was activated by physiological concentrations of ABA within 1 min after the treatment, and this activation required protein tyrosine phosphatase (Knetsch et al. 1996). This MAP kinase cascade regulates the expression of the ABA-responsive gene, *RAB16*. In *Arabidopsis*, the transcription of a MAPK gene, *ATMPK3*, is induced by drought, low temperature, salinity and touch (Mizoguchi et al. 1996). ATMPK3 is also activated by H_2O_2 and its activity is further enhanced by ectopically expressed ANP1, a MAPKKK (Kovtun et al. 2000). *ATMEKK1* (a MAPKKK), whose expression is induced within 5 min by NaCl treatment (Covic et al. 1999), can functionally complement the *ste11* (a *MAPKKK*) mutant of *S. cerevisiae* (Covic and Lew 1996, Mizoguchi et al. 1996). Yeast cells expressing *ATMEKK1* are more tolerant to osmotic stress and produce more glycerol under non-stressed conditions (Covic et al. 1999). Similarly, a pea PsMAP kinase is capable of rescuing morphological defects of yeast *hog1* mutants in hyperosmotic medium and partially restored the mutant growth (Popping et al. 1996). These results suggest functional similarity of these MAPK components with their yeast counterparts.

To further explore the role of MAPK module in stress signaling in plants, Kovtun et al. (2000) overexpressed a tobacco ANP ortholog, NPK1, which activates the H_2O_2-regulated gene expression in plants, and found that *Arabidopsis* plants overexpressing NPK1 showed an increased tolerance to freezing, heat shock and salt stress. Yet, in NPK1 transgenic cells, the expression of the stress-regulated gene *RD29A* was not altered. It is not known whether this MAPKKK could activate other abiotic stress signal transduction pathways and thus contribute to the increased tolerance to these stresses. In this context, it is worth noting that reactive oxygen species (ROS) relate to abiotic environmental stresses besides their well-known involvement in biotic stresses (for review, see Bolwell 1999). ROS could indirectly potentiate stress signaling via some unidentified branches, e.g. the generation of cADPR.

Transcriptional regulators

According to their conserved DNA-binding domains, common transcription factors in eukaryotes can be classified into several groups such as the basic region leucine zipper (bZip) protein, MYB-like proteins (containing helix-turn-helix motifs), MADS-domain proteins, helix-loop-helix proteins, zinc-finger proteins and homeobox proteins. Plants also have some transcriptional factors with unique DNA-binding domains such as the AP2/EREBP domain. In the activation of abiotic stress-responsive genes in plants, it seems that there is not a general rule regarding which class of transcriptional factors activate which class of stress-responsive genes. Instead, there could be several kinds of transcriptional factors regulating one group of stress-responsive genes, or even several transcriptional factors that can cooperatively activate the same gene.

Many ABA-responsive genes have the ABA-responsive element (ABRE) with an ACGT core and additional coupling elements (Shen and Ho 1995) to impart ABA induction. Proteins binding to this ABA-responsive complex contain bZIP motifs. These bZIP proteins include, for example, wheat EmBP1 (Guiltinan et al. 1990), the tobacco TAF-1 (Oeda et al. 1991), rice OSBZ8 and osZIP-1a (Nantel and Quatrano 1996). Some of these transcriptional activators are themselves induced at the transcriptional level by ABA or stress treatments. For example, *OSBZ8*, which codes a bZIP protein from rice that binds to the G-box in

the ABRE-responsive element, was rapidly induced by ABA treatment. OSBZ8 is probably involved in the transcription of ABA-responsive genes in rice (Nakagawa et al. 1996). Similarly, a maize bZip protein coding gene, *mLIP15*, is induced by low temperature, salt and ABA, but not by drought (Kusano et al. 1995). However, whether mLIP15 can regulate ABA-responsive genes is not known.

