Genetic Analysis of Plant Salt Tolerance Using Arabidopsis

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Soil salinity is one of the most significant abiotic stresses for plant agriculture. Apart from the practical goal of genetically improving the salt tolerance of crop plants, salt tolerance research represents an important part of basic plant biology, contributing to our understanding of subjects ranging from gene regulation, signal transduction to ion transport, and mineral nutrition. Research on two other major abiotic stresses, drought and cold, is intimately linked with salt stress work. For example, many genes that are regulated by salt stress are also responsive to drought or cold stress (Zhu et al., 1997). Because salt stress can be applied accurately and reproducibly, many "drought" stress studies in the laboratory use salt stress instead of actual drought. The widely known Hog pathway for osmotic stress perception and signaling in yeast was discovered by using NaCl stress (Brewster et al., 1993).

Salt tolerance is a complex trait involving responses to cellular osmotic and ionic stresses and their consequent secondary stresses (e.g. oxidative stress) and whole plant coordination. The complexity and polygenic nature of salt stress tolerance are important factors contributing to the difficulties in breeding salt-tolerant crop varieties. Breeding efforts have been hampered by a lack of understanding of salt tolerance mechanisms as well as a lack of field and laboratory screening tests, including physiological and molecular markers. There was much optimism when molecular approaches began to be applied to salt stress research. Nearly 2 decades later, a long but incomplete list of salt stress-responsive genes has been produced by the molecular studies. No clear salt tolerance mechanism has emerged from the expression studies (Zhu et al., 1997). The limited success of the molecular approach in elucidating salt tolerance mechanisms is primarily due to two factors. First, the approach is only correlative. It is now widely recognized that many salt-responsive genes do not contribute to tolerance, rather, their induction reflects salt stress damage. Second, so far the molecular approach has mostly identified genes or gene products based only on their expression, but many genes that are important for salt tolerance may not be induced by salt stress. One notable success of molecular studies has been the identification of promoter elements and transcription factors that control the expression of protective proteins such as RD29A/COR78 (Kasuga et al., 1999). Traditional differential screening/hybridization approaches are being replaced by more powerful methods, such as DNA microarray analysis, that provide a profile of gene expression at the genome level. Profiling at the genome level, when combined with systematic genetic analysis, promises to reveal much of the signaling networks that control stress tolerance.

This review describes some recent developments and prospects in genetic analysis of salt stress tolerance. The focus of the review is on mutational analysis in the model plant Arabidopsis and salt-specific responses, i.e. ion homeostasis aspects of salt tolerance.

USING ARABIDOPSIS FOR GENETIC ANALYSIS OF SALT TOLERANCE

The last decade has seen tremendous successes of genetic analysis using the Arabidopsis model system. The power of Arabidopsis genetics has been well recognized in dissecting developmental programs, hormonal, and environmental responses, including light regulation and plant-pathogen interactions. Mutational analysis is especially suited for making inroads to study complex systems because each component can be specifically mutated to reveal its effect on the entire system. Furthermore, genetic analysis has been very successful in elucidating salt stress responses in the budding yeast (*Saccharomyces cerevisiae*; for review, see Zhu et al., 1997). The application of Arabidopsis genetics on plant salt tolerance studies is beginning to shed light on novel tolerance mechanisms operating in plants.

Doubts may still exist about the validity of Arabidopsis as a model organism for salt tolerance studies. Since Arabidopsis is not a particularly salt tolerant plant species, the question arises as to whether it has evolved salt tolerance genes. This question is best answered by years of physiological studies with salt adaptation of glycophytic plants as well as cell cultures (Hasegawa et al., 1994), particularly during the period from 1970 through the 1980s. Although few significant mechanistic advances were made, one consistent theme that emerged from these studies was that salt-sensitive plants do have salt tolerance genes. Cell cultures derived from many different glycophytes were made salt tolerant with relative ease by gradual adaptation to higher levels of NaCl. For

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example, a salt-sensitive tobacco cell culture was adapted to grow over tens of thousands of generations in medium containing near seawater-level NaCl (Hasegawa et al., 1994). Salt-sensitive plants were similarly adapted to grow in the presence of high salinity. New crop varieties have not been obtained by salt adaptation, mainly because adapted plants and cells, although they survive well under high salinity, grow very slowly even without salt stress. Nevertheless, these studies illustrate that all plants have in their genomes genes for salt tolerance. Without adaptation, the salt tolerance genes may not be properly expressed to confer salt tolerance.

