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Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants

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Abstract

The perception of abiotic stresses and signal transduction to switch on adaptive responses are critical steps in determining the survival and reproduction of plants exposed to adverse environments. Plants have stress-specific adaptive responses as well as responses which protect the plants from more than one environmental stress. There are multiple stress perception and signalling pathways, some of which are specific, but others may cross-talk at various steps. Recently, progress has been made in identifying components of signalling pathways involved in salt, drought and cold stresses. Genetic analysis has defined the Salt-Overly-Sensitive (SOS) pathway, in which a salt stress-induced calcium signal is probably sensed by the calcium-binding protein SOS3 which then activates the protein kinase SOS2. The SOS3–SOS2 kinase complex regulates the expression and activity of ion transporters such as SOS1 to re-establish cellular ionic homeostasis under salinity. The ICE1 (Inducer of CBF Expression 1)–CBF (C-Repeat Binding Protein) pathway is critical for the regulation of the cold-responsive transcriptome and acquired freezing tolerance, although at present the signalling events that activate the ICE1 transcription factor during cold stress are not known. Both ABA-dependent and -independent signalling pathways appear to be involved in osmotic stress tolerance. Components of mitogen-activated protein kinase (MAPK) cascades may act as converging points of multiple abiotic as well as biotic stress signalling pathways. Forward and reverse genetic analysis in combination with expression profiling will continue to uncover many signalling components, and biochemical characterization of the signalling complexes will be required to determine specificity and cross-talk in abiotic stress signalling pathways.

Key words: Abiotic stress, adaptive response, expression profiling, genetic analysis, signal transduction, stress perception.

Introduction

Plants experience various environmental stresses including too little water (drought), too much salt (salinity), and extremes of temperature. Tolerance or susceptibility to these abiotic stresses is a very complex phenomenon, in part, because stress may occur at multiple stages of plant development and often more than one stress simultaneously affects the plant. Perception of stress cues and relay of the signals to switch on adaptive responses are the key steps leading to plant stress tolerance. As a result, differences in stress tolerance between genotypes or different developmental stages of a single genotype may arise from differences in signal perception and transduction mechanisms. Because stress sensors are not known and most of the signalling intermediates have not been identified, there is little definitive information regarding cross-talk between different stress signal transduction pathways in plants. The term 'cross-talk' is used here loosely to refer to situations where different signalling pathways share one or more intermediates/components or have some common outputs. Strictly speaking though, signalling pathways sharing common components may not necessarily cross-talk if the common components are scaffolded into distinct protein complexes (Park *et al*., 2003).

Various abiotic stresses result in both general and specific effects on plant growth and development. For

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example, drought limits plant growth due to photosynthetic decline, osmotic stress-imposed constraints on plant processes and interference with nutrient availability as the soil dries. Salinity interferes with plant growth as it leads to physiological drought and ion toxicity (Zhu, 2002). Chilling (temperatures below optimal but above freezing) and freezing temperatures can also cause osmotic stress in addition to their direct effect on metabolism (Thomashow, 1999). Therefore, osmotic stress and the associated oxidative stress appear to be common consequences of exposure to drought, salinity and low temperature. Prevention of osmotic stress caused by drought depends upon minimizing stomatal and cuticular water loss and maximizing water uptake (through root growth and osmotic adjustment). During salt stress, osmotic adjustment appears to play a major role in maintaining osmotic homeostasis, while survival during freezing-induced osmotic stress may depend upon prevention or delay of ice nuclei formation. As part of plant stress responses, regulation of gene expression also involves both universal and unique changes in transcript levels of certain plant genes (Shinozaki and Yamaguchi-Shinozaki, 2000). Based on the presence of these general and specific abiotic stress tolerance mechanisms, it is logical to expect plants to have multiple stress perception and signal transduction pathways, which may cross-talk at various steps in the pathways.

In this review, several abiotic stress signalling pathways and common signalling molecules that are important in stress responses are described. Specificity and potential points of cross-talk are discussed when evidence exists.

Sensory kinases

Specificity in signalling is easy to envision if each stress signal has a sensor that can specifically transduce the signal to cellular targets. At present, few stress sensors have been identified and there is not enough information to assess whether cross-talk occurs at the level of sensors. Two-component systems, consisting of a sensory histidine kinase and a response regulator, function as stress sensors in bacteria and yeast. For example, the transmembrane two-component histidine kinases, HIK33 (cyanobacterium) and DesK (*Bacillus subtilis*) have been suggested as thermosensors (Suzuki *et al*., 2000; Aguilar *et al*., 2001). In yeast, an osmosensory histidine kinase, SLN1, senses osmotic stress and activates the HOG1 (high-osmolarity glycerol response 1) mitogen-activated protein kinase (MAPK) cascade (Maeda *et al*., 1994). Based on the stress-inducibility of its transcript and its ability to complement *sln1-ts*, a yeast mutant defective in osmosensing, the *Arabidopsis thaliana* histidine kinase, ATHK1, is thought to be a candidate osmosensor (Urao *et al*., 1999). *ATHK1* gene expression is up-regulated during salt (250 mM NaCl) and low temperature (4 °C) stresses. When expressed in yeast, *ATHK1* confers a high degree of osmolarity tolerance to the double mutant *sln1*Δ *sho1*Δ that lacks both yeast osmosensors, demonstrating that ATHK1 can activate the HOG1 MAPK pathway in yeast (Urao *et al*., 1999).

Some receptor-like protein kinases have been implicated in abiotic stress responses. Transgenic analysis has shown that wound, salt and osmotic stresses induce C7 (*NtC7*) in tobacco and that this putative membrane-localized receptor-like protein may play an important role in osmotic stress tolerance (Tamura *et al*., 2003). Determination of the *in vivo* role of higher plant putative sensory kinases and the identification of signalling intermediates and targets will ultimately be required to determine whether sensor kinase signalling is specific or involved in cross-talk between stress signalling pathways.

