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Salt Tolerance

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Abstract

Studying salt stress is an important means to the understanding of plant ion homeostasis and osmo-balance. Salt stress research also benefits agriculture because soil salinity significantly limits plant productivity on agricultural lands. Decades of physiological and molecular studies have generated a large body of literature regarding potential salt tolerance determinants. Recent advances in applying molecular genetic analysis and genomics tools in the model plant *Arabidopsis thaliana* are shading light on the molecular nature of salt tolerance effectors and regulatory pathways.

Introduction

Plants need essential mineral nutrients to grow and develop. However, excessive soluble salts in the soil are harmful to most plants. In fact, no toxic substance restricts plant growth more than does salt on a world scale. It is estimated that salinity affects at least 20% of world's arable land and more than 40% of irrigated land to various degrees (Rhoades and Loveday, 1990). In extreme cases, productive agricultural land could no longer sustain agricultural production and had to be abandoned. This may have contributed to the decline of some human civilizations in history. In coastal areas periodic invasions of seawater directly add salts to the soil. Soils in other semi-arid or arid regions, especially those with ineffective drainage, accumulate salts as irrigation water evaporates, leaving deposits of soluble salts.

Based on their capacity to grow on high salt medium, plants are traditionally classified as glycophytes or halophytes (Flowers et al., 1977). Halophytes are tolerant to high concentrations of NaCl; some can withstand salts that are more than twice the concentration of seawater. Most plants, including the majority of crop species, are glycophytes and cannot tolerate high salinity. For glycophytes, salinity imposes ionic stress, osmotic stress, and secondary stresses such as nutritional disorders and oxidative stress (Zhu, 2001a). Sodium toxicity represents the major ionic stress associated with high salinity. Additionally, some plant species are also sensitive to chloride, the major anion found in saline soils. In certain saline soils, ion toxicity is worsened by alkaline pH. The low osmotic potential of saline solutions hampers plant water uptake, resulting in "physiological drought." For halophytic plants that are tolerant of sodium toxicity, osmotic stress may be the main cause of growth inhibition.

Understanding the mechanisms of plant salt tolerance will lead to effective means to breed or genetically engineer salt tolerant crops. Salt tolerance research also represents an important part of basic plant biology, contributing to our understanding of subjects ranging from gene regulation and signal transduction to ion transport, osmoregulation and mineral nutrition. Additionally, some aspects of salt stress responses are intimately related to drought and cold stress responses (Zhu, 2001b). Plant salt tolerance studies thus contribute to understanding mechanisms of cross-tolerance of abiotic stresses.

Salt stress studies generally fall into the following four categories: 1) Physiology of salt toxicity and salt tolerance. This includes cellular and metabolic responses to salt (Bohnert and Sheveleva, 1998; Hasagewa et al., 2000), as well as whole plant responses (Flowers et al., 1997; Greenway and Munns, 1980; Yeo, 1998). 2) Mechanisms

of salt transport across cellular membranes and over long distances in plants. This includes physiological and molecular characterization of various ion transporters involved in salt uptake, extrusion, compartmentalization, and in the control of long distance transport (Blumwald et al, 2000; Schachtman and Liu, 1999). 3) Survey of genes whose expression is regulated by salt stress (Zhu et al., 1997; Xiong and Zhu, 2001; Shinozaki and Yamaguchi-Shinozaki, 1997; Ingram and Bartels, 1996; Bray, 1997; Bohnert et al., 1995). More recently, research in this aspect is being accelerated by using gene chips and cDNA microarrays (Seki et al., 2001; Kawasaki et al., 2001; Bohnert et al., 2001). 4) Mutational analysis of salt tolerance determinants and salt stress signaling (Zhu, 2000; 2001a, b; Xiong and Zhu, 2001). This overview will emphasize recent progress in the genetic analysis of salt tolerance in Arabidopsis.

Salt Stress and Arabidopsis

Arabidopsis is a glycophytic plant that is sensitive to growth inhibition and damage by salt stress. Although salt sensitivity among Arabidopsis ecotypes/accessions may exist, a systematic comparison among different ecotypes has not been reported. Given that the Arabidopsis genome has been fully sequenced, this comparison between ecotypes may localize salt tolerance QTLs (quantitative trait loci) and potentially lead to the molecular identification of some major loci, if relatively large differences in salt tolerance exist between ecotypes. Since Arabidopsis is a relatively new model for salt tolerance studies, and is not an ideal subject for physiological analysis, information on salt tolerance in Arabidopsis is rather limited. Research with other glycophytic plant species has shown that upon exposure to high salinity, plants may exhibit a reduced growth rate, accelerated development and senescence or death if the stress is severe or prolonged (reviewed by Lazof and Bernstein, 1999). Like many other glycophytes, the sensitivity of Arabidopsis to salt stress is exhibited at all stages of development. For example, a brief (8 hr) treatment with 150 mM NaCl during seed development stage resulted in callose deposition and abnormal changes in ovule and embryo structures indicative of cell death (Sun and Hauser, 2001). However, in Arabidopsis salt sensitivity is most evident at the seed germination and seedling stages. Even after cold stratification of imbibed seeds to break dominancy, Arabidopsis seed germination was greatly impaired at salt concentrations at or above 75 mM (Figure 1). After germination, seedling growth is also very sensitive to NaCl. Although lower concentrations of NaCl (< 50 mM NaCl) may have a slightly stimulating effect on fresh weight gain in culture media, higher than 50 mM of NaCl clearly inhibits plant growth (Figure 2) and eventually kills the plants.

Arabidopsis As A Model Plant To Study Salt Tolerance

With their high levels of salt tolerance, halophytes would appear to be the plants of choice to search for tolerance genes and to study salt tolerance mechanisms. A number of research groups have been studying various halophytes in an effort to clone the genes responsible for salt tolerance (Bohnert et al., 1995). However, because of the complexity of salt tolerance mechanisms and the lack of a well-defined genetic system, the potential of halophytes in revealing salt tolerance mechanisms largely remains to be realized.

Because Arabidopsis is a glycophyte and is very sensitive to salt, one might assume that this plant is not suitable for studying the mechanisms of salt tolerance. However, previous studies with cultured glycophytic plant cells indicated that these cells could be adapted to tolerate high concentrations of salt that would kill un-adapted cells. For instance, normal tobacco cells cannot grow in the pres-

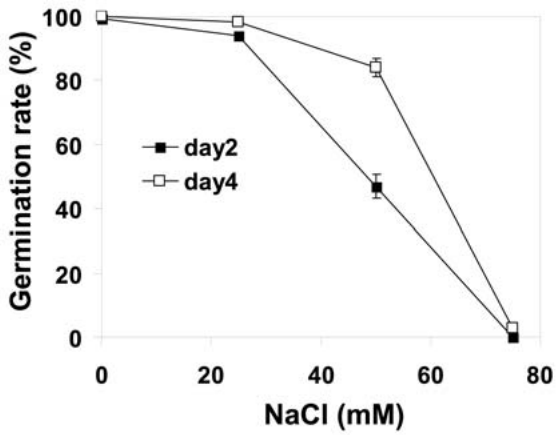

Figure 1. Germination of Arabidopsis seeds is sensitive to NaCl. Seeds of the ecotype C24 (one of the salt sensitive ecotypes) were planted on filter paper saturated with indicated concentrations of NaCl and incubated at 4°C for 2 days before being moved to room temperature (23°C) under white light for germination. Germination (radical emergence) was scored 2 (closed squares) or 4 (open squares) days after the plates being incubated at room temperature.

ence of 100 mM NaCl. After salt adaptation, some tobacco cells are able to tolerate five times that much salt (Hasegawa et al., 1994). Similarly, alfalfa cells as well as intact plants have been adapted to tolerate very high levels of NaCl (Winicov, 1991). In fact, like cold acclimation, whole plants can also become salt tolerant by being exposed to lower non-lethal concentrations of salt (e.g., Amzallag et al., 1990). Although salt adaptation process did not receive as much attention as cold acclimation did, presumably because of technical difficulties in quantitatively implementing the 'salt acclimation' treatments. Nevertheless, the fact that adaptation can increase plant salt tolerance suggests that glycophytes do have salt tolerance machinery that may not be operating effectively in un-adapted conditions. Therefore, the difference in salt tolerance between glycophytes and halophytes appears to be quantitative rather than qualitative, and basic salt tolerance mechanisms are probably conserved in all plant species. The difference in salt sensitivity/tolerance may have resulted from differences in regulatory circuits or from gene alleles coding for key salt tolerance effectors. For example, the vacuolar Na^+/H^+ antiporter gene *AtNHX1* is not as highly inducible in Arabidopsis as its homologous gene is in halophytes, and high level *AtNHX1* expression driven by the strong CaMV *35S* promoter could significantly improve Arabidopsis salt tolerance (Apse et al., 1999; Hamada et al., 2001; Shi and Zhu, 2002).