Salt tolerance genes in halophytes may have evolved from genes in glycophytes that were adapted to low levels of salt stress, a common environmental factor for most plants because Na^+ is one of the most abundant soil cations. Even at a typically low concentration of <1 mM, Na^+ could accumulate to high levels inside plants because the large volume of transpirational flow of water through plants. Accumulation of Na^+ to toxic levels may not occur because of salt tolerance genes that regulate the fluxes of Na^+. This evolutionary argument is supported by much research showing that many agronomic traits in crop species are controlled by genes that have isologs or paralogs in Arabidopsis despite an apparent lack of those agronomic traits in this model plant. For example, although Arabidopsis does not have the fruit-ripening responses typically observed in tomato, the ethylene receptor for tomato fruit ripening is the same type of molecule that mediates the triple responses to ethylene in Arabidopsis (Wilkinson et al., 1995). Therefore, glycophytes such as Arabidopsis can be expected to have salt tolerance genes that are not very different from those of halophytes. This notion is also supported by recent findings that wild tomato strains that have very small fruits are a source of genes for increasing fruit size even in cultivars that have larger fruits (Tanksley and McCouch, 1997).

ECOTYPE DIFFERENCES AND QUANTITATIVE TRAIT LOCI

Quantitative trait loci (QTL) analysis has progressed to a point where it is technically feasible to clone some of the genes involved in a particular quantitative trait. This is especially true of some major QTLs that in practice can be treated as Mendelian loci. For agronomic traits such as flowering time, plant and seed sizes, and disease resistance, ecotype differences have been used to map the responsible QTLs in Arabidopsis (Alonso-Blanco and Koornneef, 2000).

Commonly used Arabidopsis ecotypes, such as Columbia, Landsberg erecta, Ws, and C24, differ only slightly in salt tolerance. Recombinant inbred lines are publicly available for some of the ecotype pairs. However, mapping salt tolerance QTLs in common Arabidopsis ecotypes would be difficult because the differences between these ecotypes are small. A comprehensive survey of the hundreds of Arabidopsis accessions will be necessary to use the natural variations to identify salt tolerance determinants. In addition, several close relatives of Arabidopsis are extremely salt tolerant and are true halophytes. For example, *Thellungiella halophila* (*Thellungiella* is a synonym for Arabidopsis), endemic to the coastal lands of eastern China, can survive seawater-level salinity and complete its life cycle in the presence of 300 mM NaCl. *T. halophila* is very similar to Arabidopsis in terms of growth, development, and also in DNA sequence (H. Zhang and J.-K. Zhu, unpublished data). Such halophytic Arabidopsis relatives could be exploited in the future for salt tolerance studies through QTL or mutational analyses.

SALT-TOLERANT MUTANTS

Like many other cellular signaling pathways, tolerance responses to salt stress are expected to be under negative as well as positive regulation. Salt-tolerant mutants have the potential to reveal negative regulatory mechanisms that normally suppress some of the tolerance responses. Pleiotropic mutations that change ion transport characteristics, e.g. to restrict Na^+ and/or to enhance K^+ influx, may also result in salt tolerance. Several single gene mutations conferring salt tolerance in plants are known. These include soybean mutants that appear to exclude Cl^- (Abel, 1969), barley and tobacco mutants that overaccumulate Pro (Kueh and Bright, 1982; Sumaryati et al., 1992), fern mutants with altered K^+ transport (Warne and Hickok, 1987), and several Arabidopsis mutants (Saleki et al., 1993; Werner and Finkelstein, 1995; Tsugane et al., 1999).

Saleki et al. (1993) selected three *RS* (for resistant to salts) mutants of Arabidopsis that are capable of germination under saline conditions. The *RS* mutants show enhanced tolerance to not only NaCl but also KCl, K_2SO_4, LiCl, and mannitol, indicating that the mutants are primarily osmotolerant. Similarly, the *rss* (for resistant to salt stress) mutant identified by Werner and Finkelstein (1995) is also osmotolerant. The *RS* and *rss* mutations are all recessive. It is unclear whether *rss* is allelic to either of the three *RS* mutants (i.e. *RS17*, *RS19*, and *RS20*). Although the *RS* and *rss* mutants germinate under high salinity, seedlings and mature plants are not more salt tolerant than the wild type. This implies that different salt tolerance mechanisms may operate during seed germination and subsequent plant growth. It is important to note that mutants with reduced abscisic acid (ABA) contents or sensitivities are also more tolerant to salt stress at seed germination (Koornneef et al., 1984). This can be explained because salt stress increases ABA levels, and ABA is inhibitory to germination.