The direct involvement of bZip proteins in ABA-responsive gene regulation, at least during embryo development, is further supported by the identification of *ABI5* gene product as a bZIP transcription factor (Finkelstein and Lynch 2000). ABI5 regulates the expression of some *LEA* genes and it is mainly expressed in seeds. Its expression is regulated by ABA and seems also dependent on ABI1/2 (Finkelstein and Lynch 2000). The maize VP1 protein (*Arabidopsis* ABI3 homolog, Giraudat et al. 1992) is a well-studied transcription factor that regulates seed maturation and dormancy by activating genes responsive to ABA (McCarty et al. 1991). However, VP1 does not directly interact with ABRE in the promoter of these genes. Instead, a bZip factor, TRAB1, was found to interact with VP1 and directly bind with ABRE to mediate ABA-induced transcription (Hobo et al. 1999). Besides being expressed in embryos, TRAB1 transcripts were also detected in roots and leaves. TRAB1 expression is not affected by ABA. However, ABA may regulate the interaction between VP1 and TRAB1 in the activation of ABRE-containing genes. If this is true, there should exist VP1-like factor(s) in vegetative tissues because VP1 is only expressed in embryos (Hobo et al. 1999). ABI5, TRAB1 and another ABA-responsive Dc3-promoter binding factor, DPBF-1, from sunflower (Kim et al. 1997) have over 50% homology at the amino acid level, implying a probable common mechanism for gene regulation.

Besides bZIP proteins, other transcription factors can also activate ABA-responsive genes. For example, the *Arabidopsis* ABI4 is an APETALA2 (AP2) domain-containing transcription factor (Finkelstein et al. 1998). An *Arabidopsis* drought and ABA-responsive *RD22* promoter-binding protein, RD22BP1, is a MyC-like transcription factor (Abel et al. 1997). Moreover, ATMYB2, a MyB-like protein, can also activate *RD22* expression. In fact, RD22BP1 and ATMYB2 act synergistically in inducing *RD22* transcription (Abel et al. 1997). *RD22BP1* was found to be induced by drought and salt stress (Abel et al. 1997) and *ATMYB2* was also induced by dehydration stress (Urao et al. 1993). Plants also possess a unique group of transcription factors, the homeodomain-leucine zipper (HD-Zip) proteins. The HD-Zip genes, *ATHB7* and *ATHB6* from *Arabidopsis*, are constitutively expressed, but are significantly up-regulated by water stress, osmotic stress and ABA (Söderman et al. 1996, 1999). Another homologous gene, *ATHB-12*, was found also to be induced by water stress and ABA treatment (Lee and Chun 1998). Moreover, the induction of ATHB6 was mediated by ABA and required ABI1 and ABI2 (Söderman et al. 1999). It is not yet known whether these proteins play any role in the activation of ABA-responsive genes.

In addition to ABRE, the promoters of many cold or drought-responsive genes contain another *cis*-regulatory element with the core sequence CCGAC that specifically responds to drought or cold signals. This element was termed C-repeat element (Baker et al. 1994) or drought-responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki 1994). A transcriptional activator containing AP2 domain that binds to this element, C-repeat binding element-1 CBF1 (CBF1), was isolated (Stockinger et al. 1997). The expression of *CBF1* is itself cold up-regulated and precedes the activation of other cold-regulated genes (Gilmour et al. 1998). Homologous genes are found in the *Arabidopsis* genome and they are named *CBF2*/*DREB1C* and *CBF3*/*DREB1A* (Gilmour et al. 1998, Liu et al. 1998), respectively. Additionally, two similar genes encoding DRE-binding proteins, *DREB2A* and *BREB2B*, were identified (Liu et al. 1998). The expression of *DREB2* was rapidly induced by dehydration and salt stress (Liu et al. 1998). Overexpression of *CBF*/*DREB* has been shown to induce stress-responsive gene expression and increase plant tolerance to cold and drought stresses (e.g. Jaglo-Ottosen et al. 1998, Liu et al. 1998).