Calcium—a ubiquitous messenger

In plant cells, calcium (Ca^{2+}) serves as a second messenger during abiotic stress signalling (Sanders *et al*., 1999; Knight, 2000). Calcium is a major point of signalling cross-talk because it can be elicited by numerous abiotic as well as developmental, hormonal and biotic stress cues. Specific Ca^{2+} signatures (e.g. oscillations) have been implicated in numerous physiological processes, such as stomatal regulation where oscillations in cytosolic Ca^{2+} led to stomatal closure. Further evidence for the importance of Ca^{2+} in the regulation of stomatal aperture came from studies in which closure was prevented by steady and sustained or non-oscillating cytosolic Ca^{2+} signals (Allen *et al*., 2000). The Ca^{2+} signature changes depending on the particular stress (Kiegle *et al*., 2000), the rate of stress development (Plieth *et al*., 1999), previous exposure to stress conditions (Knight *et al*., 1997), and the tissue type (Kiegle *et al*., 2000). The mechanisms giving rise to the changes in cytosolic Ca^{2+} levels are just beginning to be identified. Recent studies have implicated Ca^{2+} channels in the regulation of cytosolic Ca^{2+}. For example, pharmacological studies have shown that membrane fluidity and reorganization of the cytoskeleton are essential for cold-induced cytosolic Ca^{2+} oscillations in alfalfa and *Brassica napus*, respectively (Orvar *et al*., 2000; Sangwan *et al*., 2001) suggesting the involvement of stretch/mechanosensitive Ca^{2+} channels. Pharmacological studies have also shown that cADPR-gated Ca^{2+} channels are involved in abscisic acid (ABA)-induced expression of cold-regulated genes in tomato (Wu *et al*., 1997) and *B. napus* (Sangwan *et al*., 2001). Inositol 1,4,5-*tris* phosphate (IP_3)-gated Ca^{2+} channels have been implicated in dehydration- and salt stress-induced cytosolic Ca^{2+} elevations (DeWald *et al*., 2001; Takahashi *et al*., 2001). Genetic analysis of the *FRY1* locus of *Arabidopsis* suggested the involvement of IP_3 (which in turn is expected to generate cytosolic Ca^{2+} oscillations) in ABA, salt and cold stress signalling. *FRY1*

encodes an inositol polyphosphate 1-phosphatase, which catabolizes IP_3. An ABA-induced IP_3 transient is altered in the *fry1* mutant, which leads to a more sustained accumulation of IP_3 and a plant that is hypersensitive to ABA, cold and salt stresses (Xiong *et al*., 2001*b*).

Although involvement of a heterotrimeric GTP-binding (G)- protein has been demonstrated in ABA signal transduction during guard cell regulation (Wang *et al*., 2001), the role of G-protein-associated receptors or classical stress sensors in eliciting Ca^{2+} signatures during abiotic stress has yet to be shown in plants. Specificity and/or cross-talk in Ca^{2+} signalling may depend upon the magnitude, duration and subcellular localization of the Ca^{2+} oscillation as well as Ca^{2+} sensor diversity. Experimentally induced increases in cytoplasmic Ca^{2+} (via microinjection, the use of Ca^{2+} ionophores or Ca^{2+} channel agonists/antagonists) have been shown to mediate hormone/stress responses (Sheen, 1996; Wu *et al*., 1997; Orvar *et al*., 2000; Sangwan *et al*., 2001). Because these artificial changes in Ca^{2+} may not exactly mimic stress-induced Ca^{2+} oscillations, but, nevertheless, were sufficient to regulate stress-specific responses, specificity in signalling might arise downstream of Ca^{2+}, for example, from the presence or absence of a specific Ca^{2+} responsive signalling complex.

The SOS pathway regulates ion homeostasis

Regulation of cellular ion homeostasis during salinity stress is critical for plant salt tolerance. One of the responses of plant cells to salt stress is the generation of a cytosolic Ca^{2+} transient (Knight *et al*., 1997) and the subsequent activation of Ca^{2+} sensor protein expression and/or activity. Until recently, little was known about the *in vivo* targets and downstream outputs of salt stress signalling pathways; however, the identification of the Salt-Overly-Sensitive (SOS) pathway in *Arabidopsis* has begun to reveal components and mechanisms involved in the plant's response to ionic stress. Molecular analysis of *sos* mutants of *Arabidopsis* led to the identification of components (SOS1, SOS2 and SOS3) of a pathway that transduce a salt stress-induced Ca^{2+} signal to reinstate cellular ion homeostasis (Fig. 1; Zhu, 2002).

SOS3 is a Ca^{2+} sensor essential for transducing the salt stress-induced Ca^{2+} signal and for salt tolerance in *Arabidopsis*. *SOS3* encodes a Ca^{2+} binding protein with an N-myristoylation motif and three Ca^{2+}-binding EF hands. The amino acid sequence of SOS3 shows significant similarity to the regulatory subunit of yeast calcineurin and animal neuronal Ca^{2+} sensors (Liu and Zhu, 1998; Ishitani *et al*., 2000). A loss-of-function mutation that reduces the Ca^{2+} binding capacity of SOS3 (*sos3-1*) renders the mutant hypersensitive to salt (Ishitani *et al*., 2000); a defect that can be partially rescued by high levels of Ca^{2+} in the growth medium (Liu and Zhu, 1998). SOS3 binds Ca^{2+} with low affinity when compared to other Ca^{2+}-binding proteins like caltractin and calmodulin (Ishitani *et al*., 2000). The differences in the affinity of these Ca^{2+} sensors may ultimately be useful in distinguishing variations in Ca^{2+} signals.