An Arabidopsis mutant, *pst1* (for photoautotrophic salt tolerance 1), was reported recently (Tsugane et al., 1999). The recessive *pst1* mutation increases the capacity of plants to detoxify active oxygen species and thus enhances plant tolerance to oxidative stress as well as to salt stress. Oxidative stress is a secondary effect of salt stress. It is likely that *pst1* is also more tolerant to other stresses such as heat, freezing, and drought, each of which can lead to oxidative stress. Consistent with this correlation between oxidative detoxification and multiple stress tolerance, plants genetically engineered to over-produce reactive oxygen-scavenging osmolytes show enhanced tolerance to salt, cold, and heat stresses (Hayashi et al., 1997). In contrast to the *RS* and *rss* mutants, *pst1* is not salt tolerant during seed germination. Like the *RS* and *RSS* genes, *PST1* has not yet been cloned.

sos MUTANTS

Most salt-sensitive mutants are expected to be recessive, and their dominant wild-type alleles, by definition, are necessary for salt tolerance. Salt-sensitive mutations have been instrumental in elucidating salt tolerance mechanisms in microbial model organisms such as *Escherichia coli* (Schuldiner and Padan, 1996) and yeast (Brewster et al., 1993; Mendoza et al., 1994), revealing essential roles of various transporters and signaling molecules for salt tolerance. It was not obvious initially whether salt-sensitive mutants could be isolated from Arabidopsis because the plant is salt sensitive to begin with, typical of most glycophytic plants. Nevertheless, Arabidopsis is capable of tolerating up to 100 mM NaCl depending on treatment duration and other conditions. Therefore, it is theoretically possible to have salt-sensitive mutations that make Arabidopsis even more sensitive. Several Arabidopsis mutants that are more sensitive to NaCl stress have been reported (Wu et al., 1996; Liu and Zhu, 1997a; Zhu et al., 1998). Since Arabidopsis is a relatively salt-sensitive species, the salt-sensitive mutants are more appropriately referred to as salt-hypersensitive mutants. Wu et al. (1996) have named Arabidopsis salt-hypersensitive mutants recovered from their genetic screen as *sos* for salt overly sensitive (it is easier to say *sos* than *shs*). In addition, several *los* and *hos* mutants recovered from a genetic screen for deregulated *RD29-LUC* expression (Ishitani et al., 1997) are more sensitive to salt stress inhibition (J.-K. Zhu, unpublished data).

Approximately 250,000 seedlings derived from ethylmethane sulfonate-, fast neutron-, or T-DNA mutagenesis were screened using a root-bending assay (Wu et al., 1996; Zhu et al., 1998). Over 40 *sos* mutant lines were recovered. Allelism tests by pair-wise crosses between the mutants revealed that the mutants fall into five complementation groups, defining five salt tolerance genes: *SOS1* (Wu et al., 1996), *SOS2* (Zhu et al., 1998), *SOS3* (Liu and Zhu, 1997a), *SOS4*, and *SOS5* (J.-K. Zhu, unpublished data).

By comparing the growth of the *sos* mutants on various salts, *sos1*, *sos2*, and *sos3* mutants were found to be hypersensitive to Na^+ and Li^+. It is interesting to note that hypersensitivity to Na^+ in these *sos* mutants always seems to be linked to hypersensitivity to Li^+, a more toxic analog of Na^+. The *sos* mutants are not more sensitive to high concentrations of K^+, Cs^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_3^-, or SO_4^{2-}. The lack of hypersensitivity to Cs^+ was unexpected and may indicate that mechanisms of Cs^+ transport in plants are distinct from those for K^+, Na^+, and Li^+.

The *sos2* and *sos3* mutants also do not have altered responses to osmotic stress as indicated by normal growth on mannitol media. It is interesting that *sos1* seedlings appeared to be more inhibited by low to medium levels of mannitol stress. Under high mannitol stress, the growth of *sos1* plants did not appear different from that of wild-type plants. Furthermore, *sos1* (Liu and Zhu, 1997b) as well as *sos2* and *sos3* accumulate more Pro under NaCl stress. It appears that the extent of Pro accumulation is correlated with the levels of salt sensitivity and stress damage. Consistent with this notion, the salt-tolerant mutants *rss* and *pst1* accumulate less Pro than the wild type under NaCl stress (Werner and Finkelstein, 1995; Tsugane et al., 1999).