Signaling partners: protein-modifiers, scaffolds and adapters

In addition to the modules that directly transduce abiotic stress signals, there are other molecules that regulate the activity of the signaling components in the above-mentioned modules; yet, they do not directly relay the signals. These signaling partners are protein modifiers (other than protein kinases/phosphatases) and scaffolds or adapters that provide various physical supports for many signaling events.

Posttranslational modifications of proteins play very important roles in the regulation of protein functions. Common protein modifications include phosphorylation, acetylation, methylation, ADP-ribosylation, glycosylation, myristoylation and isoprenylation. As discussed in the above sections, protein phosphorylation/dephosphorylation through protein kinases and phosphatases are the major forms of signaling transduction modes in any organisms. In both prokaryotes and eukaryotes, protein methylation has been shown to regulate gene transcription (e.g. Chen et al. 1999) and receptor activity (e.g. Falke et al. 1997).

Protein lipidation is a common form of protein modification. Lipidation facilitates membrane association and this process can be very important for the transmission of extracellular signal into the cell (for review, see Yalovsky et al. 1999). Common lipid modifications make use of fatty acids (myristate and palmitate) and isoprenoinds (farnesol and geranylegeranol). Isoprenylation of proteins is realized by the cysteinyl thioether bond formation between the cystein residue at the C-terminus of the protein (with the cognate CAAX sequence where A is an aliphatic residue) and the 15-C farnesyl or 20-C geranylgeranyl moiety by farnesyl transferase (FTase) or geranylgeranyl transferase. In human cells, approximately 0.5% of cellular proteins are isoprenylated and the majority of these are geranylgeranylated. In plants, it has been demonstrated that a chaperone, ANJ1, which is a homolog of bacterial DnaJ, is isoprenylated to be associated with microsomal membranes and this association is required for function at high temperature (Zhu et al. 1993). The involvement of isoprenylation in stress signal transduction is supported by the identification of the *ERA1*

gene from *Arabidopsis* that encodes the catalytic subunit of FTase. The germination of *era1* mutant seeds is more sensitive to ABA inhibition (Cutler et al. 1996). Moreover, guard cell anion-channel and stomatal closure in *era1* mutants are hypersensitive to ABA treatment (Pei et al. 1998). Thus, at least one of the substrates of ERA1 appears to be a negative regulator of ABA signal transduction. However, the in vivo substrate of ERA1 is still not known. In mammalian cells, a major group of isoprenylated proteins are the small GTPases belonging to the Ras superfamily. Ras activates several MAPK cascades that regulate a wide range of cellular activities, such as cell-cycle progression, apoptosis and stress responses. In plant genomes, there are quite a number of sequences that can serve as FTase targets. If the target of ERA1 is a Ras-like protein, then it should be upstream of ABI1 in the signal flow, assuming that the target of ABI1 is a MAPK component. However, *era1* mutation can suppress the phenotype of dominant *abi1* allele, and thus it was thought that ERA1 acts downstream of ABI1 (Pei et al. 1998). However, as discussed above, recessive *abi1* mutations actually confer sensitivity to ABA. Thus, this apparent epistasis between ERA1 and ABI1 should be treated with caution. Also, it has been shown that isoforms of phosphoinositol 5-phosphatases in animal cells are targeted to cell membranes by isoprenylation. These phosphatases can hydrolyze IP_3 to negatively regulate phosphoinositol signaling. However, plant inositol 5-phosphatase with potential isoprenylation consensus has not been found in released *Arabidopsis* sequences (L. Xiong and J.-K. Zhu, unpublished data).

