Cell signaling under salt, water and cold stresses

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Forward genetics and biochemical approaches to studying plant responses to salt, water and cold stresses began to bear fruit recently. Analysis of *salt overly sensitive* (*sos*) *Arabidopsis* mutants revealed a novel calcium-regulated protein kinase pathway for response to the ionic aspect of salt stress. In-gel kinase assays identified several SOS-independent protein kinases that are either activated specifically by osmotic stress or by multiple abiotic and biotic stresses. Molecular analysis revealed a transcriptional cascade in cold-regulated gene expression.

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Current Opinion in Plant Biology 2001, 4:401–406

1369-5266/01/$ – see front matter

Abbreviations

ABA	abscisic acid
CBF	CRT-binding factor
COR	*COLD-REGULATED*
CRT	*C repeat*
DRE	*DEHYDRATION RESPONSIVE ELEMENT*
DREB	DRE-BINDING FACTOR
HOS1	*HIGH EXPRESSION OF OSMOTIC RESPONSIVE1*
KIN	*COLD-INDUCIBLE*
LTI	*LOW-TEMPERATURE-INDUCED*
MAPK	mitogen-activated protein kinase
RD	*RESPONSIVE TO DESSICATION*
SIPK	salicylic-acid-induced protein kinase
sos	*salt overly sensitive*
VSP2	*VEGETATIVE STORAGE PROTEIN 2*

Introduction

One important feature distinguishing plants from other complex multicellular organisms is that plants are sessile and thus have to endure environmental challenges such as soil salinity, drought and cold temperatures. Although salt, water and cold stresses are clearly different from each other in their physical nature and each elicits specific plant responses, they also activate some common reactions in plants. The most widely studied common response is the induction of some plant genes by all three stresses [1]. Because of this and other commonalities, these stresses are often considered together in molecular studies. Plant responses to these stresses involve nearly every aspect of plant physiology and metabolism. Consequently, there exists a complex signaling network underlying plant adaptation to these adverse environmental conditions. This review highlights recent progress in understanding the signaling pathways that are either specific or common to responses to these stresses. Owing to space constraint, the complex topic of abscisic acid (ABA)-dependent and -independent gene regulation by osmotic stress is not reviewed here.

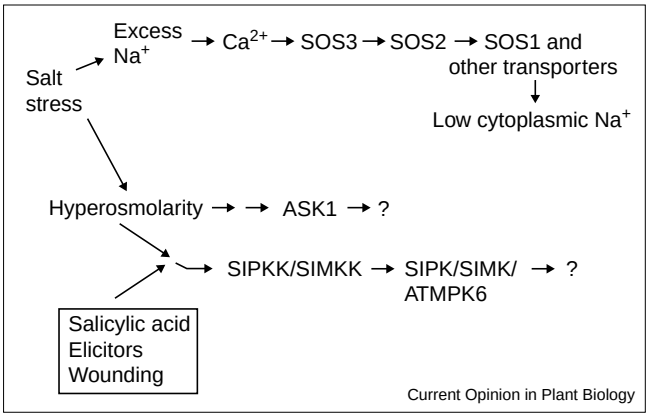
Soil salinity creates both ionic and osmotic stresses for plants. The ionic aspect of salt stress is clearly distinct from other abiotic stresses such as drought and cold, and there are signaling pathways dedicated specifically to deal with ionic stress (i.e. excess sodium and associated potassium deficiency) [2]. It has often been commented that drought and cold stresses also cause osmotic stress, and this is why salt, drought and cold stresses induce some common sets of plant genes. It is true that extracellular freezing leads to cellular dehydration, and that chilling temperatures reduce root water absorption and conduction in some plants [3]; however, under the chilling conditions typically used in laboratories to induce cold-responsive genes in plants such as *Arabidopsis thaliana*, osmotic stress is minimal or does not exist. Therefore, the common signal shared by salt, drought and cold in the induction of *RD/COR/KIN/LTI*-type (*RESPONSIVE TO DESSICATION/COLD-REGULATED/COLD-INDUCIBLE/LOW-TEMPERATURE-INDUCED-type*) genes is unlikely to be osmotic stress. Instead, the inductive signal could be derived from some common damage caused by these stresses. Accordingly, the role of the gene products that are ultimately induced by these signaling pathways could be to help repair damage or to protect plants from further stress damage. Alternatively, there might not be a common inductive signal; the different stresses might be perceived directly by different sensors with the resulting specific signals converging at the target gene promoter elements.