Significantly, *sos1*, *2*, and *3* mutants were found to grow poorly on agar medium depleted of K^+. Of the three *sos* mutants, *sos1* plants are most sensitive to Na^+ and require the highest levels of K^+ for normal growth. Experiments using $^{86}Rb^+$ indicated that *sos1* seedlings have reduced capacity for high affinity K^+ uptake (Ding and Zhu, 1997). Similar uptake studies have failed to detect significant differences in high affinity K^+ transport between wild-type plants and *sos2* or *sos3*.

The similar spectrum of phenotypes of *sos1*, *sos2*, and *sos3* suggests that the three *SOS* genes function in the same or related processes. Double mutants were constructed between these mutants and no additive effect was found (Liu and Zhu, 1997a; Zhu et al., 1998; Halfter et al., 2000). Based on these genetic results, we have proposed that *SOS1*, *SOS2*, and *SOS3* function in the same pathway leading to Na^+ tolerance.

SOS GENES AND A REGULATORY PATHWAY FOR PLANT SALT TOLERANCE

SOS3

SOS3, the first *SOS* gene cloned, encodes a Ca-binding protein with three predicted EF-hands (Liu and Zhu, 1998). Functionally characterized proteins that are most similar to the SOS3 gene product are the B-subunit of calcineurin (Mendoza et al., 1994) and animal neuronal Ca sensors (NCS) (Schaad et al., 1996). Calcineurin is a Ca^{2+}- and calmodulin-dependent protein phosphatase consisting of a catalytic A-subunit (CnA) and a regulatory B-subunit

(CnB), which has four high affinity EF-hand Ca-binding sites (Klee et al., 1988). Full activation of CnA requires Ca-CnB as well as Ca-calmodulin complexes. NCS represents a large subfamily of EF-hand Ca-binding proteins that are expressed mainly in neurons of the brain or in photoreceptor cells. In vitro experiments have shown that NCS proteins are capable of stimulating the protein phosphatase CnA or inhibiting protein kinases (Schaad et al., 1996).

Calcineurin activity is critical for many Ca-regulated processes, including T-cell activation, neutrophil chemotaxis, and apoptosis (Hemenway and Heitman, 1999). The immunosuppressants cyclosporin A and FK506 when associated with the respective binding proteins, inhibit calcineurin in lymphocytes. This inhibition prevents activation of NFAT, a transcription factor that is necessary for the proliferation of T cells. Specifically, dephosphorylation of NFAT by calcineurin results in NFAT being translocated from the cytoplasm to the nucleus where it induces expression of cytokine genes. In other cell types, calcineurin has been implicated in the control of ion homeostasis. Evidence indicates that α-adrenergic receptor activation by norepinephrine causes an increase in cytosolic Ca and an activation of calcineurin. This then may directly or indirectly lead to dephosphorylation and activation of Na^+/K^+-ATPase to bring about Na^+ retention in the mammalian kidney.

In yeast, calcineurin regulates K^+, Na^+, and Ca^{2+} homeostasis and pheromone response (Mendoza et al., 1994; Stathopoulos and Cyert, 1997). Loss of function mutations in CnB confer increased sensitivity of yeast cells to Na^+ and Li^+ inhibition (Mendoza et al., 1994). Calcineurin is required for the transcriptional induction of genes encoding a Na^+-ATPase, Ca^{2+}-ATPases, and a cell wall β-1,3 glucan synthase. A downstream zinc-finger transcription factor, CRZ1, participates in the transcriptional induction of these genes (Stathopoulos and Cyert, 1997).

The yeast calcineurin has also been implicated in the regulation of K^+ transport systems under salt stress. Under Na^+ stress, the K^+ uptake system is converted into a high affinity mode of K^+ transport that results in higher K^+/Na^+ discrimination, thereby reducing the influx of Na^+ (Mendoza et al., 1994). It has been proposed that calcineurin directly or indirectly regulates the phosphorylation status of TRK1, a high affinity K^+ transporter in yeast cells.

Despite its functional and sequence similarities with the regulatory subunit of calcineurin (CnB), the mechanism of SOS3 function may be different for the following reasons: (a) The CnA-binding region of CnB is very conserved but appears to be absent in SOS3; (b) Expression of a *SOS3* cDNA in yeast using a multicopy plasmid did not complement the yeast cnb−phenotype (P. Hasegawa, personal communication); and (c) SOS3 is required for plant survival under K^+ starvation (Liu and Zhu, 1997a), whereas CnB is not necessary for yeast to cope with K^+ starvation (Mendoza et al., 1994). In fact, SOS3 recently has been found to physically interact with and to activate a protein kinase encoded by *SOS2* (Halfter et al., 2000).