The success of Arabidopsis as a genetic model plant for developmental and disease resistance studies has attracted investigators to test its feasibility as a model to study plant salt tolerance. Screening for mutants that are tolerant to high concentrations of salt during germination represented the first such attempt. The germination salt tolerant mutants were named as *rs* (Saleki et al., 1993), *rss* (Werner and Finkelstein, 1995), or *san* (Quesada et al., 2000). Other mutant screens used the inhibitory effect of salts on root growth (*sos* mutants) (Zhu, 2000) or general seedling growth (e.g., Tsugane et al., 1999). The identification of *Salt Overly Sensitive* (*SOS*) loci has uncovered an important regulatory pathway that controls ion homeostasis and salt tolerance (Zhu, 2000). More sophisticated genetic screens such as the one using a firefly luciferase reporter driven by a salt stress-responsive promoter have been successful in identifying salt tolerance determinants that are difficult to isolate from growth inhibition-based screens (Ishitani et al., 1997). It is expected that the use of Arabidopsis as a model will continue to unravel the salt tolerance mechanisms in higher plants (Sander, 2000; Zhu, 2000).

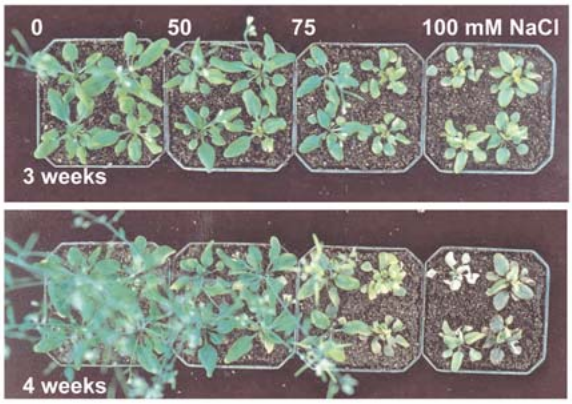

Figure 2. Sensitivity of Arabidopsis plants to salt during the vegetative stage. Two-week-old seedlings (ecotype Columbia) growing in the soil were irrigated with 0, 50, 75, and 100 mM NaCl, respectively. Pictures were taken three weeks (upper panel) and four weeks (low panel) after the treatment. Note anthancyanin accumulation in leaves, the bleaching of leaves, and retarded growth and delayed development of seedlings treated with higher concentrations of the salt.

Mechanisms of Salt Entry into Root Cells

Sodium ions are not required for the growth of most land plants. Land plants do not seem to have transport systems specifically for Na^+ uptake. However, Na^+ can still enter plant cells via several routes. Since the concentration of Na^+ in the soil solution is usually much higher than that in the cytosol of root cells, Na^+ movement into root cells is passive. Current evidence suggests that Na^+ enters root cells mainly through various cation channels. These channels could be voltage-dependent cation channels or voltage-independent cation channels (VIC). Among them, VIC channels are considered the major route for Na^+ entry into plant cells (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; White, 1999). Whereas the molecular identities of the VIC type non-selective cation channels are not known, those of voltage-dependent ion channels that facilitate Na^+ entry have been understood to some extent. Due to the similarity between Na^+ and K^+, voltage-dependent K^+ inward rectifiers or outward rectifiers appear to be one path for Na^+ entry into root cells (reviewed in Blumwald et al., 2000). For example, in wheat roots, the K^+ transporter HKT1 may function as a low affinity Na^+ transporter at high external Na^+ (Rubio et al., 1995). The homolog in Arabidopsis, AtHKT1, mediated Na^+ uptake when expressed in *Saccharomyces cerevisiae* or *Xenopus* oocytes (Uozumi et al., 2000). However, the function of AtHKT1 *in planta* is unclear. In a screen for second site suppressor mutations

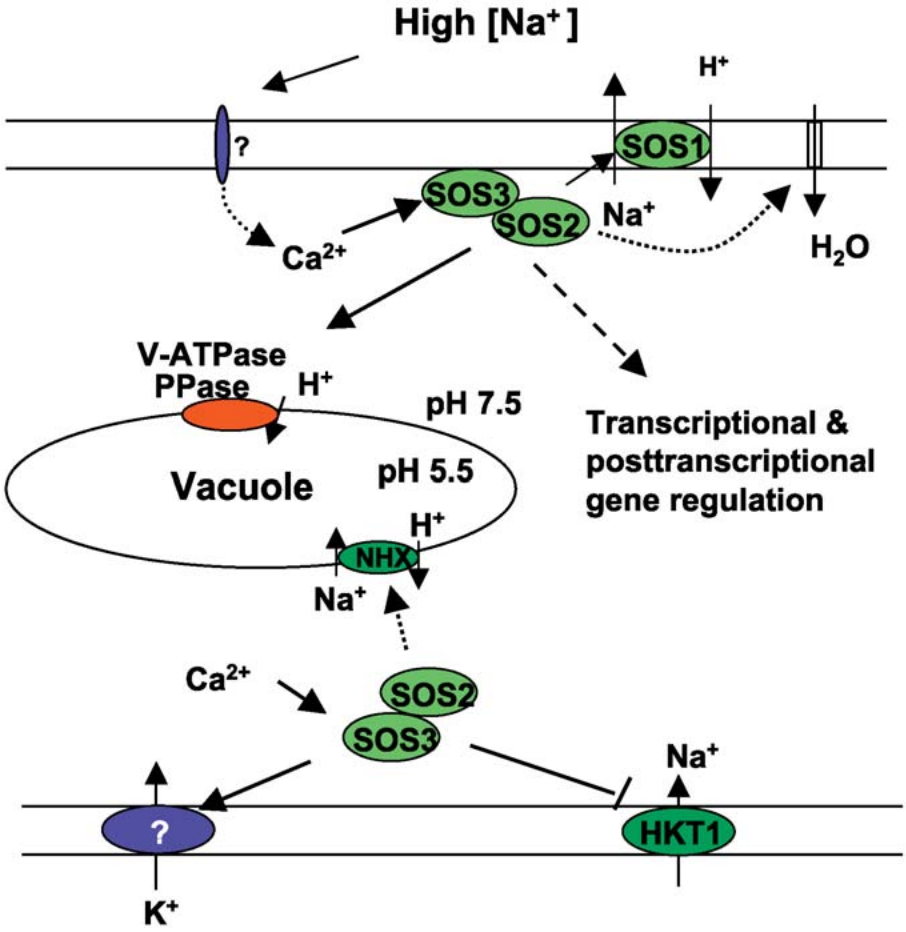

Figure 3. The SOS pathway functions in ion homeostasis under salt stress. High extracellular concentrations of salt elicit a rise in cytosolic Ca^{2+} concentrations. The Ca^{2+} sensor SOS3 upon the perception of this Ca^{2+} signal interacts with and activates the protein kinase SOS2. Activated SOS2 then regulates the activities of ion transporters or transcriptional activators to regulate ion homeostasis or gene expression. The SOS2 targets include the SOS1 Na^+/H^+ antiporter, the vacuolar Na^+/H^+ exchangers NHX, and the Na^+ transporter HKT1. Other potential targets include tonoplast ATPase and pyrophosphtases, water channels, and potassium transporters.

of salt sensitive phenotypes of *sos3* (see later sections), one of the suppressors was identified as a lesion in AtHKT1 (Rus et al., 2001). The *hkt1* suppressor mutant has a lower Na^+ content, implying that AtHKT1 mediates Na^+ uptake into plants. This study also suggests that wild type SOS3, likely by acting together with SOS2, may repress the activity of AtHKT1 in allowing Na^+ entry into root cells (Figure 3). It is thus expected that *hkt1* mutations would also suppress the salt sensitive phenotype of *sos2*.

Besides being regulated by the SOS module (see later sections), Na^+ uptake or exclusion and hence plant salt tolerance are also directly or indirectly modulated by many other factors. These factors include membrane potential, acidity of the external solution, variety and concentration of salts, and plant hormones (see below) (reviewed in Xiong and Zhu, 2002a). Here we briefly discuss the effect of one of these factors, membrane potential, on plant salt tolerance.

Under normal conditions, the plasma membrane potential (MP) of root cells is maintained at ~ -130 mV. Apparently, a more negative potential would facilitate entry of the positively charged Na^+ into cells. MP in plant cells

is generated and maintained by P-type ATPases, which pump H^+ out of the cell and create an electrochemical potential facilitating the uptake of solutes. Some transporters affect salt sensitivity indirectly by altering MP as a result of regulation of ion flux. For example, in yeast *trk1*/*trk2* mutants that are defective in K^+ uptake, the plasma membrane becomes hyperpolarized and this greatly enhances the uptake of cations and rendered the mutants more sensitive to Na^+, Li^+, and low pH (Serrano et al., 1999). An interesting observation was reported also in yeast where a deletion in the *PMP3* gene resulted in hyperpolarization of the membrane as measured by methylammonium uptake. The yeast mutant was more sensitive to salt stress due to increased uptake of Na^+ (Navarre and Goffeau, 2000). As expected, this effect on uptake was not specific to Na^+. The *pmp3* mutation also suppressed the increased K^+ requirement in the *trk1* and *trk2* mutants, presumably because membrane hyperpolarization enabled K^+ uptake through other transporters. Interestingly, Ca^{2+} can reverse the salt sensitivity in the *pmp3* mutant. PMP3 is a small hydrophilic protein predicted to be localized on the plasma membrane. It is not known how this protein can regulate membrane potential. PMP3 is homologous to the plant proteins RCI2A and RCI2B in Arabidopsis (Medina et al., 2001; Nylander et al., 2001) and BLT101 in barley (Goddard et al., 1993). In fact, the Arabidopsis RCI2A can complement the yeast *pmp3*/*sna1* salt sensitive phenotype (Navarre and Goffeau, 2000; Nylander et al., 2001). Interestingly, the expression of *RCI2A* and *RCI2B* genes in Arabidopsis was transiently induced by low temperature or salt stress (Medina et al., 2001; Nylander et al., 2001). However, Nylander et al. (2001) reported that a mutation in the yeast homologous gene *SNA1*, which is the same gene as *PMP3*, makes the yeast sensitive specifically to NaCl, since the mutant was not sensitive to either 1 M KCl or 0.6 M LiCl (Nylander et al., 2001). It would be interesting to see whether mutations in Arabidopsis *RCI2A* and *RCI2B* also yield similar salt sensitive phenotypes. Future studies on the mechanism of RCI2 function might shed light on salt stress regulation by membrane potentials.