Ionic stress signaling and tolerance: the SALT OVERLY SENSITIVE (SOS) pathway

Salt stress disrupts plant ion homeostasis, resulting in excess toxic Na^+ in the cytoplasm and a deficiency of essential ions such as K^+ [4]. Various ion transporters function to limit Na^+ entry into and exit out of plant cells, to regulate Na^+ compartmentation in the vacuole, and to selectively import K^+ over Na^+ into plant cells [5]. When plants are subjected to salt stress, some of the ion transporters need to be activated or to have their activities enhanced, whereas others (e.g. Na^+ influx transporters) may need to have their activities suppressed. In addition, the transcript levels of many of the transporters are increased or decreased in response to salt stress [4]. A signaling pathway for the regulation of ion homeostasis and salt tolerance has emerged following the recent cloning and biochemical characterization of several *SOS* genes and gene products from *Arabidopsis* (Figure 1; [6•]).

The *Arabidopsis sos* mutants were identified in a genetic screen for seedlings that were hypersensitive to growth inhibition by NaCl stress [7,8]. The *sos1*, *sos2* and *sos3* mutants are specifically hypersensitive to Na^+ and Li^+ ions. In the absence of added Na^+ or Li^+ in culture media, the mutants grow and develop just like wild-type plants, although the growth of the mutants is slightly slower. In addition to hypersensitivity to Na^+ and Li^+ inhibition, the

Figure 1



Salt stress activates several protein kinase pathways. Excess Na^+ in growth medium elicits a cytoplasmic calcium signal that is presumably perceived by the calcium-binding protein, SOS3 [13•]. SOS3 interacts with and activates SOS2, a protein kinase [10•]. The SOS3–SOS2 kinase pathway is proposed to regulate the transcript abundance and/or transport activity of ion transporters such as SOS1 (an Na^+/H^+ antiporter), thereby maintaining a low concentration of toxic Na^+ in the cytoplasm [9•,14••]. Salt stress also causes hyperosmolarity, which activates the protein kinase ASK1 [17••,18•], and multiple MAPK pathways [17••,19,23•]. Only one of the MAPK pathways is shown. This pathway is also activated by various other signals such as salicylic acid, elicitors and wounding. SIPK, SIMK and ATMPK6 are homologous MAPKs from tobacco, alfalfa and *Arabidopsis*, respectively. SIPKK and SIMKK are MAPK kinases that interact with SIPK and SIMK, respectively. Question marks indicate that the targets of the osmotic-stress-activated kinase pathways are unknown.

growth of the mutants is also impaired on media that are deficient in K^+ [8]. The *sos* mutants do not show altered responses to general osmotic stress or drought. These phenotypes suggest that *SOS* genes function specifically in coping with the ionic aspect of salt stress.

SOS1 encodes a plasma membrane localized Na^+/H^+ antiporter ([9•]; J-K Zhu, unpublished data). Thus, the biochemical and physiological function of SOS1 is to remove Na^+ from the cytoplasm and export it to the extracellular space or the root medium. The role of SOS1 in K^+ acquisition may be indirect and could possibly arise through H^+ coupling with H^+–K^+ co-transporters [9•]. *SOS1* transcript is present in *Arabidopsis* plants even without salt stress but its levels are upregulated by NaCl treatment [9•]. Unlike many other stress-regulated genes that are upregulated not only by salt stress but also by ABA or cold, *SOS1* upregulation does not occur in response to ABA or cold-stress treatment. Increased transcript level of *SOS1* as a result of salt stress is partly under the control of the *SOS2* and *SOS3* genes [9•]. In *sos2* and *sos3* mutant plants, NaCl induction of *SOS1* transcript level is substantially reduced (Figure 1).

The *SOS2* gene encodes a serine/threonine protein kinase with an amino-terminal catalytic domain and a carboxy-terminal regulatory domain [10•]. There is an interaction between the catalytic and regulatory domains within SOS2 [11••]. This intramolecular interaction appears to keep SOS2 in an inactive state. Indeed, deletion of the regulatory domain results in a constitutively active protein kinase. A constitutively active SOS2 can also be constructed by changing Thr168 in the putative activation loop to Asp. The Thr168→Asp mutation presumably mimics phosphorylation by an unknown upstream kinase.