Recombinant SOS3 protein expressed in bacteria is capable of binding $^{45}Ca^{2+}$ (Ishitani et al., 2000), although the binding is quite weak compared with other EF-hand-type Ca-binding proteins. Also unlike many other EF-hand-type Ca-binding proteins, SOS3 protein does not exhibit a Ca- or EGTA-induced mobility shift on SDS-PAGE. These unusual properties of SOS3 are not surprising in light of its unique sequence feature: The second consensus acidic amino acid residues (D or N) in all three Ca-binding loops are replaced by basic residues (K or R) in SOS3 (Liu and Zhu, 1998). The novel Ca-binding properties of SOS3 are likely important in determining the specificity of Ca signaling under Na^+ stress.

Another prominent feature of the deduced amino acid sequence of SOS3 is that it contains a myristoylation motif at the amino terminus. Both CnB and NCS contain such a motif and are myristoylated, although no functional significance has been found for the myristoylation of CnB in yeast. In vitro translation of *SOS3* mRNA in the presence of radiolabeled myristic acid and *N*-myristyl transferase showed that SOS3 can be myristoylated at Gly-2 (Ishitani et al., 2000). Myristoylation is necessary for SOS3 function because when Gly-2 was mutated to Ala to abolish myristoylation, the resulting mutant *SOS3* gene could not functionally complement the *sos3-1* mutant phenotype (Ishitani et al., 2000). No substantial difference was detected between the membrane association of myristoylated and non-myristoylated SOS3. However, these experiments may not detect subtle differences. Myristoylation could enhance the association of SOS3 with certain membrane micro domains, e.g. near a Ca^{2+} channel for more efficient and/or specific Ca signaling.

SOS2

The *SOS2* gene encodes a Ser/Thr protein kinase of 446 amino acids with an estimated molecular mass of 51 kD (Liu et al., 2000). The N-terminal approximately 270 amino acids of SOS2 comprise its kinase catalytic domain, and the remaining C-terminal region is its regulatory domain. The catalytic domain is very similar to the yeast SNF1 (sucrose nonfermenting 1) and mammalian AMP-activated protein kinase (AMPK). In yeast, SNF1 responds to Glc starvation by activating the transcription of Glc-repressed genes (Celenza and Carlson, 1986). The mammalian AMPK is a homolog of SNF1 and is involved in the protection of cells against stresses that deplete ATP, such as heat shock, hypoxia, and oxidative stress (Mitchelhill et al., 1994). AMPK is activated by elevated AMP to ATP ratio and inhibits biosynthetic pathways to preserve ATP for essential

cellular processes. Although SOS2, SNF1, and AMPK all function in stress responses, SOS2 is clearly different from SNF1/AMPK in sequence, function, and regulation. First, SOS2 functions in Na^+ and K^+ homeostasis and Na^+ tolerance but not in metabolism. Second, the regulatory domain of SOS2 is different from that of SNF1/AMPK. Third, SOS2 activity is regulated by Ca^{2+}, whereas AMPK is regulated by AMP. SNF1/AMPK homologs that are highly similar to SNF1 and AMPK throughout their entire sequence do exist in plants (Halford and Hardie, 1998).

The kinase domain of SOS2 is necessary for function because the *sos2*-5 mutant allele (Gly-197 changed to Glu), when expressed in bacteria, produces a protein SOS2(G197E) that does not show autophosphorylation (Liu et al., 2000). Because *sos2*-5 is a recessive mutation (Zhu et al., 1998), this suggests that kinase activity is required for SOS2 function in plant salt tolerance.

The C-terminal regulatory domain of SOS2 is also essential for the protein to function in plant salt tolerance (Liu et al., 2000). Mutations in several loss-of-function alleles of *SOS2* disrupt only its regulatory domain and appear to leave the catalytic domain intact. In the *sos2-1* mutant allele, 29 amino acids were inserted between Glu-390 and Ile-391. The *sos2-2* and *sos2*-3 mutations result in truncated polypeptides of 287 and 262 amino acids, respectively.