The search for salt-tolerant determinants also led to the identification of the *SOS2* locus in *Arabidopsis*; the *sos2* mutant is also hypersensitive to NaCl stress. *SOS2* encodes a serine/threonine protein kinase with an N-terminal kinase catalytic domain similar to SNF1/AMPK, and a unique C-terminal regulatory domain (Liu *et al*., 2000). Under normal cellular conditions, the catalytic and regulatory domains interact with each other, probably preventing substrate phosphorylation by blocking substrate access. In the presence of Ca^{2+}, SOS3 activates the SOS2 kinase (Halfter *et al*., 2000). A FISL-motif in the regulatory domain of SOS2 is necessary and sufficient for interaction with SOS3 and deletion of this FISL-motif constitutively activates the SOS2 kinase (Guo *et al*., 2001). Replacement of Thr^{168} in the kinase domain by Asp also led to the production of a constitutively active SOS2 kinase. Overexpression of active forms of SOS2 under the control of the CaMV 35S promoter rescued the salt-sensitive phenotype of both *sos2* and *sos3*, supporting the idea that SOS3 and SOS2 are functioning in the same Ca^{2+}-signalling pathway during salt stress (Zhu, 2002).

The first target of the SOS3–SOS2 pathway was identified by molecular genetic analysis of the *sos1* mutant of *Arabidopsis*. As with *sos2* and *sos3*, *sos1* is hypersensitive to salt and all three mutants accumulate higher levels of Na^+ than is found in wild-type plants. Genetic analysis confirmed that *SOS3*, *SOS2*, and *SOS1* function in a common pathway of salt tolerance (Zhu *et al*., 1998). *SOS1* encodes a plasma membrane Na^+/H^+ exchanger (antiporter) with a very long predicted cytoplasmic tail (Shi *et al*., 2000). The SOS3–SOS2 kinase complex appears to control both the expression and activity of *SOS1* (Fig. 1). During salt stress, up-regulation of *SOS1* transcript levels is diminished in the *sos3* and *sos2* mutants (Shi *et al*., 2000). The Na^+/H^+ antiport activity of SOS1 was demonstrated in comparisons of activity in plasma membrane vesicles isolated from wild-type and *sos1* plants; where activity in *sos1* was significantly lower than that in the wild type. A demonstration that SOS1 is a target of the SOS2–SOS3 regulatory pathway and that the antiport activity of SOS1 is regulated by the SOS2 kinase came from experiments in which plasma membrane Na^+/H^+ antiport activity was compared in vesicles isolated from *sos2* and *sos3* plants; activity was reduced in both mutants compared to that in wild-type plants and activity in these two mutants could be restored when activated SOS2 protein kinase was added *in vitro* (Qiu *et al*., 2002). The SOS3–SOS2 kinase complex was found to phosphorylate SOS1 directly (Quintero *et al*., 2002). The myristoylation of SOS3 has been shown to be critical for salt tolerance; a mutation in

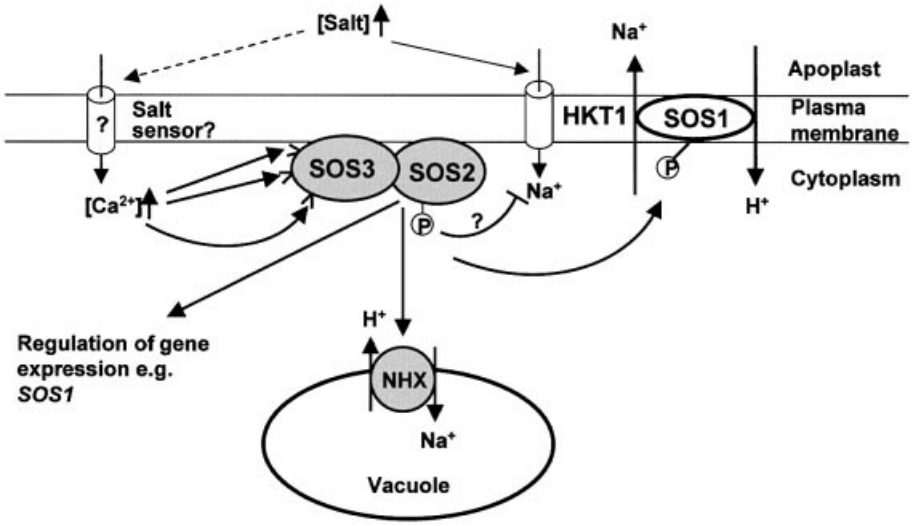

Fig. 1. Regulation of ion homeostasis by the SOS pathway during salt stress. Salt stress-induced Ca^{2+} signals are perceived by SOS3 which activates the SOS2 kinase. The SOS3–SOS2 kinase complex regulates cellular Na^+ levels by stimulating Na^+ transport out of the cytoplasm (e.g. by increasing the expression and activity of *SOS1*) and conceivably by restricting Na^+ entry into the cytosol (e.g. inhibiting HKT1 activity). An additional target of the SOS2 kinase, NHX (vacuolar Na^+/H^+ exchanger), also contributes to Na^+ ion homeostasis by transporting Na^+ from the cytoplasm into the vacuole.