The *SOS3* gene encodes a myristoylated calcium-binding protein [12,13•]. It was proposed that SOS3 senses cytosolic calcium changes that are elicited by salt stress [12]. Salt stress has been known to trigger a transient increase in cytosolic free calcium concentration. How this calcium signal differs from other stimuli-activated calcium signals, and how SOS3 specifically senses salt-stress-elicited calcium signals, is unclear.

SOS3 physically interacts with and activates SOS2 [14••]. SOS3 activation of SOS2 requires calcium, consistent with calcium being the second messenger in salt stress responses (Figure 1). SOS2 interaction with SOS3 is mediated through the FISL (using the single-letter code for amino acids) motif, a 21-amino-acid sequence located in the SOS2 regulatory domain [11••]. The FISL motif exists in 23 other *Arabidopsis* putative protein kinases that have amino-acid sequences that are similar to SOS2 [11••]. These SOS2-like protein kinases (PKSs) appear to interact with certain SOS3-like calcium-binding proteins (SCaBPs) [11••]. Therefore, certain PKS proteins may pair with particular SCaBPs to form specific kinase complexes that function in various calcium signaling pathways. In essence, these kinase complexes are rather similar to calmodulin-dependent (CaM) kinases in non-plant systems. This family of calcium-regulated kinases adds complexity to the already complicated calcium regulation of protein kinases through the large family of calcium-dependent protein kinases (CDPKs).

Besides regulating the transcript levels of *SOS1* under salt stress, the SOS3–SOS2 kinase complex also seems to activate the Na^+/H^+ antiporter activity in isolated plasma membrane vesicles (Q Qiu, K Schumaker, J-K Zhu, unpublished data). Future genetic suppressor and enhancer screens in *sos* mutant backgrounds, protein interaction screens with the SOS proteins, and gene expression profiling of the *sos* mutants will identify other components and targets of the SOS regulatory pathway. Recently, through differential subtraction screening and comparative RNA-blot analysis, Gong *et al.* [15•] found several genes that are expressed at higher or lower levels in the *sos3* mutant than in wild-type plants. Except for *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), all of these genes show similar salt-induced expression in *sos1* and *sos3*, implying that their altered expression is probably a consequence of reduced salt tolerance in these mutants. Because salt induction of *VSP2* is impaired by mutations in *sos2* or *sos3* but not by mutations in *sos1*, the results suggest that *VSP2* expression is a target of the SOS3–SOS2 kinase pathway. Whether and how VSP2 functions in salt tolerance remains to be determined.

The ion homeostasis pathway defined by SOS3/SOS2/SOS1 appears to be unique to plants. In yeast, salt stress may also involve calcium signaling but the downstream target of calcium in yeast is calcineurin — a type-2B protein phosphatase with a regulatory subunit that has sequence similarity to SOS3 [4]. The *Arabidopsis* genome does not seem to encode a protein that is similar to the catalytic subunit of calcineurin. Nevertheless, other types of protein phosphatases could be involved in the plant SOS pathway.

Osmotic stress signaling: SOS-independent protein kinases get activated

In yeast and mammalian systems, protein phosphorylation is central to osmotic stress signaling. In *Saccharomyces cerevisiae*, the osmoregulatory pathways begin with either an Src-homology 3 (SH3)-domain-containing membrane protein or a two-component histidine kinase, which activates a mitogen-activated protein kinase (MAPK) cascade and leads to increased osmolyte synthesis and accumulation [16]. Although plants are known to accumulate compatible osmolytes for osmotic adjustment, it is unclear whether plants use membrane sensors and MAPK cascades similar to those found in yeast to regulate osmolyte synthesis. Studies in plants have recently identified several protein kinases that are activated by osmotic stress. Whether any of these protein kinases regulate osmolyte accumulation remains an important question.

A broad specificity 42-kDa kinase in cultured tobacco cells is activated rapidly in response to high concentrations of NaCl or sorbitol treatments [17••]. Partial peptide sequences from this kinase suggest that it is an ortholog of *Arabidopsis* SERINE/THREONINE KINASE1 (ASK1), a SUCROSE NONFERMENTING1 (SNF1)-related kinase with no known function. In *Arabidopsis* seedlings, a 40-kDa kinase was also identified as being rapidly activated by hyperosmotic stress in a calcium- and ABA-independent manner [18•]. Although the amino-acid sequence of this 40-kDa *Arabidopsis* protein is not known, it is most likely to be the homolog of the 42-kDa kinase of tobacco. Osmotic stress activation of the *Arabidopsis* 40-kDa protein kinase is not impaired by the *sos3* mutation, implying that its activation is independent of the SOS pathway. Because these 40/42-kDa plant kinases are clearly not MAPKs either, these results may suggest a novel mechanism for osmotic stress signaling in plants (Figure 1).