SOS1

Like *SOS3* and *SOS2*, the cloning of *SOS1* was accomplished through a map-based approach. Even though several *sos1* mutant lines were recovered from a T-DNA insertion population, the T-DNA did not cosegregate with the *sos1* mutant phenotype (Zhu et al., 1998). *SOS1* encodes a putative Na^+/H^+ antiporter with a predicted molecular mass of 127 kD (Shi et al., 2000). The N-terminal region of SOS1 is hydrophobic and may contain 10 to 12 transmembrane domains, depending on the prediction program used for the analysis. The transmembrane region has sequence similarities with Na^+/H^+ antiporters from microorganisms and animals. A putative Na^+-binding region is conserved between SOS1 and other Na^+/H^+ antiporters. No amiloride-binding site can be discerned in the SOS1 sequence. SOS1 is distinct from the AtNHX family of tonoplast Na^+/H^+ antiporters that were recently characterized (Apse et al., 1999; Gaxiola et al., 1999). SOS1 appears to represent a new class of Na^+/H^+ antiporters that may function at the plasma membrane (Shi et al., 2000).

A unique feature of the SOS1 sequence is its long hydrophilic tail that is predicted to reside in the cytoplasm. Mammalian Na^+/H^+ antiporters contain relatively shorter cytoplasmic tails that are known to interact with a variety of proteins including protein kinases, molecular chaperones, and Ca-binding proteins (Schmitt et al., 1996). The long tail of SOS1 would provide ample opportunity for interaction with a multitude of proteins that are expected to regulate its antiport activity.

The steady-state level of *SOS1* transcript is up-regulated by NaCl stress (Shi et al., 2000). Unlike many other salt stress-responsive genes, this regulation is very specific to NaCl and does not occur under ABA or cold stress treatment (Shi et al., 2000). It is not known if the NaCl up-regulation is at the transcriptional level.

Studies on the tissue-specific expression of *SOS1* using a β-glucuronidase reporter under control of *SOS1* gene promoter have provided very interesting results (H. Shi and J.-K. Zhu, unpublished data). β-Glucuronidase activity is primarily detected in cells bordering the xylem elements. This expression pattern suggests that SOS1 functions in either loading Na^+ into the xylem or retrieving Na^+ from the xylem. SOS1 expression in cells surrounding the xylem also helps explain the K^+ transport phenotype of *sos1* mutants. It is known that K^+ and Na^+ transport at the symplast/xylem boundary is tightly linked, perhaps through H^+ cycling between K^+/H^+ symporters and Na^+/H^+ antiporters (Lacan and Durand, 1996). Therefore, it is probable that the transport and growth defect of *sos1* on low K^+ is caused by defective K^+ loading into the xylem.

A REGULATORY PATHWAY FOR Na^+ TOLERANCE

Besides identifying genes and gene products, an important goal of salt stress research should be to try to connect the various gene products to learn about the pathways and networks that underlie salt stress responses. These pathways and networks are not only necessary for a comprehensive understanding of salt stress tolerance, but also form the necessary basis for rationale approaches to genetic improvement of plant salt tolerance.

After *SOS3* was cloned, its gene product was used as a bait to identify its interacting proteins via the yeast two-hybrid approach (Halfter et al., 2000). A large number of related protein kinases were found to interact with SOS3. However, such interactions in the yeast two-hybrid system or in vitro may not necessarily reflect any in planta interaction or function. It was fortunate that when the *SOS2* gene was cloned later, it became clear from its sequence that it belongs to the family of SOS3-interacting protein kinases. However, SOS2 was not among those protein kinases discovered by two-hybrid library screening. Even though SOS2 does interact with SOS3, the *SOS2* transcript was under-represented in the two-hybrid library because of its low level of expression (Halfter et al., 2000). In fact, the interaction between SOS2 and SOS3 is stronger than that between SOS3 and any of the other protein kinases. SOS2-SOS3 interaction is mediated through the regulatory domain of SOS2.

An outcome of SOS3 binding to SOS2 is that SOS2 protein kinase activity is activated (Halfter et al., 2000). Without SOS3, SOS2 has virtually no activity with several peptide substrates. In the presence of SOS3, SOS2 becomes capable of phosphorylating the peptides. Although the binding between SOS2 and SOS3 appears to be independent of free Ca^{2+}, SOS2 phosphorylation of the peptide substrates requires Ca^{2+} (Halfter et al., 2000). It is likely that SOS3 and SOS2 always form a protein kinase complex and, in this sense, they should be considered as subunits of a multisubunit enzyme. Whether this enzyme complex contains other proteins remains to be determined.