Protein ubiquitination is an important modification that removes the tagged proteins for degradation in the proteasome. For example, Src family tyrosine kinases in yeast are involved in modulating various signal transduction pathways leading to the induction of DNA synthesis and cytoskeleton reorganization in response to cell-cell or cell-matrix adhesion. It was shown that Src itself is degraded in a ubiquitin-dependent manner and that the active form is specifically targeted for degradation (Harris et al. 1999b), presumably due to a change in the confirmation after phosphorylation which serves as the degradation signal. Besides ubiquitin, there are several other ubiquitin-like small proteins such as SUMO1/SMT3 and RUB that attach to specific proteins and function in nuclear localization and cell-cycle regulation, respectively. In mammalian cells, SUMO-1 modifies homeodomain-interacting protein kinase 2 (HIPK2), and this modification results in HIPK2 localization to nuclear speckles (Kim et al. 1999). In plants, the homologs of SUMO-1 proteins are also found (Hanania et al. 1999, Vierstra and Callis 1999) and regulation of signal transduction by protein degradation has been implicated in auxin signaling (del Pozo and Estelle 1998), light signaling (Clough and Vierstra 1998) and pathogenesis signaling (Boyes et al. 1998, Hanania et al. 1999). Although ubiquitin is suggested to be involved in desiccation tolerance in some desiccation-tolerant plants and accumulation of its transcripts increases during dehydration and rehydration (O'Mahony and Oliver 1999), in mesophytes, enhanced de novo synthesis of ubiquitin may be unnecessary during temperature or drought stress. Additionally, most plant ubiquitin genes are also not regulated by cold, salt or ABA. Thus, in abiotic stress signal transduction, the potential role of ubiquitination would more likely be the regulation of the lifetime of signaling molecules.

Spatial separation of signal components in a cascade by the formation of complexes with the aid of adapter proteins and scaffolds is a common way to control signaling specificity. Moreover, 'Piggybacking' of early signal components to membrane receptors by scaffolds and adapters also concentrates the signaling proteins to achieve an increase in steady signaling levels (Kholodenko et al. 2000). Thus, scaffolds and adapters play multiple roles at all steps of signal transduction ranging from signal reception to the activation of transcription. They can be an 'anchor' that tethers recruited proteins to specific subcellular locations or they may have additional biochemical functions. Some scaffolds such as Ste5 in the yeast pheromone pathway have both functions (Mahanty et al. 1999). Besides these specific proteins, cytoskeleton elements may function as a general physical frame to facilitate the aggregation of signaling complexes and the movement of vesicles and thus may also affect specific signaling processes. The role of scaffolds in signal transduction has been established in many systems. For example, in *Drosophila*, the scaffold InaD protein that has 5 PDZ domains pulls together transient receptor potential (TRP) calcium channel, PLCβ, eye PKC, the light receptor rhodopsin and calmodulin. Rhodopsin, when activated by light, in turn activates G-protein-coupled PLCβ and leads eventually to the activation of the TRP ion channels. Null *inaD* mutants cannot bind all these components and are completely defective in the signaling (Tsunoda et al. 1997). The A-kinase anchoring proteins (AKAP) are another group of well-studied scaffolds. AKAPs can simultaneously anchor protein kinase A and its phosphatase in a close vicinity to ion channels to regulate ion flux (for review, see Fraser and Scott 1999). In another example, AKAP was shown to anchor PKA, which phosphorylates BAD and binds with the 14-3-3 adapter protein to prevent apoptosis by blocking the association of BAD with pro-apoptotic factors. Disruption of this anchoring prevents BAD phosphorylation and results in mitochondrial dysfunction and apoptosis (Harada et al. 1999).

Regarding the 14-3-3 protein family, evidence suggests that one such protein, GF14, interacts with VP1 and Em1a to form a transcriptional complex (Schultz et al. 1998) and this complex, including TRAB1, may regulate *LEA* gene expression in embryos. A database search indicates that there are a number of signaling genes containing 14-3-3 binding motifs (Finnie et al. 1999). Interestingly, two *Arabidopsis* cold-inducible genes were found to encode 14-3-3 proteins and their expression is specifically regulated by low temperature, but not by ABA or drought stress (Jarillo et al. 1994), implying that they may function in low-temperature responses. The low-temperature-specific transcriptional factor, CBF1, also requires yeast adapter proteins ADA2, ADA3 and GCN5 for activation in yeast (Stockinger et al. 1997). The ADA adapters and the histone acetylase GCN5 may constitute part of a transcriptional complex (for review, see Struhl 1998) with CBF1 to initiate the transcription of cold-responsive genes. This also brings up the issue of