SOS3 that disrupts myristoylation (G2A) causes salt hypersensitivity (Ishitani *et al*., 2000). One interpretation of these data is that the constitutive myristoylation of SOS3 allows it to recruit SOS2 to the plasma membrane, helping to bring SOS2 to its target SOS1 (Quintero *et al*., 2002). Recent studies in which the SOS pathway has been functionally reconstituted in *Saccharomyces cerevisiae* further demonstrate that a salt stress-induced Ca^{2+} signal is transduced by the SOS3–SOS2 kinase complex to activate SOS1 and to re-establish cellular ion homeostasis (Quintero *et al*., 2002). Together these experiments suggest that Na^+ efflux from root cells experiencing salinity stress may be achieved by the activation of SOS1 by the SOS3–SOS2 complex and that SOS1 probably also retrieves Na^+ from the xylem, thereby preventing excess Na^+ accumulation in the shoot (Shi *et al*., 2002, 2003).

Additional targets of the SOS regulatory pathway are emerging. For example, in *Arabidopsis*, Na^+ entry into root cells during salt stress appears to be mediated by AtHKT1, a low affinity Na^+ transporter (Uozumi *et al*., 2000). The *athkt1* mutation suppresses the *sos3* mutation (Rus *et al*., 2001), suggesting that the SOS3–SOS2 kinase complex may prevent Na^+ influx by inactivating the HKT1 protein or down-regulating *HKT1* gene expression during salt stress (Zhu, 2002). In addition, the activity of vacuolar Na^+/H^+ antiporters may also be activated by SOS3–SOS2 (Fig. 1) (Qiu *et al*., 2003). Available evidence suggests that the SOS pathway is specific for ion homeostasis responses under salt stress (Fig. 1; Zhu, 2002). Future investigations will determine whether it controls other plant processes and cross-talk with other pathways.

SOS3-like calcium-binding proteins (SCaBPs)

The *Arabidopsis* genome encodes at least eight SOS3-like Ca^{2+}-binding proteins (SCaBPs) and 23 SOS2-like protein kinases (PKSs, Guo *et al*., 2001). Since Ca^{2+} acts as a second messenger in a number of signalling pathways including those initiated by abiotic stresses and hormones, one interesting question is whether the SOS3- and SOS2-like proteins are components of these signalling pathways. Genetic studies to 'knock-down' SCaBP and PKS levels using RNA interference (RNAi) have revealed that *scabp5* and *pks3* mutants are impaired in their response to ABA and that the SCaBP5–PKS3 complex specifically senses and transduces ABA-specific Ca^{2+} signals (Guo *et al*., 2002). In these mutants, germination, seedling growth, stomatal regulation, and gene expression were hypersensitive to ABA. ABA-induced expression levels of the cold and drought-responsive genes *COR47*, *COR15A* and *RD29A* were substantially higher in the *scabp5* and *pks3* mutants when compared with their expression in the wild type, and these mutants expressed higher levels of *COR47* and *COR15A* even without exogenous ABA. In the *scabp5pks3* double mutant, ABA sensitivity was not additive, supporting a role for these two proteins in the same ABA signalling pathway. *SCaBP5* and *PKS3* are specific to ABA-induced Ca^{2+} signalling, as *scabp5* and *pks3* were defective in their response to ABA but not in their response to salt and cold stresses or to other plant hormones including auxin, cytokinin, brassinolides, and gibberellins (Guo *et al*., 2002). As was found for SOS2, PKS3 also contains an autoinhibitory FISL motif, deletion of which (PKS3ΔF) constitutively activated PKS3; trans-

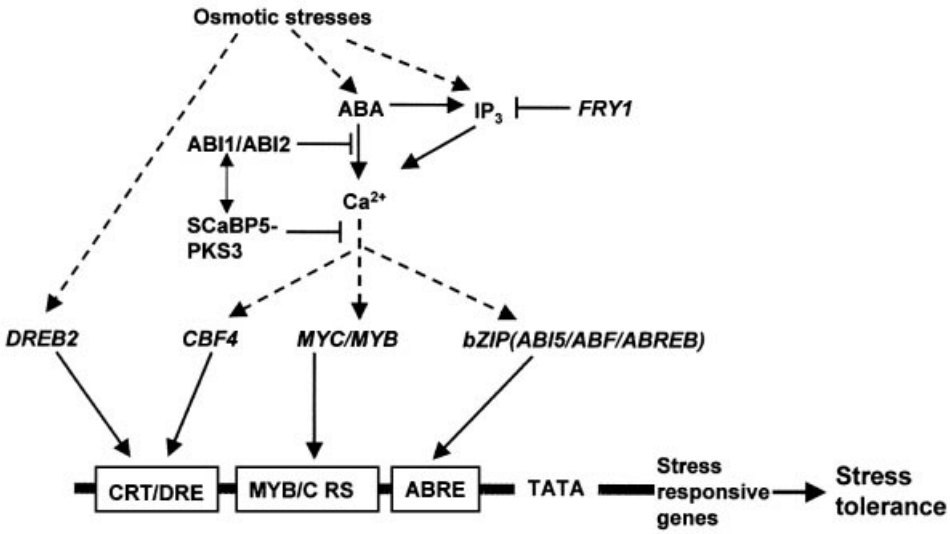

Fig. 2. Transcriptional regulation of stress-responsive genes in response to osmotic stress. Osmotic stress-induced DREB2 transcription factors induce ABA-independent transcription of stress responsive genes. ABA-dependent pathways regulate stress-responsive gene expression through CBF4, MYC/MYB and bZIP-type transcription factors, which bind to the CRT/DRE, MYB/C Recognition Sequences (MYB/C RS) and ABA Responsive Elements (ABRE) promoter elements, respectively. ABA-dependent abiotic stress signalling is mediated in part through IP_3 and Ca^{2+}. FRY1 negatively regulates IP_3 levels. ABA-induced Ca^{2+} signalling is negatively regulated by the ABI1/2 protein phosphatase 2Cs and the SCaBP5–PKS3 complex.

genic *Arabidopsis* ectopically expressing PKS3ΔF showed ABA insensitivity. In yeast two-hybrid assays, SCaBP5 physically interacts with PKS3 which, in turn, strongly interacts with the 2C-type protein phosphatase ABA-insensitive 2 (ABI2) and to a lesser extent with the homologous ABA-insensitive 1 (ABI1) protein. Genetic studies have shown that *abi1-1* and *abi2-1* are suppressors of the *scabp5* and *pks3* mutations. The current model is that SCaBP5–PKS3 and ABI2 form part of a Ca^{2+}-responsive negative regulatory loop controlling ABA sensitivity (Fig. 2; Guo *et al*., 2002).