SOS3 interaction with and activation of SOS2 kinase is very consistent with genetic evidence that the two genes are both positive regulators of salt tolerance and function in the same pathway (Zhu et al., 1998; Halfter et al., 2000). These two proteins together define a previously unknown regulatory pathway for plant Na^+ tolerance (Fig. 1). Since it is known that salt stress elicits a rise in the concentration of cytosolic-free Ca^{2+} (Läuchli, 1990; Knight et al., 1997), we anticipate that Ca^{2+} will be a direct input signal for this SOS pathway. Such a Ca^{2+} signal does not appear to be very different from that elicited by drought (Knight et al., 1997). However, specificity for Na^+ stress might be found in subtle differences between the Ca^{2+} signals, e.g. subcellular location, frequency, and amplitude of the Ca^{2+} oscillation. The fact that the *sos3* mutation (i.e. mutation in the Ca^{2+} sensor) impairs Na^+ tolerance but has no effect on osmotic stress tolerance strongly suggests the existence of a Ca^{2+} signal that is specific for Na^+ stress (Liu and Zhu, 1998). The receptor(s) for Na^+ stress is not known in either plants or yeasts. The connection between Na^+ sensing and the generation of a Ca^{2+} signal are equally unknown. It is even unclear whether Na^+ is sensed inside or outside the cell. In *E. coli*, Na^+ appears to be sensed internally by the *NhaR* gene product, a transcription factor that binds to the promoter of the NhaA Na^+/H^+ antiporter (Schuldiner and Padan, 1996). In plant cells, it is possible that Na^+ is sensed by a transporter(s) for K^+, Na^+, or Ca^{2+}.

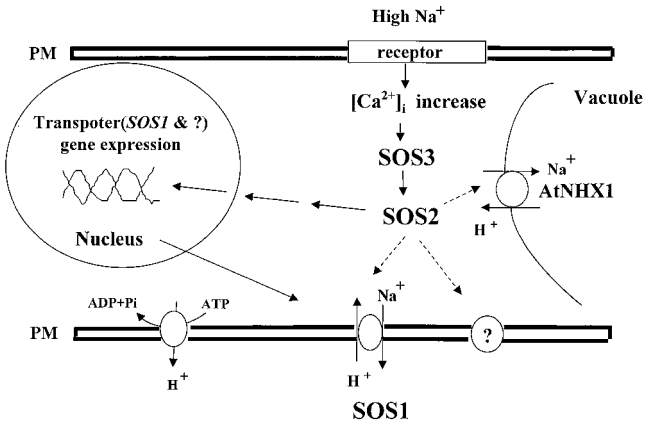

Figure 1. Diagram of the SOS pathway for plant Na^+ tolerance. High Na^+ stress is sensed either externally or internally (not shown) and somehow leads to an increase of cytosolic free Ca^{2+} concentration. SOS3 binds to this Ca^{2+} and activates the protein kinase SOS2. Activated SOS3-SOS2 kinase complex is necessary for increased expression of *SOS1* and perhaps other transporter genes under salt stress. The SOS3/SOS2 pathway may also regulate the activities of SOS1 and other transporters at the post-translational level. This gene expression and transporter activity regulation brings about homeostasis of ions such as Na^+ and K^+ and consequently plant tolerance to Na^+ stress.

Based on the phenotypes of the *sos2* and *sos3* mutants, the output of the SOS3/SOS2 regulatory pathway is expected to be modulation of abundance and/or activity of certain K^+ and Na^+ transporters. Many K^+ and Na^+ transporters have been cloned. Because there is little information about their function in Na^+ tolerance, it is difficult to tell which ones might be regulated by the SOS pathway.

One output of the regulatory pathway is up-regulation of *SOS1* Na^+/H^+ antiporter gene expression by salt stress (Shi et al., 2000). In the *sos3-1* mutant background, no such up-regulation could be found. Mutations in *SOS2* also seem to prevent NaCl induction of *SOS1* transcript in the shoot but not in the root. In the root, there may be a functionally redundant SOS2-like protein kinase. How the pathway modulates *SOS1* gene induction remains to be determined. If the induction is transcriptional, then a transcription factor in the pathway needs to be identified. The SOS3/SOS2 regulatory pathway may also control the expression of other genes, particularly other transporter genes. This awaits comprehensive gene expression profiling of *sos2* and *sos3* mutants and the wild type using DNA micro-arrays or DNA chips.