histone acetylation and chromatin modification in gene regulation. Basically, all the DNA sequences in eukaryotic genomes are associated with histones. However, not all genes are affected in transcription by acetylation/deacetylation of histones. It is speculated that the outcome of histone modification on transcription may be gene-specific (Struhl 1998). In plants, it seems that environmental stresses not only initiate specific signaling cascades, they may also affect the basic transcription processes. For example, the expression of histone linker genes in *Arabidopsis* and in tomato was found to be induced by drought stress and ABA (Wei and O'Connell 1996, Ascenzi and Gantt 1997). However, the significance of this stress induction is not known. Altered expression of a histone linker gene by overexpressing the sense transcript or expressing the antisense transcript did not alter the expression of several drought-responsive genes (Ascenzi and Gantt 1999).

The above examples illustrate a potentially general role of adapter proteins in mediating transcription in plants. Meanwhile, these adapters may also contribute to signaling specificity. As the ABRE and the G-box element found in many light-responsive genes share similar core sequences, it would not be surprising if ABRE-binding factors and G-box-binding factors are functionally exchangeable in activating ABA-responsive genes in vitro. Nevertheless, most of the ABA- and stress-responsive genes were not reported to be regulated by light. It is anticipated that adapter proteins may help to realize signal specificity in plants. Yet, in certain circumstances, light and ABA are in fact related to each other as it has been shown that one barley ABA-responsive gene, *HVA1*, is regulated both by ABA and by light (Sheen 1996) and that dark-treatment of light-grown plants triggered an obvious increase in the endogenous ABA level (Weatherwax et al. 1996). As such, light-regulated expression of a phytochrome-responsive gene in *Lemna gibba* was found to be mediated through changes in ABA levels (Weatherwax et al. 1998). These direct connections, however, may be exceptions rather than rules. Nevertheless, comparison of light signaling and ABA signaling may be helpful, as the former is a little better understood at the moment. The importance of adapter proteins in plant signal transduction is best illustrated by the identification of NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3*)* as an adapter protein that may facilitate the interaction between the blue-light receptor kinase NPH1 and immediate downstream signal components (Motchoulski and Liscum 1999). Mutation at the NPH3 locus simply blocks the signaling downstream of NPH1. NPH3 belongs to a small gene family and it would be of interest to investigate whether its homologs participate in stress signal transduction.

Analysis of stress signaling in plants

From the above outline of common themes in signal transduction, it can be seen that most of the research in plant environmental stress signaling is conducted by a candidate gene approach, i.e. identify candidate genes based on knowledge in heterologous systems and then characterize their expression or biochemical functions in plants under stress conditions. These studies are sometimes strengthened by altering the expression of these genes to result in phenotypic alterations in plants under various stress conditions. Gene expression profiling has also identified many potential signaling molecules that change in expression in response to stresses. The rationale that a putative signaling component probably functions in stress signaling if it is induced by stress has little experimental or theoretical support. Pharmacological studies using various agonists/antagonists against target proteins have also yielded considerable information regarding the involvement of the proteins in signal transduction; however, much of the evidence must be considered circumstantial because the specificity of the pharmacological compounds in plants is virtually unknown. Heterologous complementation in yeast mutants is also not reliable and the potential problems are often ignored. As has been demonstrated in a few cases in yeast, when one gene is knocked out in a mutant, another gene that does not function in that pathway in the wild type may fill in the position and complement the defect (Sprague 1998). Conversely, failure in heterologous complementation does not necessarily exclude the plant gene from functioning within a pathway. For example, the *SOS3* gene did not complement the yeast CNB defective mutant (Pardo et al. 1998); yet, SOS3 does have analogous function as the yeast CNB (Zhu 2000). Thus, circumstantial evidence obtained from expression and biochemical studies in many cases requires the confirmation from genetic studies. However, until now, no mutations in abiotic stress receptors, phosphoinositol module, CDPKs and MAPK module as related to abiotic signaling have been reported in plants. A limited number of genetically defined components in intracellular phosphoproteins (i.e. ABI1/2 and SOS2) are not sufficient to construct the signal network to any certainty. The transcriptional factors identified from mutations in the ABA signal transduction pathway seem mainly functioning in seed development. Clearly, genetic studies still have many gaps to bridge in our understanding of abiotic stress signaling in plants.