Plants also have several MAPKs that are activated by hyperosmotic stress (Figure 1). In alfalfa cells, a 46-kDa MAPK, named SALT-STRESS-INDUCIBLE MAP KINASE (SIMK) becomes activated upon hyperosmotic stress treatments [19]. In tobacco cells, a similar MAPK named SIPK (salicylic-acid-induced protein kinase) is activated by hyperosmotic stress [17••]. This tobacco MAPK is also known to be activated by hypoosmotic stress, salicylic acid, or fungal elicitors [20•]. In addition, an *Arabidopsis* protein that cross-reacts with antibodies against the tobacco MAPK was also activated by hyperosmotic stress [18•]. A MAPK kinase that interacts with and activates the alfalfa SIMK has been found [21]. Similarly, a tobacco MAPK kinase that interacts with SIPK has been cloned [22]. In *Arabidopsis*, at least three MAPKs have been found that are enzymatically activated by salt as well as by cold, wounding and other environmental signals [23•]. It has also been shown that MAPKs known as WIPK (wound-induced protein kinase) in tobacco and SAMK (stress-activated MAP kinase) in alfalfa are activated by cold, drought, wounding, and biotic signals [24,25].

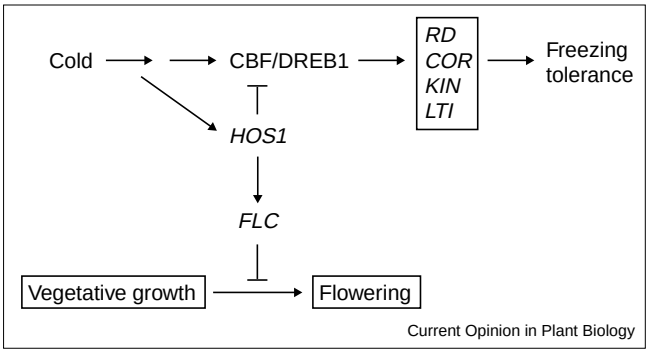
Transcript levels of a number of protein kinases, including a two-component histidine kinase, a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK), have been found to increase in response to osmotic stress and other stress treatments [26]. Whether these and the osmotic-stress activated kinases discussed above function in osmotic stress adaptation in plants awaits functional genetic studies. Of particular importance is the need to identify the input and output of the putative kinase pathways (Figure 1). The input signals could be osmotic stress itself (e.g. turgor changes) or could be derived from osmotic stress damage. The output could be osmolyte accumulation, which helps to establish osmotic homeostasis, or damage repair mechanisms.

Cold stress signaling: a transcriptional cascade negatively regulated by a RING-finger

Low temperatures induce the expression of a diverse array of plant genes [27•]. The products of these genes help plants adapt to subsequent freezing stress. Substantial progress has been made in the past several years in understanding the transcriptional regulation of some of these genes. Many cold-regulated genes have in their promoters one or several copies of the *DEHYDRATION RESPONSIVE ELEMENT* (*DRE*)/ *C-repeat* (*CRT*) *cis*-element, which has the core sequence CCGAC [28,29]. Transcription factors in the AP2/EREBP (APETALA2/ETHYLENE RESPONSE ELEMENT BINDING PROTEIN) family bind to this element and activate transcription of the downstream genes. These transcription factors are known as CRT-binding factors (CBFs) or DRE-BINDING FACTORS (DREBs) [29,30]. Importantly, the *CBF*/*DREB1* genes are themselves induced by low temperature [30]. This induction is transient and precedes that of downstream cold-regulated genes containing the *DRE*/*CRT cis*-element. Therefore, there is a transcriptional cascade that leads to the expression of the *RD*/*COR*/*KIN*/*LTI* genes under cold stress (Figure 2). A related transcription factor gene, *DREB2A*, is induced specifically by osmotic stress. Unlike the *CBF*s/*DREB1*s, which, when ectopically expressed in plants, turn on downstream cold-responsive genes even at warm temperatures, ectopic expression of *DREB2A* is not sufficient to activate the *RD*/*COR*/*KIN*/*LTI* genes [30,31].