Dealing with Ion Toxicity

Restoring ion homeostasis in plants disturbed by salt stress represents an acute response, both in terms of time and the importance in dealing with overall salt stress effects. Plants employ several ways to combat ionic stress imposed by high salinity. These include restricting salt uptake, increased extrusion and compartmentalization, and controlled long distance transport to aerial parts. Additionally, to avoid cellular damage and nutrient deficiency, plant cells need to maintain adequate K^+ nutrition and a favourable K^+/Na^+ ratio in the cytosol (Niu et al., 1995; Serrano et al., 1999).

Excessive sodium ions at the root surface may disrupt plant potassium nutrition that is vital for the maintenance of cell turgor, membrane potential, and the activities of many enzymes (reviewed by Lazof and Bernstein, 1999). Because of the similar physic-chemical properties of sodium and potassium ions, sodium at a high concentration has a strong inhibitory effect on potassium uptake by the root. For example, K^+ uptake via Arabidopsis KUP1 (Kim et al., 1998; Fu and Luan, 1998), which mediates both high and low affinity K^+ transport, was inhibited by 5 mM or higher concentrations of NaCl (Fu and Luan, 1998). Plants use both low and high affinity systems for potassium uptake. Sodium ions may have a more damaging effect on the low affinity system that has low potassium/sodium selectivity. Under sodium stress, it is necessary for plants to operate the more selective high affinity potassium uptake system in order to maintain adequate potassium nutrition. It is a general phenomenon that salt treatment of plants causes a decrease in cellular K^+ content (Figure 4), which may be partly responsible for reduced growth and vigour under salt stress.

Sodium, once enters into the cytoplasm, has a strong inhibitory effect on the activity of many enzymes. This inhibition is also dependent on potassium level in the cytoplasm: a high sodium/potassium ratio is most damaging. Even for halophytes that accumulate large quantities of sodium inside the cell, their cytosolic enzymes are just as sensitive to sodium as enzymes of glycophytes. This implies that halophytes have to compartmentalize sodium into the vacuole, away from cytosolic enzymes, which is precisely what has been found (Flowers et al., 1977).

An important determinant for plant salt tolerance that is particularly relevant to sodium and potassium homeostasis is calcium. It has long been observed both in solution culture and in soils that increased calcium supply has a protective effect on plants under sodium stress. Yet these experiments did not distinguish whether Ca^{2+} acted extracellularlly or intracellularly. Recently, experiments with altered cellular Ca^{2+} homeostasis imply that internal/cytosolic Ca^{2+} is important to salt sensitivity regulation. For example, when the Arabidopsis *ACA4* gene that codes for a vacuolar Ca^{2+}-ATPase was expressed in yeast, it increased the salt tolerance of the yeast cells (Geisler et al., 2001). Arabidopsis vacuolar Ca^{2+}/H^+ antiporter gene *CAX1*, when overexpressed in tobacco, led to increased sensitivity to ionic stress in the transgenics. These transgenic plants appeared to be Ca^{2+}-deficient despite a higher total Ca^{2+} content (Hirschi, 1999). Similarly, overexpression of the Arabidopsis ionotropic glu-

tamate receptor GluR2 reduced the utilization efficiency of Ca^{2+} and resulted in Ca^{2+}-deficient and salt sensitive phenotypes (Kim et al., 2001). The resulting physiological deficiency of Ca^{2+} may have been due to an alteration in the threshold requirement for this cation. Since different organelles within the cell may have different tolerance to Na^+, it is of interest to know whether the tolerance has to do with Ca^{2+} concentration in organelles.

Calcium also sustains potassium transport and potassium/sodium selectivity in sodium-challenged plants. The underlying mechanism for maintaining at least a threshold level of cytosolic potassium in plant cells under sodium stress seems to be similar to that operating in the unicellular yeast *Saccharomyces cerevisiae*. Sodium stress is sensed by an as yet unknown receptor(s). An early detectable response to sodium stress is a rise in the cytosolic free calcium concentration (Knight, 2000). This calcium signal serves as a second messenger that is believed to turn on machineries that control, for example, potassium uptake and potassium/sodium discrimination. In Arabidopsis, genetic studies suggested that the sensor protein for this salt-induced calcium signature is the Ca^{2+}-binding protein SOS3. A loss-of-function mutation in this protein renders the plant hypersensitive to salt stress. SOS3 is functionally similar to the B subunit of the protein phosphatase calcineurin (Liu and Zhu, 1998), although SOS3 does not function through a phosphatase (Halfter et al., 2000). In yeast cells, calcineurin upon activation by calcium modifies potassium transporters (Mendoza et al., 1994). As a result, low affinity potassium transport that cannot sufficiently discriminate potassium from sodium is turned off, while high affinity potassium transport is turned on. High affinity potassium transporters have the ability to take up potassium in the presence of higher concentrations of Na^+. Although it has not been demonstrated experimentally, similar processes are believed to occur in plant cells following calcium activation of SOS3. Besides the regulation of potassium transport, the SOS3 pathway in plants or calcineurin pathway in yeast is also important in activating transporters that remove sodium from cells. Such transporters in Arabidopsis include for example, sodium/proton antiporters such as SOS1. Consistent with this, the Na^+ efflux rate in *sos3* was much slower than that in the wild type (Elphick et al., 2001).

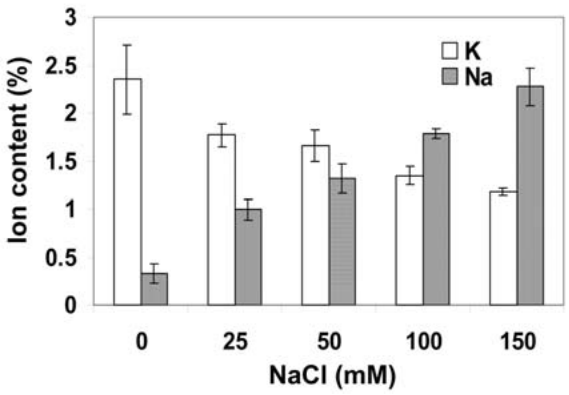

Figure 4. Salt stress impairs K nutrition in Arabidopsis. With increased concentration of NaCl in the culture medium, Na^+ content in plants increases whereas K content decreases. Arabidopsis seedlings (ecotype Columbia) growing in ? strength of the MS solution (pH 5.3) for two weeks were treated with NaCl by adding NaCl into the culture solution to the indicated final salt concentrations. Seedlings were allowed to grow for 3 days before harvesting and analyzing ion contents (dry weight basis). Data are means ± SE (n=3).

Extrusion of sodium out of the cell is a straightforward way to avoid Na^+ accumulation in the cytosol. It is expected that sodium extrusion may be more important in certain cells, for example, root epidermal cells. This is because most other cells in the plants are surrounded by neighbouring cells and sodium extruded by one cell would become a problem for its neighbouring cells. Indeed, SOS1 promoter-GUS analysis showed that the plasma membrane Na^+/H^+ antiporter gene is preferentially expressed in epidermal cells surrounding the root tip, and in parenchyma cells bordering the xylem throughout the plant (Shi et al., 2002a).

Sodium extrusion is achieved by sodium/proton antiporters on the plasma membrane. In Arabidopsis, this is accomplished by the plasma membrane-localized Na^+/H^+ antiporter SOS1 (Shi et al., 2000; 2002a). Mutations in *SOS1* rendered the mutant plants very sensitive to Na^+ (Wu et al., 1996). Recent studies (Qiu et al., 2002) indicate that plasma membrane vesicles from Arabidopsis plants have a Na^+/H^+ antiporter activity. This activity is enhanced by pre-treatment with salt stress. More importantly, the *sos1* mutation reduces the antiporter activity drastically, demonstrating that SOS1 does function as a plasma membrane Na^+/H^+ antiporter.

To further explore the role of SOS1 in combating salt stress, the gene was overexpressed in wild type Arabidopsis. It was found that plants overexpressing SOS1 have markedly improved tolerance to salt stress (H. Shi and J.-K. Zhu, unpublished). Analysis of the transgenic plants revealed that these lines had a lower Na^+ content in the shoot upon treatment with Na^+ (H. Shi and J.-K. Zhu, unpublished). These results provide evidence that SOS1 as a Na^+ efflux carrier is vital for plant salt tolerance.

Salt Compartmentalization and Long Distance Transport

Another important mechanism to reduce accumulation of cytosolic Na^+ is to store it in the vacuole where the Na^+ no longer contacts cytosolic enzymes. Compartmentalization of sodium into the vacuole not only separates Na^+ from cytosolic enzymes, it also helps balance against the low extracellular osmotic potential created by salt stress. The ability to compartmentalize Na^+ in the vacuole is likely the principle determinant of salt tolerance capacity in most plant species. Vacuolar compartmentalization of Na^+ is achieved by the action of Na^+/H^+ antiporters on the tonoplast - the vacuolar membrane. The proton gradient that drives the antiporter is generated by tonoplast H^+-ATPases and pyrophosphatases (Figure 3). Transgenic Arabidopsis plants overexpressing the vacuolar H^+- pyrophosphatase AVP1 showed increased tolerance to salt stress presumably by increasing the sequestration of salt in vacuoles (Gaxiola et al., 2001). Similarly, overexpression of one of the vacuolar Na^+/H^+ antiporters, AtNHX1, increases the salt tolerance of the transgenic Arabidopsis plants (Apse et al., 1999).