Calcium-dependent protein kinases (CDPKs)

Besides the PKS family of protein kinases that are responsive to Ca^{2+} via the SCaBPs, plants also contain a large family of Ca^{2+}-dependent protein kinases (CDPKs). CDPKs have been implicated in signalling pathways in response to stresses such as drought, wounding and cold. Transgenic analyses, studies of stress-responsive gene expression and characterization of CDPK activation suggest that some stress-induced Ca^{2+} signals are perceived and transduced by CDPKs (Table 1). The involvement of CDPKs in stress-induced gene transcription was demonstrated using a maize leaf protoplast transient expression system (Sheen, 1996). In these studies, a constitutively active form of an *Arabidopsis* CDPK (AtCDPK1) activated the expression of a barley ABA-responsive promoter fusion (*HVA1::LUC*; Sheen, 1996). Ectopic expression of CDPK induced the expression of a rice stress-responsive gene, *RAB16* (Saijo *et al*., 2000) and *HVA1* in the maize protoplasts (Sheen, 1996). *RAB16* and *HVA1* have a G-box type ABA-responsive *cis* element (ABRE), which can be activated by a bZIP transcription factor (Leung and Giraudat, 1998). Overexpression of the catalytic domain of *ABI1* inhibited ABA- and AtCDPK1-induced *HVA1* transcription (Sheen, 1996). These results suggest that the up-regulation of *RAB16* and *HVA1* may be mediated by CDPKs, which are under the negative control of ABI1. In the common ice plant *Mesembryanthemum crystallinum*, CDPK substrate protein 1 (CSP1) has been identified as a substrate protein for McCDPK1. McCDPK1 phosphorylates CSP1 *in vitro* in a Ca^{2+}-dependent manner and salt stress induces co-localization of McCDPK1 and CSP1 in the nucleus of ice plants (Patharkar and Cushman, 2000). While many more CDPKs exist in the plant genome, little is known about the targets or specificity of most of these kinases.

Diverse signals converge at MAPK cascades

Perhaps some of the strongest evidence for cross-talk during abiotic stress signalling in plants comes from studies of MAPK cascades. The *Arabidopsis* genome encodes approximately 60 MAPKKKs, 10 MAPKKs and 20 MAPKs. Signals perceived by the 60 MAPKKKs have to be transduced through 10 MAPKKs to 20 MAPKs which offer scope for cross-talk between different stress signals. MAPKs are implicated in developmental, hormonal, biotic, and abiotic stress signalling (Ligterink and Hirt,

Table 1. *CDPKs implicated in abiotic stress signalling*

Plant	Name of the CDPK	CDPK expression	Remarks	Reference
Arabidopsis	AtCDPK1 AtCDPK2	Induced by salinity and drought but not by low/high temperatures		Urao *et al*., 1994
Arabidopsis	AtCDPK1	ABA-inducible	HVA1 promoter was activated by AtCDPK1	Sheen, 1996
Rice	OsCDPK7	Gene expression induced by cold and salt stresses but not ABA. Protein levels did not change	Rice transgenics over-expressing OsCDPK7 were more tolerant to salt and drought stress. More RAB16 expression	Saijo *et al*., 2000
Common ice plant	McCDPK1	Salt stress induction	Salt stress induced co-localization of McCDPK1 and CSP1 in the nucleus	Patharkar and Cushman, 2000

Table 2. *Activation of MAPK cascades by diverse stresses*

Plant	Component of MAPK cascade	Remarks	Reference
MAPKs			
Alfalfa	SIMK (salt-induced MAPK)	Activated by salt stress and pathogen-derived elicitors	Munnik *et al*., 1999 Baluska *et al*., 2000 Cardinale *et al*., 2000
Tobacco	SIPK (salicylic acid induced MAPK)	Activated by salicylic acid and salt stress; activation of SIPK is Ca^{2+}- and ABA-independent	Hoyos and Zhang, 2000; Mikolajczyk *et al*., 2000
Arabidopsis	ATMPK3	Salt stress	Mizoguchi *et al*., 1996
	ATMPK4 and ATMPK6	Salt stress, low humidity, cold and wounding	Ichimura *et al*., 2000
	ATMPK6	ROS, cold	Yuasa *et al*., 2001
	ATMPK4	Negative regulator of SAR	Peterson *et al*., 2000
Rice	OsMAPK5	ABA, biotic and abiotic stresses (wounding, drought, salt, and cold) induce gene expression and kinase activity; overexpression in transgenic plants increased tolerance, while suppression led to hypersensitivity to drought, salt, and cold stresses but increased resistance to biotic stress	Xiong and Yang, 2003
MAPKKs			
Alfalfa	SIMKK	Salt stress and pathogen elicitor- induced activation of SIMK by SIMKK, which does not require an upstream MAPKKK	Kiegerl *et al*., 2000; Cardinale *et al*., 2002
Arabidopsis	ATMKK2 and MEK1	MEK1 complemented growth defects of the yeast *bck1* mutant during salt stress	Ichimura *et al*., 1998
MAPKKKs			
Arabidopsis	ATMEKK1	Salt stress activates expression and activity; yeast complementation studies suggest that ATMEKK1, ATMKK2/MEK1, and ATMPK4 may form a MAPK cascade	Ichimura *et al*., 1998
Arabidopsis	ANP1	Activated by H_2O_2; targets are AtMPK3 and AtMPK6	Kovtun *et al*., 2000
Tobacco	NPK1 (ANP1 orthologue)	Transgenic overexpression increases tolerance to salt and other stresses, and activates the expression of *GST6* and *HSP* but not *RD29A*	Kovtun *et al*., 2000