Conventional genetic analysis of plant environmental stress responses

Genetic analysis of cellular signal transduction requires the isolation of mutations that alter the signal flux, which may result in observable phenotypes. With the model plant *Arabidopsis*, which is particularly amenable for genetic studies, mutational analysis has been applied to many signal transduction pathways. For example, plants exhibit unique photomorphogenesis in response to light regimes, and this morphogenesis provides a reliable marker for screening mutants defective in light signal transduction. Application of genetic analysis in plant stress tolerance, however, is rather limited. This is mainly because of the scarcity of suitable phenotypes for mutant screening. Under drought, low temperature or salt stress, plants show a reduced growth rate or are killed by prolonged or severe stress conditions. However, retarded growth or stress damage by themselves are not reliable criteria for screening a mutagenized population

as many other factors also affect plant growth and responses to stresses.

One phenotype that has been very successfully exploited for mutant-screening is inhibition of seed germination by exogenous abscisic acid (or gibberellin biosynthesis inhibitors). Mutants defective in ABA biosynthesis or signal transduction have been isolated from *Arabidopsis* and other plants using this effect of ABA. Some of these mutants have provided us with valuable information regarding ABA-related stress signal transduction. However, other mutants isolated this way seem to only affect late embryogenesis and are seed-specific. Furthermore, the role of ABA in abiotic stress signaling is not so straightforward. Not all stress signaling pathways employ ABA and the intertwined relation of ABA and stress signaling is obscure.

Several groups have also used seed germination to screen salt-tolerant mutants in *Arabidopsis* [the *rs* mutants (Saleki et al. 1993) and *rss* mutants (Werner and Finkelstein 1995)]. However, the salt tolerance of the resistant mutants thus isolated was not extended to vegetative stages, implying that the mechanism of germination tolerance to salt is different from that of vegetative salt tolerance. Recently, Quesada et al. (2000) conducted a more large-scale screen for salt-tolerant mutants, *san*, and 3 new loci were defined. Like the *rs* or *rss* mutants, the salt resistance in *san* at germination also was not observed at seedling stages, and these mutants are not defective in the expression of stress-induced genes examined. It thus seems that the isolation of mutations in salt stress signal transduction pathways by salt germination tolerance is difficult.

To isolate genes that are important for vegetative tolerance to NaCl, a root-bending screen for salt sensitivity was devised (Wu et al. 1996). Identification of the *Salt-Overly-Sensitive* genes revealed a fine-tuned SOS module that controls potassium and sodium homeostasis and, therefore, sodium tolerance (reviewed by Zhu 2000). Due to the constraints imposed by the screening, it seems that the *sos* mutants are primarily affected in ion homeostasis. A screen for salt-tolerant growth at seedling stage was conducted by Tsugane et al. (1999). Two *Arabidopsis pst* mutants were isolated and it was shown that salt-tolerance in the *pst1* mutant was attributable to enhanced scavenging of ROS. However, it is not known if this salt-tolerant trait extends to adult plants.

Under drought stress, it was reported that plant roots undergo drought rhizogenesis. Water stress caused root hairs to become short and bulbous, and ABA treatment was shown to mimic the phenotypes of water stress on root hairs (Schnall and Quatrano 1992). It seems that this rhizogenesis may be drought/ABA-specific. However, there have been no reports using this phenotype to screen drought-related mutants.