The ability to restrict Na^+ transport from the root to the aerial parts is another important determinant of salt tolerance. It was thought that growth inhibition is mainly restricted to the shoot under moderate salt stress (Lazof and Bernstein, 1999). One related process in the aerial parts is to retain Na^+ in older leaves, which reduces transport to young organs. Amongst several steps that control the long distance transport of Na^+ are the radial transport of Na^+ across the root cortical cells and the loading of Na^+ into the xylem vessel. One component of radial transport of Na^+ in Arabidopsis root is *SAS1*. Mutations in *SAS1* resulted in several times increased Na^+ content in aerial parts while the Na^+ content in roots remained unchanged (Nublat et al., 2001). Cloning of the *SAS1* locus has not been reported. Another component of Na^+ xylem loading is SOS1. In *sos1* mutants, Na^+ concentration in the xylem sap is higher than that in the wild type, suggesting that SOS1 controls Na^+ loading into and/or retrieval from the xylem, in addition to its role in extruding Na^+ in root epidermal cells (Shi et al., 2002a). *SOS1* is expressed around vascular tissues, consistent with its function in Na^+ loading/retrieval (Shi et al., 2002a).

Dealing with Osmotic Stress and Salt Stress Injuries

When plants are challenged with hyperosmolarity, accumulation of ions such as Na^+ in the vacuoles can serve as a means to lower osmotic potential of the cells, and this process is perhaps cost-effective with regard to the amount of energy and resources spent (Yeo, 1983). A related strategy used to lower the osmotic potential of the cell cytosol is to accumulate compatible osmolytes. For glycophytes, the capacities for Na^+ compartmentalization and accumulation of osmolytes are both limited. Various compatible osmolytes such as proline, glycine betaine, and polyols can greatly reduce stress damage to plant cells. An increased production of osmolytes is a general phenomenon found in all plants in response to salt stress. This is clearly an adaptive strategy, and transgenic plants with increased osmolyte production or reduced degradation (e.g., Nanjo et al., 1999) show improved salt tolerance. Besides reducing the osmotic potential of the cytosol to facilitate water uptake, many compatible osmolytes have additional functions such as protecting proteins from misfolding and alleviating the toxic effect of reactive oxygen species generated by salt stress (Smirnoff and Cumber, 1989; for reviews see Nuccio et al, 1998; Hong et al., 2000). In fact, the direct protective effect of osmolytes on cellular structures against oxidative stress or protein misfolding may outweigh their role in osmotic adjustment, since their concentration in the transgenic plants overexpressing osmolyte biosynthetic genes is generally very low and thus not very significant for osmotic adjustment (Zhu, 2000).

The beneficial role of compatible osmolytes was suggested by early studies showing that the activities of certain enzymes were less inhibited by excessive salts in the presence of compatible osmolytes (Greenway and Munns, 1980). The major compatible osmolyte in Arabidopsis is proline. One interesting Arabidopsis mutant with altered proline accumulation is *eskimo1* that exhibits increased freezing tolerance (Xin and Browse, 1998). The *eskimo1* mutant has a high content of proline, resulting from both enhanced production and reduced degradation (Xin and Browse, 1998). It was not reported in that study whether this mutant also has enhanced tolerance to salt stress. Nonetheless, the role of osmolytes in plant salt tolerance was supported with experimental evidence that salt tolerance was increased to various extents in transgenic plants engineered to produce new osmolytes absent in the parental lines or in plants that overexpressed the genes whose products limit the production of these osmolytes (Nuccio et al., 1999; Bohnert and Sheveleva, 1998).

As osmolytes have general protective effects, the benefit seen in transgenic plants goes beyond salt stress toler-

ance. For example, expression of a bacterial choline oxidase gene *CodA* in Arabidopsis conferred accumulation of glycinebetaine and increased tolerance to cold and salt stress (Hayashi et al., 1997). In another example, Holmstrom et al. (2000) expressed bacterial glycine betaine biosynthesis genes in tobacco, a species that does not accumulate glycine betaine. The transgenic plants accumulated glycine betaine, and were more tolerant to salt stress, to photoinhibition by high light, as well as to low temperature stress. Nevertheless, it should be pointed out that an enhanced production of osmolytes is not the whole story of salt tolerance. As discussed in earlier sections, there are many other determinants that are also important. In fact, it is likely that osmolyte accumulation may initially result from cellular damage (Delauney and Verma, 1993). For example, it was found that there is an increased accumulation of proline in the *sos1* mutant under salt stress, which probably reflects increased cellular damage by salt stress in the mutant (Liu and Zhu, 1997).

Whereas the types of osmolytes that plants produce are plant species-dependent, the magnitude of the accumulation is influenced by other factors including environmental conditions such as salt stress. Although the signal transduction pathways leading to the production of osmolytes are not understood at the moment (Hare et al., 1999), it is possible that these pathways are similar to those operating in yeast, i.e., the HOG1 pathway. Upregulation by salt or drought stress of genes in proline biosynthesis such as P5CS was widely reported (Savoure et al. 1997; Strizhov et al., 1997; Yoshiba et al., 1999; Xiong et al., 2001c). In yeast cells, genes that encode glycerol transporters are also down regulated by osmotic stress with an outcome of reduced glycerol loss. In Arabidopsis, genes that encode enzymes in proline catabolism are also down regulated by osmotic stress. For example, expression of the proline dehydrogenase gene is up-regulated by proline and down-regulated by dehydration (Kiyosue et al., 1996; Reymond et al., 2000). Additionally, the production of osmolytes is modulated by feedback regulation. For example, the biosynthesis of proline in plants involves a feedback inhibitory regulation. Under salt stress, the P5CS protein, which is responsible for the limiting step in proline biosynthesis, may lose this negative regulation due to conformational changes in the protein. Tobacco plants expressing a mutated form of P5CS protein that disabled this negative regulation accumulated more proline under both stress and unstressed conditions and showed increased salt tolerance (Hong et al., 2000).

Secondary Stresses

The ionic and osmotic stresses imposed by high salinity on plants may create secondary stresses. These secondary or derived stresses include for example the accumulation of toxic or unwanted compounds, perturbance in cellular metabolism, and nutritional disorders. Among these, oxidative stress is an important constraint for salt tolerance.

Studies implicated that salt stress could generate reactive oxygen species (ROS) in plants (e.g., Burdon et al., 1996; Shen et al., 1997; Tsugane et al., 1999; Hong et al., 2000), which include, for example, hydrogen peroxide, hydroxyl radicals, and superoxide anions. The mechanism responsible for the generation of ROS upon abiotic stress in plants is not clear. In phagocytic cells, H_2O_2 generation is via NADPH oxidase that is triggered by growth factors (Rhee et al., 2000). In plants, NADPH oxidases are likely also responsible for the generation of H_2O_2 in hypersensitive responses (Kawasaki et al., 1999). ROS can have damaging effects on cellular structures and macromolecules such lipids, enzymes and DNA. Detoxification of these compounds therefore contributes to salt and other stress tolerance. As mentioned above, one major function of compatible osmolytes is to alleviate oxidative stress damage, as suggested by transgenic studies (Reviewed in Bohnert et al., 1998; Nuccio et al., 1999). Thus, transgenic plants overexpressing ROS scavenging enzymes such as superoxide dismutase, catalase, and GST showed somewhat increased tolerance to salt stress and other stresses (reviewed in Bohnert et al., 1998). The role of ROS scavengers in plant salt tolerance is also supported by genetic evidence. For example, a genetic screen for salt tolerant growth in Arabidopsis resulted in the isolation of several salt tolerant *pst* mutants. The enhanced salt tolerance in *pst1* mutants is associated with increased ROS detoxification as a result of activation of superoxide dismutase and ascorbate peroxidase (Tsugane et al., 1999).

Salt stress, like some other stresses, may also cause genotoxicity, i.e. DNA damage. In murine kidney cells, it was reported that hyperosmolarity from NaCl causes DNA double strand breaks within 15 min, which is not attributable to apoptosis (Kultz and Chakravarty, 2001). In plants, although direct observation on DNA damage caused by salt stress has not been reported, a connection between DNA damage and salt stress is demonstrated by the isolation of the Arabidopsis *uvs66* mutant. This mutant is hypersensitive to UV-C and to the DNA damaging agents methyl methane sulfonate and mitomycin (Albinsky et al., 1999). Interestingly, root growth of the mutant seedlings is also sensitive to NaCl and ABA. The salt sensitivity is specific to NaCl and not to general osmotic stress. This suggests that the genotoxic signal

pathway and salt stress signal pathways may use some common components, for example, the MAPK (mitogen activated protein kinase) module.