2000). Members of MAPK cascades are activated by more than one types of stress, which suggests that MAPK cascades act as points of convergence in stress signalling (Table 2). Two-hybrid analysis and the use of *Arabidopsis* kinases for complementation studies in yeast led to the identification of a putative *Arabidopsis* MAPK cascade consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4 (Ichimura *et al*., 1998). Salt stress induces the expression and activity of AtMEKK1 (Ichimura *et al*., 2000), which activates AtMPK4 *in vitro* (Huang *et al*., 2000). AtMPK4 is activated by cold, low humidity, osmotic stress, touch, and wounding (Ichimura *et al*., 2000). The *Arabidopsis* mutant *atmpk4* is insensitive to jasmonic acid, accumulates high levels of salicylic acid and shows constitutive Systemic Acquired Resistance (SAR, Peterson *et al*., 2000). The identification of a MAPK phosphatase 1 mutant (*mkp1)* in *Arabidopsis* further demonstrated the complexity of the AtMPK4 signalling cascade. *mkp1* is resistant to salinity but hypersensitive to genotoxic stress induced by UV-C. A yeast two-hybrid screen showed that MKP1 could interact with AtMPK4 (Ulm *et al*., 2002). Although the inputs and outputs of the AtMPK4 pathway have yet to be defined, microarray analysis of *mkp1* indicated that the transcript level of a putative Na^+/H^+-antiporter (*AT4G23700*) is negatively regulated by AtMKP1 (Ulm *et al*., 2002). Reactive oxygen species have been shown to activate a MAPK cascade in *Arabidopsis* that appears to include ANP1 (a MAPKKK), AtMPK3 and AtMPK6 and its positive regulator, Nucleoside Diphosphate Kinase 2 (Kovtun *et al*., 2000; Moon *et al*., 2003). Transient expression studies in *Arabidopsis* mesophyll protoplasts showed that MPK3

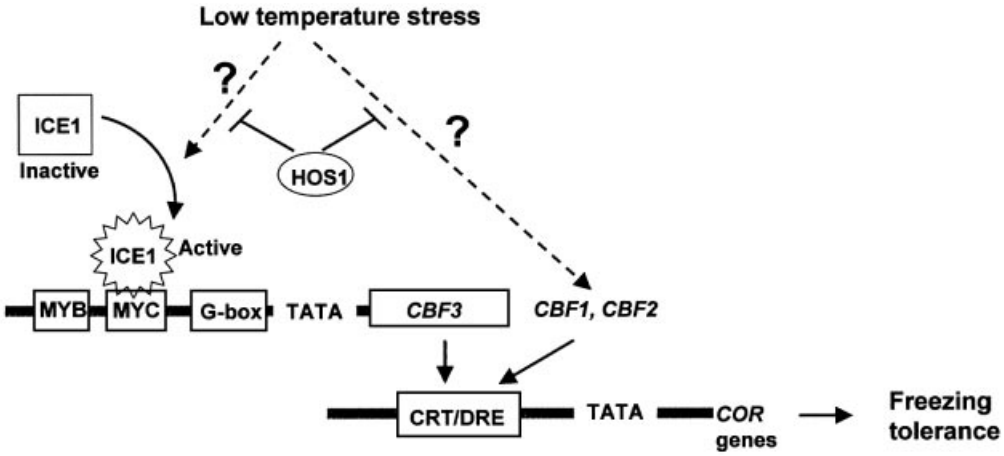

Fig. 3. Regulation of a cold-stress-responsive transcriptome and freezing tolerance. ICE1 is a constitutively expressed myc-like bHLH transcription factor, which is inactive under non-stress conditions. Low temperature stress presumably activates ICE1, which binds to *myc cis*-elements of the *CBF3* promoter and induces *CBF3* expression. CBFs binds to the CRT/DRE *cis*-elements on cold-stress-responsive (*COR*) genes inducing their expression and leading to acquired freezing tolerance. HOS1, a putative E3 ubiquitin-conjugating enzyme, appears to target a positive regulator of CBFs for degradation.

and MPK4 function downstream of the flagellin receptor FLS2 (Asai *et al*., 2002). Studies from alfalfa and tobacco also showed that a MAPK cascade is a node of cross-talk in biotic and abiotic stress signalling (Table 2). Thus biotic and abiotic stress signals converge at the level of MAPK cascades.

The ICE1 pathway regulates cold-stress-responsive genes

Many plant species with origins in temperate regions can acquire tolerance to freezing temperatures by a prior exposure to low non-freezing temperatures, a process called cold acclimation. The expression of *COR* (cold responsive) genes has been shown to be critical in plants for both chilling tolerance and cold acclimation (Thomashow, 1999; Xiong *et al*., 2002*b*). *Arabidopsis COR* genes (*COR78/RD29A, COR47, COR15a, COR6.6*) encode LEA-like stress proteins. These genes are induced by cold, dehydration (due to water deficit/high salt/freezing) or ABA. Promoter analysis of the *COR* genes showed that they contain dehydration responsive elements (DRE) or C-Repeats (CRT) and some of them contain ABREs as well (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997). The expression of *COR* genes is regulated by both ABA-dependent and -independent pathways (Ishitani *et al*., 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). A family of transcription factors known as C-Repeat binding factors (CBFs) or dehydration responsive element binding factors (DREBs) that control ABA-independent expression of *COR* genes in response to cold stress has recently been identified. These transcription factors belong to the ethylene-responsive element binding protein/APETELA2 (EREBP/AP2) family (Stockinger *et al*., 1997; Liu *et al*., 1998). Ectopic expression of *CBF*s/*DREB1*s in transgenic plants turns on downstream cold-responsive genes even at warm temperatures and confers improved freezing, drought and salt tolerance (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998; Kasuga *et al*., 1999). An RNA helicase mutant of *Arabidopsis*, *los4* (low expression of osmotically responsive genes), is impaired in the cold-regulated expression of *CBF* genes and their downstream target genes and, consequently, is sensitive to chilling stress (Gong *et al*., 2002).