Plant chilling and freezing tolerances are different but related traits. *Arabidopsis* is chilling-tolerant, but its tolerance to freezing is moderate, and this tolerance can be enhanced by cold acclimation. Under prolonged chilling stress, wild-type *Arabidopsis* plants do not show visible damage. Alterations in cold acclimation, low-temperature sensitivity and freezing tolerance can be used for screening mutations by scoring visible damages as well as freezing-induced electrolyte leakage. Several *Arabidopsis* mutants sensitive or tolerant to cold/freezing stress were isolated this way (Warren et al. 1996, Xin and Browse 1998). Due to the nature of these screens, the relevance of these mutations to stress signal transduction is uncertain as there are many factors (e.g. sugars, soluble carbohydrates, proline and ROS) contributing to freezing tolerance and any changes in the biochemical pathways leading to alterations in the synthesis and turnover of these compounds may result in altered chilling or freezing sensitivity (e.g. Xin and Browse 1998, Araki et al. 2000). An additional fact that dictates the inefficiency of visible phenotypic screens for abiotic stress signaling is that there is extensive connectedness in the signal pathways (Ishitani et al. 1997). Therefore, the effect of mutations in a single component in the pathway may well be compromised by signaling redundancy or connectedness. To overcome these limitations of visible phenotypic screens, alternative approaches are required. Accordingly, molecular genetic approaches are increasingly being applied for dissecting abiotic stress signaling networks in plants.

Molecular genetic analysis of stress signal transduction

The use of transgene as a reporter in genetic analysis of signal transduction has many advantages especially in cases where visible phenotypes fall short. A chimeric gene consisting of the promoter of a stress-responsive gene and a convenient reporter gene is introduced into plants and the transgenics are used as starting material for mutagenesis. In the M_2 generation, individual mutants with de-regulated reporter gene responses to stress treatments are isolated. These mutations likely define components in the signal transduction pathways under study. By using a series of marker genes (promoters) in different positions of the signal network, one may be able to dissect the whole network genetically.

Reporters that are in common use are the bacterial β-glucuronidase (GUS), firefly luciferase (LUC) and green fluorescent protein (GFP). GFP may not be sensitive enough to find wide applications in reporting promoter activities in genetic analysis. The GUS reporter has the advantage that its detection does not need special equipment. GUS reporter has been used for screening signal transduction mutations [e.g. pathogenesis signaling (Bowling et al. 1994) and light signal transduction (Jackson et al. 1995)]. Attempts to use *GUS* as a reporter driven by the Dc3 promoter to screen *Arabidopsis* ABA-responsive mutants were also reported (Ng et al. 1998). However, the limitations of GUS as a reporter including a requirement for tissue-staining, interference from stress-induced anthocyanin, the high stability of the GUS protein and, last but not the least, laborious work in large-scale screens prevent its wide application in high-throughput isolation of signal transduction mutants.

Compared with the GUS reporter, LUC has many advantages. Imaging analysis of LUC can be done in vivo without damaging the plant sample and hundreds of seedlings can be analyzed simultaneously. The half-life of luciferase is long enough for accurate assay of its activity, while short enough

to allow repeated stress treatments of the same sample over a short period of time (Xiong et al. 1999a). The activity of luciferase can be conveniently monitored by using a cooled charge-coupled device (CCD) camera. Technical advances and the wide application of luciferase in biology have now made a CCD imaging device affordable for most laboratories. LUC reporter has been used in the study of light-regulated gene expression in plants (Millar et al. 1992).

To study environmental stress signal transduction, we have used LUC as a reporter in a genetic screen to recover mutations that affect the perception and transduction of low temperature, drought, salinity and abscisic acid signals. The promoter chosen to drive the LUC reporter is that of the *RD29A* gene (also known as *lti78* or *COR78*). The *RD29A* gene is strongly induced by cold, drought and salinity, as well as ABA. The promoter of this gene contains both the ABRE and drought-responsive element (DRE/C-repeat) (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994). It is thus likely that the *RD29A-LUC* transgene can reflect both ABA-dependent and ABA-independent stress signaling events. Mutagenesis of the transgenic plant seeds containing the chimeric gene was initially conducted with EMS and mutants with altered *RD29A-LUC* responses to stress and ABA treatments were screened (Ishitani et al. 1997, Xiong et al. 1999a). At a moderate scale of screening, hundreds of mutants were readily isolated. Compared with wild-type plants, these mutants exhibit either a constitutive (*cos*), high (*hos*) or low (*los*) level of expression of the *RD29A-LUC* gene in response to various stress and ABA treatments. The occurrence of mutations with differential effects on various combinations of stress and ABA responses reveals an outline of the signal transduction network. The genetic evidence obtained with the recovery of these unique mutations suggests that there are extensive connections among cold, drought, salinity and ABA signal transduction pathways (Ishitani et al. 1997). Map-based cloning of these mutations is expected to uncover important components in stress signal networks.