Studies in yeast and animal cells have implicated connections between oxidative stress and osmotic stress, both of which use common components of the MAPK module (for review, see Kyriakis and Avruch, 1996; Xiong and Zhu, 2002b). Similar connections were recently also revealed in Arabidopsis. A number of studies showed that several MAPK components are activated or their gene expression induced by salt and other stresses (reviewed in Meskiene and Hirt, 2000; Xiong and Zhu, 2001). The role of MAPK modules in plant tolerance to abiotic stresses was further illustrated by the study where a tobacco MAPK kinase kinase (MAPKK) ANP orthologue, NPK1, was expressed in an active form in Arabidopsis. NPK1 mediates H_2O_2- regulated gene expression in plants (Kovtun et al., 2000). The engineered Arabidopsis plants showed increased tolerance to freezing, heat shock and salt stress (Kovtun et al., 2000), yet the expression of the stress-responsive gene *RD29A* was not affected. This suggests that in addition to the connections among MAPK modules used by osmotic stress and ROS signaling, there is specificity in the individual pathways (Knight and Knight, 2001). For example, an Arabidopsis mutant defective in a MAPK kinase phosphatase exhibited hypersensitivity to UV-C, but not to salt stress (Ulm et al., 2001).

Salt Stress Regulation of Gene Expression

The expression of numerous plant genes is regulated by salt stress. Investigations of salt induced transcript changes in fact opened the era of molecular biology of plant salt stress. By studying which genes are regulated and how they are regulated, investigators are most interested in the functions of the gene products in plant stress tolerance. A general assumption has been that stress upregulated genes may be important for stress tolerance. Early works employed several model plants such as the common ice plant, the resurrection plant, several crop plants, as well as Arabidopsis. These studies have been comprehensively reviewed (Skriver and Mundy, 1990; Ingram and Bartel, 1996; Bray, 1997; Zhu et al., 1997) and readers are referred to these reviews for details. Here we briefly outline the main findings in Arabidopsis and also discuss some recent progress.

Genes that are upregulated by salt stress mainly belong to several groups, based on their possible functionality. These genes encode the LEA proteins, enzymes (involved in the biosynthesis of osmolytes, hormones, detoxification, and general metabolism), transporters (ion transporters, ABC transporters, and aquaporins), and regulatory molecules such as transcription factors, protein kinase and phosphatases. The most common and widely reported genes that are stress-regulated are perhaps the *LEAs* or *LEA*-like genes. *LEA* genes encode late embryogenesis abundant proteins (Baker et al., 1988; Dure et al., 1989). These genes are highly expressed in seeds during the desiccation stage following maturation, and in vegetative tissues in response to water deficit. They have been described in many plant species. *LEA*-like genes are also found in bacteria, yeast and animals (Close and Lammers, 1993; Garay-Arroyo et al., 2000; Shen et al., 2001), suggesting that they may represent some common stress-responses since the *LEA*-like genes in these non-plant systems are also induced by osmotic stress (Garay-Arroyo et al., 2000). Despite their wide occurrence, the functions of this group of polypeptides are ill defined except in a few cases where overexpression of individual *LEA* genes resulted in some degree of stress protection (e.g. Xu et al., 1996).

One group of genes in Arabidopsis that are termed *RD* (responsive to dehydration), *COR* (cold-regulated) or *LTI* (low temperature-induced), or KIN (cold induced), are strongly induced by salt, drought, low temperature, and ABA. The products of many of these genes may not be structurally related to but share some features with LEA proteins and are therefore can be loosely classified as LEA-like proteins. These proteins are highly soluble and remain soluble even after being boiled. Because these genes are not expressed under normal (non-stress) growth conditions, they may not be required for normal cellular functions. Since they are strongly expressed following stress treatments such as salt, drought, or low temperature, their products are thought to have some function in protecting cellular structures under stress. One major hypothesis is that these proteins could prevent the denaturation of key proteins by acting as chaperones. The finding that enhanced expression of the transcription factors that regulate the expression of these *LEA*-like genes can increase the tolerance of transgenic plants to cold, drought, or salt stress demonstrates that these proteins do have a protective effect against abiotic stresses (Jaglo-Ottesen et al, 1998; Liu et al., 1998; Kasuga et al., 1999).

Traditional expression profiling approach to search for salt-regulated genes is being replaced with cDNA microarrays or oligo chips. Microarray analysis of gene expression under salt stress was reported for the yeast *S. cerevisae* (Yale and Bohnert, 2001; Rep et al., 2000; Posas et al., 2000). Microarray has the potential to monitor gene expression profiles on a genome scale, yet current application of this technology in Arabidopsis still falls short of this expectation, partly because only a limited number of cDNAs are available. Seki et al. (2001) examined

Arabidopsis 1,300 genes under drought or cold stresses. Kawasaki et al. (2001) and Bohnert et al. (2001) examined the expression of more genes in rice and Arabidopsis and several other organisms, respectively. The significance of the results generated can only be appreciated with a careful consideration of the experimental conditions and after confirmatory experiments.

With the availability of the microarray technology, the large number of genes that are regulated may at first please the investigators. However, caution should be exercised in interpreting the results. For example, salt stress treatment results in a rapid decline in photosynthesis within minutes (e.g., Kawasaki et al., 2001). Therefore, genes that are regulated by photosynthetic activities may be affected but they are not regulated by salt stress per se. In an example, Reymond et al. (2000) studied the expression of 150 genes in response to wounding, pathogen inoculation and drought treatment. It was found that many of the wounding-induced genes are also up-regulated by drought treatment. Therefore, some of these genes may be primarily induced by drought stress instead of the mechanical stimulus intended. The dosage of stress and the time course of gene induction are also important factors to be considered. The primary stresses and secondary stresses may induce genes in different time frames.

ABA and Other Plant Hormones in Salt Stress Responses

The inhibitory effect of salt stress on plant growth is exhibited at several levels and involves an array of cellular processes. Salt stress affects the expression of cell cycle progression genes (e.g., Burssens et al., 2000) and thus affects cell division. A perturbed turgor may also affect cell expansion and inhibits growth. All these cellular processes may be regulated by an altered hormone homeostasis under salt stress. One well-documented observation is that abscisic acid (ABA) level increases upon salt stress. ABA may have to do with cell cycle regulation (e.g., Wang et al., 1998). However, a major physiological effect of ABA is the stimulation of stomatal closure. As a result, photosynthesis declines (e.g., Kawasaki et al., 2001) and photoinhibition and oxidative stress occur. Another factor that contributes to decreased photosynthesis is the inhibitory effect of salt stress on the efficiency of translocation and assimilation of photosynthetic products; both processes are regulated by plant hormones. Therefore, a perturbed growth, evidenced particularly by inhibition of cell expansion under salt stress, may be controlled to a large extent by hormone homeostasis. Accordingly, quite a number of reports have indicated that applications on crops of growth regulators, such gibberrelic acid and cytokinin, had some benefits in alleviating the adverse effects of salt stress.

ABA is involved in many aspects of plant responses to salt stress. Besides inducing the expression of many salt-responsive genes (Rock, 2000; Bray, 1997; Skriver and Mundy, 1990; Ingram and Bartel, 1996; Bray, 1997; Zhu et al., 1997; Chandler and Robertson, 1994), exogenous ABA application was shown in some experiments to increase salt tolerance of the treated plants or plant tissues. ABA may regulate plant salt responses at several levels. ABA synthesized in the root under drought or salt stress may be an endogenous signal that ascends with transpirational flow to regulate shoot growth, stomatal aperture and hence stress tolerance (reviewed in Davies and Zhang, 1991). At the whole plant level, ABA can relieve the growth inhibition caused by decreased water potential (Øw) under salt or drought stress. Under these conditions, root activity is very important for coordinating the whole plant to combat stress. Increased endogenous ABA levels were shown to be important to maintain maize primary root elongation at low Øw (Saab et al., 1990). This effect may have to do with ABA inhibition of ethylene production (Sharp, 2002). Spollen et al. (2000) reported that at a Øw of –1.6 MPa, inhibition of maize primary root elongation by the ABA synthesis inhibitor fluoridone was prevented by applying inhibitors of ethylene biosynthesis or of ethylene signaling. As in ABA deficient mutants, the production of ethylene in fluoridone-treated seedlings was increased. Re-application of ABA can reduce ethylene production back to the non-fluoridone-treated control levels. Similarly, ABA also stimulates leaf expansion and this is not related to the control of water potentials in the leaf, but instead to the control of ethylene production in the leaf, as was demonstrated with the tomato ABA deficient mutants *flacca* (Sharp et al., 2000). These results convincingly demonstrated that one role of ABA in promoting root and shoot growth at low Øw is to restrict the production of ethylene, and thereby reduce its inhibitory effect on root elongation and leaf expansion. In Arabidopsis, one interesting observation regarding the relation of ethylene production and root elongation was made with the *sos4* mutants. While root and shoot growth of *sos4* seedlings were more inhibited by salt stress than found in wild type, root growth of *sos4* was more tolerant to mannitol (Shi et al., 2002b). Because SOS4 catalyzes the production of pyridoxal phosphate (Shi et al., 2002b), a co-factor required by ACC (aminocyclopropane-1-carboxylic acid) synthase in ethylene synthesis, *sos4* mutant plants may have a reduced ethylene production relative to the wild type under the osmotic stress imposed by high concentrations of mannitol.