The *CBF/DREB1* genes are transiently induced by cold stress, and this induction precedes that of downstream genes with the *DRE/CRT cis*-element (Thomashow, 1999). Three cold-inducible *CBF* genes (*CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A*) have been identified in *Arabidopsis*. The CBF cold-response pathway appears to be conserved in *B. napus,* wheat, rye, and tomato (Jaglo *et al*., 2001; Hsieh *et al*., 2002), suggesting that regulation of the CBF transcriptional cascade during cold stress is an important strategy for plant cold tolerance. Molecular genetic analysis of the *HOS1* (high expression of osmotically responsive genes) locus of *Arabidopsis* showed that cold-signalling components upstream of the CBFs might be regulated by specific ubiquitin-mediated degradation (Lee *et al*., 2001). The *hos1* mutation results in sustained and super-induction of *CBF2, CBF3*, and their target regulon genes specifically during cold stress. Therefore, HOS1 negatively regulates the *COR* genes by modulating the expression level of the *CBF*s. *HOS1* encodes a ring finger protein, which has been implicated as an E3 ubiquitin-conjugating enzyme. *HOS1* is constitutively expressed but drastically down-regulated within 10 min of cold stress and recovers to basal levels by 1 h of cold stress. HOS1 protein is present in the cytoplasm at normal growth temperatures and accumulates in the

nucleus upon cold stress. Thus a positive regulator of *CBF* expression is probably tagged by HOS1 for protein degradation during cold stress (Fig. 3; Lee *et al*., 2001).

Since *CBF* transcripts begin accumulating within 15 min of plant exposure to cold, it has been proposed that there is a transcription factor already present in the cell at normal growth temperatures that recognizes the *CBF* promoters and induces *CBF* expression upon exposure to cold stress (Gilmour *et al*., 1998). The unknown activator(s) has been called 'ICE' (Inducer of *CBF* Expression) protein(s) and it has been suggested that, upon exposing a plant to cold, modification of either ICE or an associated protein would allow ICE to bind to the *CBF* promoter and to activate *CBF* transcription (Gilmour *et al*., 1998). In order to identify ICE proteins, systematic forward genetic analysis using a reporter gene (*CBF3::LUC*, the luciferase gene under the transcriptional control of the *CBF3* promoter)-based genetic screen was performed in *Arabidopsis*. Several mutants identified from the screen showed abnormal cold regulation of *CBF3::LUC* expression as determined by bioluminescence imaging. Molecular genetic analysis of one of the mutants led to the identification of ICE1 (inducer of *CBF* expression 1), a transcriptional activator of the *CBF* genes (Chinnusamy *et al*., 2003). *ice1* mutant plants are impaired in cold acclimation and defective in cold-regulated expression of *CBF3* and its target *COR* genes. *ICE1* encodes a constitutively expressed and nuclear localized MYC-like basic helix-loop-helix transcription factor. DNA binding assays showed that ICE1 specifically bound to the MYC recognition sequences on the *CBF3* promoter, but not to a putative MYB recognition sequence. Transient expression assays indicated that ICE1 is a transcriptional activator. Transgenic lines constitutively overexpressing *ICE1* did not express *CBF3* at warm temperatures but showed a higher level of *CBF3* expression, as well as enhanced expression of the target genes *RD29A* and *COR15A* at cold temperatures, suggesting that cold-induced modification of ICE1 protein is necessary for it to act as a transcriptional activator of *CBF3 in planta*. Taken together, these studies suggest that cold-stress-activated ICE1 binds to the MYC *cis*-elements of the *CBF* promoter and induces *CBF* expression, which, in turn, regulates target *COR* genes and cold acclimation (Fig. 3; Chinnusamy *et al*., 2003). The signalling components that transduce the cold stress signal to ICE1 remain to be identified. It is also unclear whether ICE1 also functions in other abiotic stress response pathways.