Perspectives on molecular genetic analysis of abiotic stress signaling

With a given reporter, the usefulness of transgenes in dissecting signal transduction is dependent to a large extent on the characteristics of the promoter used. To obtain mutations both with enhanced expression as well as reduced expression, a strong inducible promoter is required. Moreover, there are several factors one should be aware of when using this approach. Firstly, a mutant with strong transgene phenotypes may not have strong physiological phenotypes. As noted above, the connectivity between branches of signal networks may well attenuate the expected outcome of a particular mutation. For example, whereas the mutant *hos1* has both enhanced cold-regulated gene expression and strong defects in freezing tolerance (Ishitani et al. 1998), the *hos5* mutation that resulted in a more than 10-fold enhanced expression of *RD29A-LUC* reporter gene in response to osmotic stress and ABA treatment, however, showed rather subtle alterations in plant sensitivity to osmotic stresses and ABA (Xiong et al. 1999b). A similar observation was also reported by Foster and Chua (1999). Furthermore, the expression level of the corresponding endogenous gene is often found to be lower than that of the reporter gene. This phenomenon may result from a tight regulation of the endogenous gene in vivo and the regulatory information may be not fully encoded in the promoter fragment used.

Once the mutants are isolated, the next step is to clone the mutations and this is usually done by map-based cloning. Genetic mapping of the mutations with LUC imaging has proven to be fairly straightforward. Seedlings with mutant transgene phenotypes in the F_2 segregating population can be correctly identified even if the other ecotype used for crossing does not have the transgene. Although the *Arabidopsis* genome is being fully sequenced, the identification of mutations in some chromosome regions may not be very easy, since many genes can be considered as candidate genes for signal transduction or, they may encode novel proteins that are not described in any organisms. To facilitate the mutation cloning, we have also mutagenized the *RD29A-LUC* plants with T-DNA.

The availability of the *Arabidopsis* genome sequence not only will greatly facilitate the isolation of mutations identified by the above molecular genetic screen, it will also offer many other opportunities for plant biology in large and abiotic stress signal transduction in particular. Genome-wide expression profiling using cDNA microarray is an efficient way to survey genes whose expression is regulated by stresses. cDNA microarraying of plant mutants may also identify the role of particular regulatory components in the regulation of all or subsets of downstream genes. Moreover, a reverse-genetics approach can be used for dissecting stress signal transduction networks. This alternative candidate gene approach may provide immediate access to the function of a particular gene with the isolation of knockout mutants from the *Arabidopsis* Knockout Facility (www.biotech.wisc.edu/Arabidopsis/) or by using novel gene-silencing techniques. Heterozygous T-DNA knockout mutants also offer the opportunity to study genes whose mutations are lethal. Nevertheless, identification of T-DNA-inserted mutations in small genes such as ubiquitin-like tags and adapters is more difficult. In this regard, genome-wide protein interaction studies will help to identify all the interactions among signaling components including these small molecules. The global protein interaction analysis eventually will be instrumental for 'constructing' the signal networks 'dissected' with the above genetic analysis. Genome-wide interaction surveys have been carried out with the yeast genome (Ito et al. 2000, Uetz et al. 2000) and the data (http://portal.curagen.com) are available to the public. Similar studies in *Arabidopsis* are expected to yield exciting new insights into abiotic stress signaling in the years to come.

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