The role of ethylene in inhibiting primary root elongation

may not be the whole story of ethylene in drought or salt stress responses. Root elongation obviously helps plants explore further soil regions which may have untapped water, and is therefore important against severe water shortages. However root hairs may be more relevant to combating less severe or temporary water shortages. Although ethylene inhibits primary root elongation, it also aids root hair differentiation and promotes root hair elongation. Interestingly, the *sos4* mutation diminishes root hair elongation, possibly through diminishing ethylene production. In support of this concept, supplying the ethylene precursor ACC can overcome the short root hair phenotype in this mutant (J.-K. Zhu, unpublished). Further studies on the relationship between root hair elongation, primary root elongation and salt or drought tolerance by using *sos4* may help understand the fine balance between root development and plant drought and salt tolerance.

The interaction between ABA and ethylene in vegetative growth as well as in seed germination was further demonstrated by genetic analysis of ABA signal transduction in Arabidopsis. Characterization of mutants with altered responses to ABA during germination and analysis of ethylene response mutants indicated that ethylene counteracts ABA signaling during seed germination, but it positively regulates ABA action in root inhibition (Beaudoin et al., 2000; Ghassemian et al., 2000; Gazzarrini and McCourt, 2001).

At the molecular level, ABA induces the expression of numerous plant genes (reviewed by Rock, 2000). Some of these genes encode various signal transduction components such as putative receptors, protein kinases/phosphatases and transcription factors that may participate in salt stress signaling; others encode effectors for stress tolerance. These effectors include, for example, H^+-ATPases, P5CS, and aquaporins (e.g., Kaldenholf et al., 1993; Tyerman et al., 1999). ABA-regulated and salt stress-regulated pathways that lead to the activation of these genes are at least partially separate because the level of gene expression for combined treatment with ABA and salt could be additive (e.g. Chak et al., 2000). Additionally, these two pathways appear to interact at certain points since in some experiments the signaling output from combined treatment with ABA and salt is synergistic relative to individual treatments (Bostock and Quatrano, 1992; Xiong et al., 1999b; Xiong et al., 2001c).

Plant mutants defective in ABA and other hormone production or signal transduction are excellent tools for analyzing the function of the plant hormones in salt stress tolerance. In a genetic study of abiotic stress signal transduction, we recovered a group of mutants that exhibit reduced expression of the luciferase reporter gene under control of the ABA- and salt-stress responsive promoter *RD29A* (Ishitani et al., 1997; Xiong et al., 2001c). Expression of the endogenous *RD29A* and many other stress-responsive genes are also changed in these mutants. This is particularly evident in the *los5* mutant. In *los5*, the expression of several genes such as *RD29A, COR15, COR47, RD22*, and *P5CS* under salt stress was severely reduced or even completely blocked (Xiong et al., 2001c). Surprisingly, several of these mutants are found to be ABA deficient (Xiong et al., 2001a; Xiong et al., 2002). When ABA was re-introduced, the expression of *RD29A-LUC* was restored to the wild type level, demonstrating that ABA-deficiency is responsible for blocking the expression of these stress responsive genes (Xiong et al., 2001c). Positional cloning of two of mutant loci, *LOS6* and *LOS5*, identified that they are allelic to *ABA DEFICIENT 1* (*ABA1*) and *ABA3* respectively (Xiong et al., 2001c; Xiong et al., 2002). These findings suggest that salt induction of these stress-responsive genes is almost completely dependent on ABA, which contradicts with the previous model that osmotic stress signaling uses both ABA-dependent and ABA-independent pathways.

The studies on *los5* and *los6* mutants demonstrated a critical role of ABA in regulating salt-regulated gene expression. However, there has been ambiguity in the relationship between ABA and salt tolerance. Like other ABA-deficient or ABA-insensitive mutants, both *los5* and *los6* are more tolerant to NaCl during germination. This is because salt induces ABA biosynthesis, which contributes to the salt sensitivity of seed germination. In *aba* or *abi* mutants, the biosynthesis or the responses to ABA is defective, and hence salt stress has less inhibition on germination. Genetic screens that attempted to isolate salt tolerant mutants using the seed germination phenotype also support this notion, since some of the salt tolerant mutants are alleles of *abi* mutants (Quesada et al., 2000). The tolerance to salt found in mutants isolated by seed germination screening often does not extend to seedling or adult stages (Saleki et al., 1993; Quesada et al., 2000). Thus salt tolerance must involve some different processes at different stages of plant development.

In the *aba* mutants, salt stress responses are also complicated. Using *los5*/*aba3* and *los6*/*aba1* mutants, we tested their responses to salt in vegetative growth. We found little difference in root elongation between *los6*/*aba1* and the wild type at various concentrations of NaCl. However, root elongation in *los5*/*aba3* plants was more sensitive to NaCl. With prolonged exposure to high concentrations of salt (e.g. 100 mM NaCl for 3 weeks), *los5*/*aba3* plants showed a higher sensitivity to NaCl, as indicated by leaf yellowing or eventual death after longer exposure (Xiong et al., 2001c), whereas *los6*/*aba1* did not differ much from the wild type in appearance. The reason for the difference in salt sensitivity between *los6*/*aba1* and *los5*/*aba3* is not very clear, although both mutants have similar ABA contents as measured in bulk leaf samples. One possibility is that the ABA levels thus measured may not be accurate in

reflecting cell or tissue-specific ABA contents, which may be important for salt tolerance. Another possibility is that *los5/aba3* might have other roles unrelated to ABA biosynthesis (Xiong et al., 2001c). These additional roles could be related to the fact that the sulfurylated MoCo, the LOS5/ABA3 catalyzed product, is required both by aldehyde oxidases and xanthine dehydrogenase. It is known that aldehyde oxidases also participate in the synthesis of auxin and that xanthine dehydrogenases function in nitrogen metabolism. Currently, it is not clear whether these enzymatic functions affect salt stress signaling/ tolerance or not. Additionally, it is likely that MoCo synthases may be required for the anchoring of certain receptors to membrane patches.

Like *aba* mutants, Arabidopsis *abi* mutants also showed an increased tolerance to NaCl during seed germination, yet their sensitivity or tolerance to salt stress at vegetative stages was not reported. Our study indicates that the salt responses in root and shoot growth in *abi1* or *abi2* mutants are rather complicated: both sensitivity and tolerance to salt relative to the wild type seedlings were observed, depending on the severity and duration of salt stress and seedling ages (J.-K. Zhu, unpublished).

Aspects of Salt Stress Signal Transduction in Arabidopsis

We refer to the processes involved in the perception of salt stress and subsequent initiation of physiological responses as salt stress signal transduction. For abiotic stresses such as low temperature, drought, and salinity, the nature of the primary signals that are recognized by plants is unclear. Salt stress may have either an ionic, osmotic, or a mechanical impact on plant cells. One possibility is that all these different signals have their own specific receptors (Xiong and Zhu, 2001). These receptors can either operate independently or cooperatively to initiate downstream signaling events. This multiplicity of signals originated from one stress may account for part of the complexity of the signaling pathway that is ascribed as 'crosstalk' or connectedness (Xiong and Zhu, 2001). For ionic signals, candidate receptors could be various ion transporters that harbor ion-binding sites on either the extracellular and/or intracellular side. Along with the primary ionic and osmotic signals, secondary signals such as stress hormones (ABA and ethylene) and ROS are generated. These secondary signals may act as ligands and bind to cognate receptors. These receptors include the receptor-like protein kinases, two-component sensor kinases, and G-protein coupled receptors (Xiong and Zhu, 2001). Despite this complexity, three types of signaling pathways are apparent. These are the ionic stress signaling pathway, the osmolyte accumulation pathways, and the stress-responsive LEA-like gene regulation pathways. Here we mainly discuss the ionic stress signal transduction pathway under salt stress.

It has been well documented that salt stress, like many other abiotic stresses, can elicit a transient increase in cytosolic Ca^{2+} (for review, see Knight, 2000; Sanders et al., 1999). Higher plants, like yeast, also use Ca^{2+} to signal and combat salt stress (Bressan et al., 1998). The source of this Ca^{2+} could be extracellular or intracellular, both of which have been demonstrated experimentally (Bush, 1995; Muir and Sander, 1997). For ligand-mediated intracellular Ca^{2+} release, the three ligands known in animal cells, i.e., inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) have now been implicated to release Ca^{2+} from intracellular stores in plant cells as they do in animal cells (NaVazio et al., 2000; Wu et al., 1997; Allen et al., 1995; Cote and Grain, 1994). Evidence suggests that intracellular Ca^{2+} transient upon hyperosmotic stress of plant cells involves inositol 1,4,5- trisphosphate (IP_3) gated Ca^{2+} release from internal stores such as endoplasmic reticulum (ER) and vacuoles. It has been shown previously that exogenous IP_3 was able to release Ca^{2+} from vacuolar vesicles or isolated vacuoles (e.g., Schumaker and Sze, 1987) and to mediate transient increases in cytosolic Ca^{2+} (e.g., Allen et al., 1995). Both IP_3 and Ca^{2+} were implicated in ABA and environmental stress responses (for review, see Munnik et al., 1998; Sanders et al., 1999). In addition, the transcript levels of several enzymes involved in the generation of IP_3 are induced by ABA, osmotic stress or cold (Hirayama et al., 1995). PIP_2 levels as well as IP_3 levels increased during salt stress of Arabidopsis plants and the time frame for these changes correlates with the transient increase in cytosolic Ca^{2+} (DeWald et al., 2001). Microinjection as well as pharmacological experiments showed that increased cytoplasmic Ca^{2+} can activate the expression of stress responsive genes such as *RD29A*, *KIN1*, and *cas15* (Monroy and Dhindsa, 1995; Sheen, 1996; Wu et al., 1997). In animal cells, the d forms of phosphatide-specific phospholipase C (PLC), which are structurally similar to the plant PLCs, is activated by G-protein á subunit (Gá), resulting in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3. Whereas plant G-protein associated receptors are poorly characterized (Xiong and Zhu, 2001), recent identification of Arabidopsis *gpa1* mutant with lesions in the prototypic Gá gene showing impaired ABA-regulated stomatal aperture (Wang et al., 2001) may provide a clue to the identification of G protein-associated receptors in plants. It has not been reported whether this sole prototypic Gá in Arabidopsis also mediates salt and other stress signal

transduction.