Whether the role of *CBF*/*DREB1* genes is restricted to cold acclimation and the activation of *DRE*/*CRT* type genes has yet to be resolved. Using cDNA microarray technology, 12 downstream target genes of the CBF/DREB1 regulon

Figure 2



Low temperature signaling and the regulation of cold acclimation and vernalization. Cold temperatures activate the transcription of the *CBF*/*DREB1* family of transcriptional activators. CBF/DREB1 proteins bind to the *DRE*/*CRT cis*-element in the promoters of *RD*/*COR*/*KIN*/*LTI* genes, which is sufficient to activate the expression of these targets. The RD/COR/KIN/*LTI* gene products function in freezing tolerance. *HOS1* encodes a variant RING-finger protein and negatively regulates *CBF*/*DREB1* expression. *HOS1* also positively regulates the expression of *FLC*, a floral repressor that has been proposed to play a central role in vernalization.

have been identified [32•]. Eleven of these genes have an obvious *DRE/CRT cis*-element in their promoters. In transgenic *Arabidopsis* plants ectopically expressing *CBF3*, multiple biochemical changes have been found that can be associated with cold acclimation [33•]. *CBF3*-expressing plants had higher concentrations of proline and soluble sugars, which are generally correlated with increased freezing tolerance.

CBF1 physically interacts with a histone acetyltransferase and a putative transcriptional adaptor protein [34]. The transcription factor responsible for the activation of *CBF* expression has not been identified; however, a negative regulator of cold-induced *CBF* transcription was cloned recently [35••]. This regulator, *HOS1* (*HIGH EXPRESSION OF OSMOTIC RESPONSIVE 1*), was identified in a genetic screen for *Arabidopsis* mutants with deregulated *RD29A* expression [36]. In homozygous recessive *hos1* mutant plants, cold-induction of *RD29A* and its upstream regulators, the *CBF*s, is stronger than in wild-type plants (Figure 2). *HOS1* encodes a variant RING-finger, which normally exists in the cytoplasm but appears in the nucleus when plants are subjected to temperatures of 0–4°C [35••]. As some RING-finger proteins can serve as ubiquitin E3 ligases, which help degrade specific target proteins, HOS1 could function by targeting the transcriptional activator of *CBF*s for degradation.

The role of HOS1 in low temperature signaling is not restricted to gene regulation and cold stress tolerance. This protein is also important in vernalization, a process by which long-term exposure to low temperature promotes flower initiation (Figure 2). Mutant *hos1* plants flower considerably earlier than wild-type plants [36]. This early flowering phenotype correlates with reduced expression of *Flowering Locus C* (*FLC*), a key negative regulator of vernalization [35••].

Several other *Arabidopsis* mutants that are impaired in cold gene regulation or freezing tolerance have been reported but have not been cloned [37,38,39•]. Clearly, our understanding of cold sensing and signaling is still rudimentary. Cold stress also elicits a cytosolic calcium signal [40,41]. Pharmacological studies suggest the possible involvement of membrane fluidity, cytoskeletal arrangement and protein phosphorylation in plant cold responses [42•]. Two histidine kinases were reported as potential cold sensors in the cyanobacterium *Synechocystis* [43••]. In *Synechocystis* mutants disrupted in either one of the potential sensor genes, cold-induction of a desaturase gene is reduced.

Conclusions

Both physiological studies of stress adaptation and molecular analysis of diverse stress gene regulation patterns have suggested a network of multiple signaling pathways that mediate salt, water and cold stress responses in plants. Only recently the molecular components of these pathways have begun to be identified. Genetic dissection of salt tolerance in *Arabidopsis* established the involvement of the SOS pathway in the response to the ionic aspect of the salt stress (Figure 1). This novel protein kinase pathway is activated by calcium signaling and regulates ion transporters, which bring about ion homeostasis. Biochemical studies identified several protein kinases that are activated by the osmotic aspect of salt and water stress (Figure 1). The *in vivo* function of these osmotic stress-activated kinases and, in particular, the identification of the proteins that they regulate await future studies. Cold induction of gene expression and acclimation is intertwined with salt, water and ABA regulation. Nevertheless, the CBF/DREB1-based transcriptional cascade is triggered specifically by low temperature signals (Figure 2). The most important task in the next few years remains to identify pathway components and to establish their function by genetic approaches.

Acknowledgements

Work in my laboratory has been supported by grants from the US National Science Foundation, the National Institutes of Health and the Department of Agriculture's National Research Initiative.

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