ABA-dependence of stress responsive gene expression

ABA regulates several aspects of plant development including seed development, desiccation tolerance of seeds and seed dormancy and plays a crucial role in the plant's response to abiotic (drought, salinity, cold, and hypoxia) and biotic stresses. Although the phenomenon of ABA biosynthesis up-regulation in response to osmotic stress is well known, the signalling pathway by which ABA biosynthetic genes are up-regulated is unknown. A Ca^{2+}-dependent signalling pathway appears to regulate the expression of ABA biosynthetic genes such as *ZEP* (zeaxanthin epoxidase), *NCED* (9-*cis*-epoxycarotenoid dioxygenase), *AAO* (ABA-aldehyde oxidase), and *MCSU* (molybdenum cofactor sulphurase) (Xiong *et al*., 2002*b*). Genetic analysis of ABA-deficient mutants established the necessity of ABA signalling in stomatal control of water loss from plants (Schroeder *et al*., 2001). Here the focus is on ABA-mediated stress-responsive gene expression during abiotic stresses. Stress-responsive genes have been proposed to be regulated by both ABA-dependent and ABA-independent signalling pathways (Fig. 2; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Genetic analysis of the ABA-deficient mutants *los5/aba3* and *los6/aba1* of *Arabidopsis* showed that ABA plays a pivotal role in osmotic stress-regulated gene expression. The expression of stress-responsive genes such as *RD29A*, *RD22*, *COR15A*, *COR47*, and *P5CS* was severely reduced or completely blocked in the *los5* mutant (Xiong *et al*., 2001*a*), while in *los6*, the expression of *RD29A*, *RD19*, *COR15A*, *COR47*, *KIN1*, and *ADH* was lower than in wild-type plants (Xiong *et al*., 2002*a*). Therefore, although ABA-independent pathways may exist, an ABA-dependent signalling pathway plays an essential role in stress-responsive gene expression during osmotic stress (Xiong *et al*., 2001*a*). Stress-responsive genes contain DRE, ABRE, MYC recognition sequence (MYCRS), and MYB recognition sequence (MYBRS) *cis*-elements in their promoters (Zhu, 2002). ABA-dependent signalling activates basic leucine zipper transcription factors called ABFs/AREBs to induce stress-responsive gene expression (Xiong *et al*., 2002*b*). Recently, Narusaka et al. (2003) showed that the DRE and ABRE elements are interdependent in the expression of the *RD29A* gene.

Overexpression of a soybean cold- and ABA-inducible C2H2-type zinc finger protein (SCOF1) in *Arabidopsis* resulted in constitutive expression of stress-responsive genes and freezing tolerance. Although SCOF1 is unable to directly bind to ABRE or DRE/CRT motifs, it enhances the DNA binding activity of SGBF1, a bZIP transcription factor, to ABRE *cis*-elements of stress-responsive genes (Kim *et al*., 2001). Thus SCOF1-SGBF1 appears to regulate the ABA-dependent pathway of stress-responsive gene expression through ABRE during cold stress (Kim *et al*., 2001). ABI5-related (bZIP) transcription factors, AREB1 and AREB2 were found to promote ABA-activation of target gene expression which was repressed by either protein kinase inhibitor treatment of wild-type

cells or a dominant-negative *abi1-1* mutation (Fig. 2; Uno *et al*., 2000).

ABA-independent pathways of stress-responsive gene expression

ABA-independent stress-responsive gene expression has been thought to be regulated through DRE *cis*-elements, while ABA-dependent pathways activate gene expression through ABRE *cis*-elements. In ABA-independent pathways during osmotic stress signalling, AP2-type transcription factors, DREB2A and DREB2B, trans-activate the DRE *cis*-element of stress-responsive genes (Fig. 2; Liu *et al*., 1998). However, cloning and transgenic analysis of a DREB1-related transcription factor, CBF4 in *Arabidopsis*, showed that regulation of DRE elements may also be mediated by an ABA-dependent pathway. *CBF4* gene expression is up-regulated by drought and ABA, but not by cold stress. Overexpression of *CBF4* in *Arabidopsis* resulted in constitutive expression of CRT/DRE containing stress-responsive genes and enhanced tolerance to drought and freezing stresses (Fig. 2; Haake *et al*., 2002).

Arabidopsis stress-responsive genes may also be regulated by a zinc finger transcriptional repressor *STZ/ZAT10* (Lee *et al*., 2002). *Arabidopsis los2*, a chilling-sensitive mutant, is impaired in stress-responsive gene expression during cold but not under ABA, salt or osmotic (PEG) stress treatment. However, no apparent difference in *CBF* expression was observed between the wild type and *los2*. Cold stress up-regulates *LOS2* expression, with *LOS2* encoding an enolase that is localized in both the cytoplasm and nucleus. In animals, enolase functions as transcriptional repressor of the c-*myc* gene (Subramanian and Miller, 2000). Electromobility shift assays showed that the *Arabidopsis* LOS2 binds to the promoter element of *STZ/ZAT10*. *STZ/ZAT10* expression is induced rapidly and transiently by cold in the wild type, and this induction is stronger and more sustained in the *los2* mutant, suggesting that LOS2 acts as a repressor of *STZ/ZAT10* expression during cold stress (Lee *et al*., 2002). Thus stress-responsive proteins act as effectors of cold, drought and salt tolerance, but different signalling pathways control the expression of stress-responsive genes during cold and osmotic stresses. The components of both ABA-dependent and -independent signalling pathways that activate the transcription factors need to be genetically defined in order to assess whether a signalling pathway is specific to a stress or is involved in cross-talk with other pathways.

Conclusions and perspectives

Several abiotic stress signalling pathway components have been identified based on mutant analysis or their stress induction, ability to complement the growth of relevant yeast mutants and ectopic expression studies. Genetic analysis has been successfully exploited to define the SOS pathway of ion homeostasis during salt stress. Some of the events involved in the regulation of freezing tolerance by the ICE1–CBF pathway and osmotic stress tolerance by ABA-dependent and -independent pathways are becoming clearer. However, there is still only a fragmentary view of abiotic stress signalling pathways. Forward and reverse genetics approaches will continue to be imperative to dissect these complex pathways. Although conventional genetic screens have yielded valuable insight into stress signal transduction, this approach may ultimately be limited because of the functional redundancy of components within the signalling pathways. Reporter gene-based molecular screens offer a way systematically to identify upstream signalling components that control subsets of responses, which may not manifest as visible tolerance phenotypes. Thorough characterization of mutant phenotypes will provide an indication whether a signalling component functions in a specific pathway or is involved in multiple pathways. Cell biological analysis of spatial and temporal expression patterns, combined with biochemical characterization of the components, particularly identification of signalling complexes, will be required to firmly establish specificity or cross-talk of the signalling pathways.

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