Although molecular and pharmacological studies have implicated phosphoinositides in many cellular responses in plants, genetic evidence on the involvement of phosphoinositide signaling in abiotic stress signal transduction has been lacking until recently. In a genetic screen using the stress-responsive *RD29A* promoter driven firefly luciferase as a reporter (Ishitani et al, 1997; Xiong et al., 1999a), we isolated a mutant, *fiery1* (*fry1*) that exhibits much enhanced reporter gene expression in response to cold, salt, drought, and ABA (Xiong et al., 2001b). The *FRY1* gene encodes an enzyme with both 3'(2'),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. This gene was identical to the previously characterized *SAL1* gene that was isolated by its ability to confer increased salt tolerance when expressed in yeast cells (Quintero et al., 1996). Because the *fry1* mutant plants did not show sulfur deficiency symptoms, the bisphospahte nucleotidase activity of FRY1 in sulfur assimilation appears dispensable. Therefore, it is hypothesized that lack of the inositol polyphosphate 1-phosphatase activity is responsible for the enhanced gene expression in response to stress and ABA treatments. In animal cells, in vitro studies suggest that IP_3 is degraded through either a 5-phosphatase pathway or a 3-kinase pathway (Figure 5) (Majerus, 1992; Berridge and Irvine, 1989). These pathways in plant cells are not yet demonstrated. In a recent study, overexpression of an inositol polyphospahte 5-phosphatase resulted in reduced ABA- and stress-responsive gene expression in transgenic plants (Burnette et al., 2001). Two independent studies reported that feeding plants with [^{3}H]-labeled IP_3 yielded inositol 4,5-bisphosphate as the primary and immediate catabolite (Brearly et al., 1997; Drøbak et al., 1991), demonstrating that in these plant cells IP_3 was likely first hydrolyzed through a 1-phosphatase pathway. However, the inositol polyphosphate 1-phosphatase responsible for this early termination of IP_3 signal has not been isolated or characterized. Added to this complexity is the fact that the inositol polyphosphate 1-phosphatase characterized in animal cells is not active against IP_3 (Inhorn et al., 1987; Majerus, 1992). Whereas the FRY1/SAL1 activity against Ins(1,4)P_2 and Ins(1,3,4)P_3 was demonstrated previously (Quintero et al., 1996), its activity on IP_3 was not known. We measured the activity against IP_3 of the FRY1 recombinant protein and found that it had a measurable although limited activity against IP_3 (~13% of that against Ins (1,4)P_2 or Ins (1,3,4)P_3) (Xiong et al., 2001b). The in vivo activity of FRY1 on IP_3 and its significance in overall metabolism of IP_3 are still unknown. Nevertheless, loss of FRY1 activities would inevitably slow down IP_3 degradation (Figure 5) and lead to enhanced stress gene expression. To test the impact of *fry1* mutations on IP_3 metabolism, we measured IP_3 levels in *fry1* and wild type plants treated with ABA, and found that whereas the ABA-induced IP_3 rise was transient in wild type plants, it was higher and more sustained in *fry1* mutant plants (Xiong et al., 2001b).

Whatever the mechanisms involved, transient increases in cytosolic Ca^{2+} must be coupled with downstream signaling events to mediate stress adaptation. In Arabidopsis salt stress signaling, the Ca^{2+} signal is perceived by the calcineurin B-like Ca^{2+} sensor SOS3 (Liu et al., 2000). However, different from the calcineurin B in yeast that acts through activation of a protein phosphatase, SOS3 interacts with and activates the protein kinase SOS2 (Halfter et al., 2000). Thus, SOS3 in some aspects resembles an adaptor or scaffold protein that mediates the interaction of SOS2 with other proteins such as ion transporters. This property of SOS3 is suggested by the requirement of myristoylation for full function in salt tolerance (Ishitani et al., 2000). It appears that SOS3, by anchoring SOS2 to particular plasma membrane patches, may facilitate SOS2 phosphorylation of ion transporters or regulatory partners of transporters. This is further supported by the study that introducing SOS1, SOS2 and SOS3 all together into a yeast mutant deleted of endogenous Na^+ transporters greatly improved salt tolerance of the yeast transformants, whereas expressing SOS1 alone only slightly improved salt tolerance of the yeast cells (Quintero et al., 2002).

To understand the interaction between SOS3 and SOS2, the functional domains of both proteins were analyzed. The regulatory and catalytic domains of SOS2 interact, resulting in autoinhibition of the kinase (Guo et al., 2001) (Figure 6). A 21 amino acid motif (FISL motif, refers to the conserved amino acid residues) in the regulatory domain mediates SOS2 interaction with SOS3. It seems that the interaction between SOS3 and SOS2 results in the release of autoinhibition and provides substrate access to the SOS2 kinase domain (Figure 6) (Guo et al., 2001). Presumably through phosphorylation, the activated SOS2 can modulate the activity of ion transporters such as SOS1 and HKT1 (Figure 3), as discussed before.

Strategies for Finding Salt Tolerance Determinants in Arabidopsis

Several approaches are commonly used to identify genes important for plant salt tolerance (Xiong and Zhu, 2001). They can be classified as either biochemical, expression profiling or genetic. The biochemical approach relies on previous knowledge that a particular enzyme or biochemical pathway is important for salt tolerance. These studies are typically supported by transgenic studies where the genes of interest are overexpressed and/or underex-

pressed and the resultant transgenic plant phenotypes analyzed. The gene expression profiling approach has been the most popular one for the last decade and a half. In this approach, gene expression under stress conditions is compared to that in the absence of stress. Genes that show increased expression under stress are cloned by using a variety of differential or subtractive screening techniques. More recently, microarray or gene chip analysis is employed to probe gene expression on larger scales as described in earlier sections. A major limit of the expression profiling approach is that many genes critical for plant salt tolerance are not induced by salt stress. In addition, many of the induced genes may not be important for salt tolerance, e.g., their increased expression under salt stress may be a consequence of stress injury. Eventually, functional analysis through transgenic overexpression or under-expression, or through reverse genetics to identify knockout mutants is needed to establish whether an induced gene is functionally important for plant salt tolerance.

The genetic approach utilizes natural or induced variations in stress tolerance or stress gene regulation. Gene mutations in Arabidopsis plants can be easily created by using one of several mutagens such as ethylmethane sulfonate, fast neutrons, transposons or T-DNA. Mutations in salt tolerance genes are expected to cause decreased salt tolerance in plants, unless they are functionally redundant. Therefore, one can search for plant mutants that are hypersensitive to salt stress, and these mutants will lead to the identification of salt tolerance genes. The *sos* mutants were isolated in such a screen. Of course, screening for the opposite phenotype (i.e. increased salt tolerance) would identify negative regulators of stress tolerance or gain-of-function mutations. To overcome the shortage of

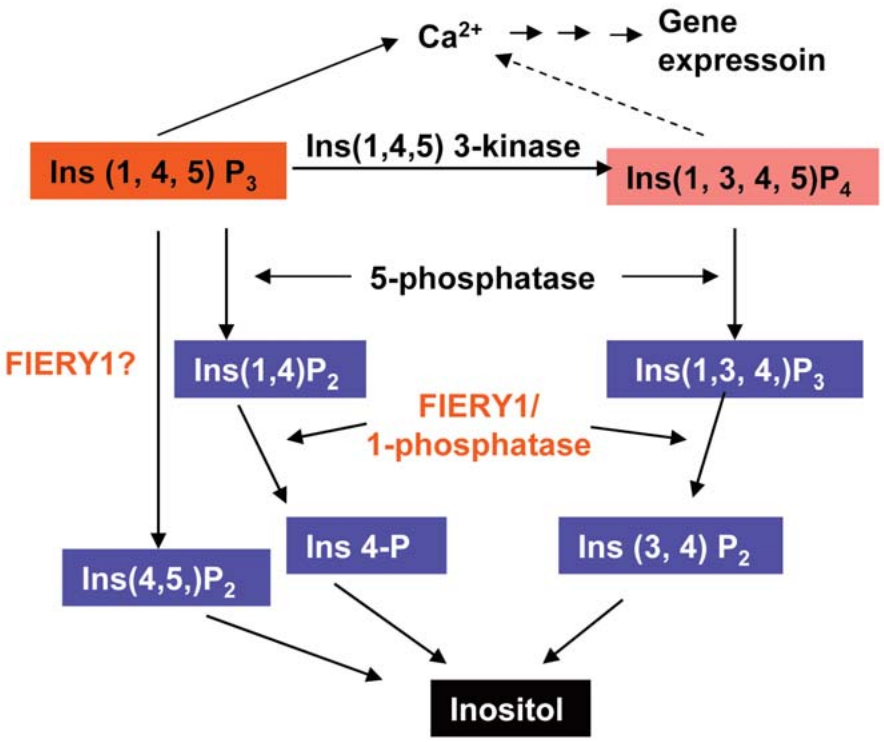

Figure 5. Pathways for the degradation of inositol 1,4,5-trisphosphate (IP_3). In animal cells, IP_3 can be catabolized by either Ins (1,4,5) 3-kinase or inositol polyphosphate 5-phosphatase (5-phosphatase). The products Ins (1,3,4,5)P_4 and Ins(1,4)P_2 are further dephosphorylated by FIERY1-like inositol polyphosphate 1-phosphatase (1-phosphatase) and finally to inositol. In plants, it is purposed that FIERY1 dephosphorylates both Ins (1,4)P_2 and Ins (1,3,4)P_3 and thus mediates the degradation of IP_3. Additionally, there may be a 1-phosphatase(s) that can directly dephosphorylate the IP_3 at the 1-position. The in vivo ability of FIERY1 in this direct catabolism of IP_3 is yet to be determined. A higher and sustained level of IP_3 may contribute to a higher level of gene expression.