In addition to controlling gene expression under NaCl stress, the SOS3/SOS2 pathway might be expected to regulate SOS1 and other transporter activities at the post-translational level. Regulation of activity would be faster and may be important for more rapid responses needed to cope with salt stress. Whether SOS3 and/or SOS2 directly interact with and modulate SOS1 antiport activity should be tested. Additional unidentified components in the pathway may be required for such regulations. When any of the other Na^+ or K^+ transporters are determined to function in Na^+ tolerance, they would also be good candidates to directly test for gene expression or activity regulation by the SOS3/SOS2 pathway.

RELATIONSHIP WITH OTHER PATHWAYS

High concentrations of salts cause hyperosmotic as well as ionic stresses. The osmotic and ionic stresses in turn may generate secondary effects such as oxidative stress. The SOS3/SOS2 pathway mediates specifically tolerance to ionic stress, particularly Na^+ stress. This pathway is therefore distinct from those defined by gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997) or genetic analysis (Ishitani et al., 1997) for osmotic, cold, and ABA re-

sponses. It is also different from other protein kinase pathways for stress, such as CDPK, AtDBF2, or mitogen-activated protein kinase (for review, see Halfter et al., 2000). None of the other kinases function specifically in Na^+ tolerance. Notwithstanding, the SOS3/SOS2 pathway may "cross-talk" with the other stress-signaling cascades. The SOS pathway may also interact with pathways for cell division and expansion, because it eventually regulates plant growth under salt stress.

SOS3 AND THE EFFECT OF EXTERNAL Ca^{2+}

External Ca^{2+} enhances plant salt tolerance (Läuchli, 1990). High levels of extracellular Ca^{2+} exert numerous effects on plant cells, many of which may be correlated with alleviating Na toxicity. These effects include, for example, improved K and Ca^{2+} nutrition, and reduced cellular Na content. Many of the effects of extracellular Ca^{2+} in relieving salt toxicity are likely achieved by activating signaling pathways for K^+ and Na^+ transport, which includes regulation of influx, efflux, and compartmentation of these ions. The *SOS3* gene has been proposed to be involved in mediating the beneficial effect of Ca^{2+} (Liu and Zhu, 1997a, 1998). A loss-of-function mutation in this gene (i.e. *sos3-1*) increases the level of extracellular Ca^{2+} required to relieve salt stress (Liu and Zhu, 1997a). Whereas the exact mechanism underlying the connection between extracellular Ca and the SOS3 protein, an apparently intracellular signaling molecule (Liu and Zhu, 1998), is unclear, the dramatic effect of *sos3-1* mutation on the Ca requirement unequivocally reveals such a connection. Na^+ stress is known to cause Ca depletion in the extracellular space and the outer surface of the plasma membrane (Läuchli, 1990). An important function of high levels of external Ca might be to compensate for this depletion, thus ensuring Ca influx for the activation of intracellular signaling pathways that control adaptive mechanisms such as the regulation of K and Na transport. The *sos3-1* mutation is a deletion in a Ca-binding domain and it reduces Ca binding of the SOS3 protein (Ishitani et al., 2000). Increased extracellular Ca has been shown to elevate the cytosolic-free Ca to higher levels, which presumably overcomes the Ca-binding defect of *sos3-1*, thereby partially rescuing the mutant phenotype. It is also possible that at higher levels of external Ca, cells use an alternative signaling mechanism that by-passes the SOS3 pathway. Furthermore, Ca may also exert its protective effect against Na^+ stress in ways that are independent of SOS3.

CONCLUSION AND PERSPECTIVES

Mutational analysis in Arabidopsis has yielded a number of single gene mutations conferring salt tolerance or hypersensitivity. Cloning and characterization of some of the *SOS* genes has uncovered a novel Ca-dependent protein kinase pathway for the regulation of ion homeostasis and plant salt tolerance. It is important that new mutations affecting salt tolerance continue to be isolated by using variations of existing genetic screens or completely new screens. As more mutations are discovered and cloned, gaps in the SOS pathway can be filled, and additional pathways may emerge. Increasingly, this endeavor will benefit from the use of reverse genetics as the Arabidopsis genome sequencing approaches completion and more gene knock-out lines become available and are examined under salt stress. Mutants resulting from the forward and reverse genetics should be used in genome scale gene expression profiling and proteome scale protein expression profiling, to increase our understanding of the complex topics of salt tolerance.

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