salt stress-specific visible phenotypes, a stress-responsive promoter driven reporter can be used to facilitate genetic screening based on molecular phenotypes. Our studies have found this promoter-reporter approach to be efficient, and especially powerful in identifying gene mutations that have only subtle visible or tolerance phenotypes (Ishitani et al, 1997; Xiong et al., 1999a). In Table 1, we summarize some of the cloned genetic loci that affect plant sensitivity to salt stress.

Several other methods such as transgenic plant analysis and yeast complementation can be used to complement mutational studies. RNAi knockout of candidate genes is a new and much more efficient way to suppress the expression of genes of interest, compared to the traditional antisense approach (Chuang and Meyerowitz, 2000; Smith et al., 2000).

Transgenic plants expressing candidate genes under the control of a constitutive promoter (such as CaMV *35S*) have been widely used to examine gene function in salt and other stress tolerance. The use of inducible promoters or tissue-specific promoters may avoid the adverse side effects of constitutive or ubiquitous expression. Kasuga et al (1999) found that plants expressing *CBF/DREB* under the stress-responsive *RD29A* promoter were more stress-tolerant but were not compromised in growth, unlike CaMV *35S* promoter-driven expression. These studies are exciting in providing leads for the genetic engineering of stress tolerance in crop plants. However, caution should be taken when the overexpression results are used to infer the native function of the endogenous plant genes. For example, overexpression of a transcription factor in a tissue or condition where it is normally not expressed may activate other target genes and thus lead to phenotypes unrelated to its normal function.

It is also worth noting that high expression of stress-responsive genes in some plant mutants does not necessarily mean that the mutants are more stress-tolerant. In our screen of signal transduction mutants using the *RD29A-LUC* reporter, not only mutants with reduced stress gene induction were more sensitive to respective stresses (e.g., *los5*, Xiong et al., 2001c), but also some mutants with a high level of stress gene induction are stress-sensitive. The latter mutants include *hos1* (Ishitani et al., 1998; Lee et al., 2001), *hos2* (Lee et al., 1999), *hos5* (Xiong et al., 1999b), *sad1*(Xiong et al., 2001a), and *fry1* (Xiong et al., 2001b). One explanation is that the stress gene products may in fact provide some protection against stress damage in the wild type background, but in the mutant, their high level of expression may be a compensatory response to reduced stress tolerance as a result of the primary mutation, and thus the enhanced stress gene expression response may be triggered by stress injury. In the mutant backgrounds, the protective effect of the stress gene products simply cannot overcome stress sensitivity caused by the primary lesions.

Yeast complementation often has been the method of choice in identifying plant genes with similar functions. This approach was very successful in the isolation of plant ion transporters, some of which are related to salt tolerance. Examples of putative plant salt tolerance determinants isolated by yeast complementation are presented in Table 2. Readers are referred to the references for details.

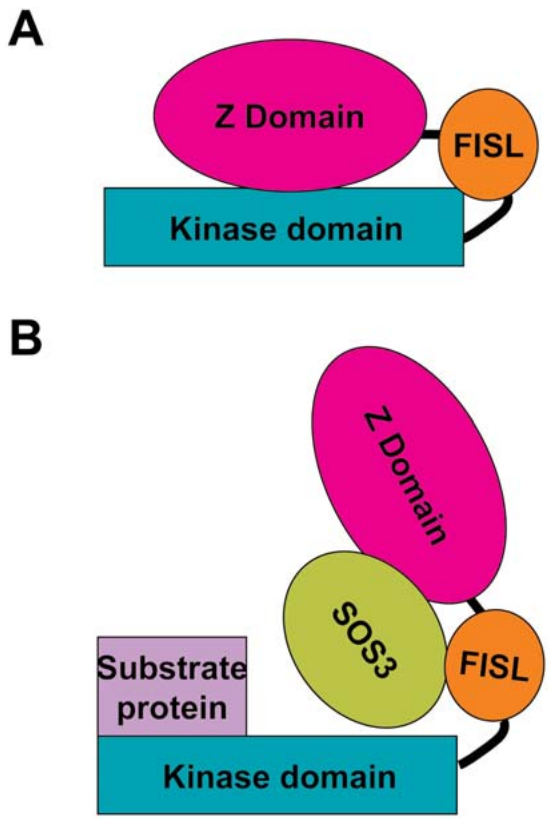

Figure 6. Activation of SOS2 protein kinase by SOS3 Ca^{2+} –binding protein. (A) In the absence of activated SOS3, the kinase activity of SOS2 is inhibited by interaction between the C-terminus and the kinase domain through the FISL motif. (B) Upon binding to Ca^{2+}, SOS3 becomes active and then interacts with the FISL motif and releases its inhibition of SOS2 kinase activity. This also provides substrate accessibility to SOS2 kinase domain. Through protein phosphorylation, SOS2 regulates the activity of ion transporters and restore ion homeostasis under salt stress. By myristoylation, SOS3 may also help to recruit SOS2 to specific membrane localization (not shown). The Z domain of SOS2 does not have a defined function yet. Figure is a courtesy of Dr. Yan Guo.

Table 1. Selected Arabidopsis genetic loci that affect plant responses to salt stress. Please refer to the text for details.

Locus symbol	Encoded product	GeneBank accession	TAIR accession
HKT1	Na^+ transporter	AF237672	At4g10310
SOS1	Na^+/H^+ antiporter	AF256224	At2g01980
SOS2	Ser/thr protein kinase	AF237670	At5g35410
SOS3	Ca^{2+} sensor	AF060553	At5g24270
SOS4	pyridoxal 5-kinase	AF400125	At5g37850
ABA1/LOS6	zeaxanthin epoxidase	AY093145	At5g67030
ABA3/LOS5	molybdenum cofactor sulfurase	AY034895	At1g16540
ABI1	Type 2C protein phosphatase	X77116	At4g26080
ABI2	Type 2C protein phosphatase	Y08965	At5g57050
FRY1	Inositol polyphosphate 1-phosphatase/3'(2')5'-bisphosphate nucleotidase	AY034894	At5g63980
HOS1	novel protein with a RING finger domain	AAB87130	At2g39810
SAD1	Lsm5-like snRNP	AY034896	At5g48870

Table 2. Some Arabidopsis genes that compliment salt sensitive phenotypes of yeast mutants or increase yeast salt tolerance when expressed in respective yeast strains.

Genes	Products	References
ACA4	Vacuolar membrane Ca^{2+}-ATPase	Geisler et al., 2001
NHX1	Vacuolar Na^+/H^+ antiporter	Gaxiola et al., 1999
AVP1	Vacuolar H^+-pyrophosphate	Gaxiola et al., 1999
SAL1	3Õ5Õ-bisphosphate nucleotidase and inositol polyphospahte phosphatase	Quintero et al., 1996
STO, STZ	Zinc finger-like transcription factors	Lippuner et al., 1996
SLT1	Unknown protein	Matsumoto et al., 2001
GSK1	GSK2/Shaggy-like kinase	Piao et al., 1999.
CBL1	Calcineurin B-like	Kudla et al., 1999.
HK1	Histidine kinase	Urao et al., 1999.
MEKK1	MAP kinase kinase kinase	Covic and Lew, 1996; Mizoguchi et al., 1996; Covic et al., 1999.
RCI2A	Unknown membrane protein	Nylander et al., 2000.
HAL3	FMN-binding protein	Espinosa-Ruiz et al., 1999

Salt Tolerance beyond Arabidopsis

What this review on salt tolerance is all about is Arabidopsis, a plant after all very sensitive to salt. With growing knowledge on salt tolerance in Arabidopsis, it is high time to tackle the salt tolerance mechanisms in halophytes. An ideal halophytic model organism has been sought after by many researchers. Commonly used salt tolerant organisms such as the common ice plant *Mesembryanthemum crystallinum*, and the unicellular alga *Dunaliella salina* are not amenable to genetic analysis. One genetically tractable halophyte is *Thellungiella halophila* that is native to the coastal regions of eastern China. This plant can complete its life cycle in the presence of 300 mM NaCl. More importantly, its genome size is small, and its cDNAs share over 90% identity to their counterparts from Arabidopsis (Zhu, 2001). Genetic analysis of *Thellungiella halophila* in comparison with that of Arabidopsis should shed light on how the ionic and osmotic homeostasis and detoxification mechanisms are fine tuned to achieve extreme salt tolerance in halophytic plants.

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