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A new TIP homolog, *ShTIP*, from *Salicornia* shows a different involvement in salt stress compared to that of TIP from *Arabidopsis*

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Abstract

To obtain an insight into the comprehensive molecular characteristics of the salt tolerance mechanism, we performed a screening for salt inducible genes in a halophytic plant, *Salicornia herbacea*, using mRNA differential display. A comparative analysis of gene expression in *Salicornia* grown in control and salt-stressed conditions led to the detection of a gene that was induced by salt. Both sequence analysis and a subsequent database search revealed that this gene was highly homologous to tonoplast intrinsic proteins (TIPs) from a variety of plant species. This gene, designated as *ShTIP*, is 1014 bp in size and contains a coding region of 762 nucleotides, which encodes a protein of 254 amino acids. Northern blot analysis revealed that *ShTIP* was predominantly expressed in shoots under normal conditions. However, salt stress induced high expression of *ShTIP* in both the shoots and roots. The expression of *ShTIP* in a salt-sensitive calcineurin-deficient yeast mutant (*cnbΔ*) resulted in a resistance to the high salt conditions. In addition, we compared the expression of a *TIP* gene in *Arabidopsis* with that of *ShTIP* under different conditions and found that the *Salicornia TIP* has a different regulatory mechanism for adapting to salt stress conditions compared with the glycophyte *Arabidopsis TIP*. These results indicate that *ShTIP* plays an important role in salt tolerance.

Additional key words: halophyte, glycophyte, *Salicornia herbacea*, salinity, tonoplast intrinsic protein.

Introduction

The environmental stress caused by salinity is a serious factor that limits the productivity of agricultural crops, which are very sensitive to high concentrations of salts in the soil. Salt stress may reduce plant growth by causing water deficiency, ion toxicity, ion imbalance or a combination of these factors (Serrano and Rodriguez 2002). Plants can survive and grow in saline environments by adjusting their osmotic potential through intracellular compartmentation (Blum *et al*. 1996), or through the accumulation of organic solutes in cytoplasm, such as glycinebetaine, proline, and soluble sugars for the maintenance of pressure potential (Karimi *et al*. 2005). Plants can also manage the water uptake by controlling the hydraulic conductivity of root membranes (Boursiac *et al*. 2005).

Water channels in membranes, aquaporins, facilitate the movement of water across the membrane by increasing its osmotic water permeability of the membrane (Chrispeels *et al*. 2001). Both physiological and genetic studies have provided evidence for a role of aquaporins in the regulation of water transport. Using the *Xenopus*-oocyte expression system (Miller and Zhou 2000), water transport activity has been demonstrated for several plant aquaporins (Kirch *et al*. 2000, Frangne *et al*. 2000, Martre *et al*. 2002). However, water is not the only molecule capable of being transported through aquaporins. Some aquaporins specifically facilitate the passage of other small solutes, such as glycerol (Dean *et al*. 1999), urea (Liu *et al*. 2003), ammonium, and carbon dioxide (Tyerman *et al*. 2002).

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Abbreviations: ABA - abscisic acid; MS - Murashige and Skoog medium; PEG - polyethylene glycol; ShTIP - *Salicornia* tonoplast intrinsic protein; SITIP - salt-inducible tonoplast intrinsic protein.

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Down-regulation of PIP1 aquaporins in *Arabidopsis* (Kaldenhoff *et al*. 1998) and tobacco (Siefritz *et al*. 2002) reduced the osmotic water permeability of both leaf and root. A decline in the pepper hydraulic conductivity of roots after saline stress could be related to either the absence or the inactivation of aquaporins (Carvajal *et al*. 1999). In rice, the mRNA levels of 10 aquaporin genes were markedly reduced in roots during chilling, but the expression of these genes recovered after warming. These changes were correlated with changes in bleeding sap volume, which suggests that there is a relationship between root water uptake and the mRNA levels of several aquaporins (Sakurai *et al*. 2005). Taken together, the results suggested that the activation or inactivation of aquaporins may be one of the mechanisms involved in homeostasis under changing environmental conditions (Kjellborn *et al*. 1999).

Salicornia herbacea is a succulent halophyte plant belonging to the *Chenopodiaceae* family and grows optimally under 100 to 500 mM NaCl. These plants may have acquired specific genes that are essential for tolerating salt. Using mRNA differential display, we isolated a salt-induced gene encoding tonoplast intrinsic protein from *Salicornia* (*ShTIP*). In this study, we compare the characteristics of TIP from the halophyte *Salicornia* with that from the glycophyte *Arabidopsis* (*SITIP*) which is salt-stress inducible (Pih *et al*. 1999), and examine the expression of *ShTIP* in mutant yeast cells grown under salt stress conditions.

Materials and methods

Salicornia herbacea L. seeds were sown in *Vermiculite* and germinated in a naturally illuminated greenhouse. Thirty-day-old plants were transferred to a growth chamber in an aerated hydroponic system and subjected to 16 h of light at 30 °C and 8 h of dark at 20 °C. Plants were supplied with Hoagland's nutrient solution. After two weeks of adaptation, plants were treated with 0.1, 0.3, 0.5, or 0.7 M NaCl for 15 d or 0.3 M KCl for 7 d. For ABA and PEG 6000 treatments, plants were transferred into Hoagland's nutrients supplemented with 100 μM ABA or 15 % PEG 6000 and harvested after 0, 1, 2, 4 and 8 h. The leaves and roots were rinsed with distilled water and frozen immediately in liquid nitrogen and stored at -80 °C until use. *Arabidopsis thaliana* (ecotype Columbia) seeds were surface-sterilized and then sown onto MS agar medium. One-week-old seedlings were transferred to MS liquid medium and grown for a further 2 weeks. The plants were subsequently treated with 0.3 M NaCl, 0.3 M KCl, 15 % PEG or 100 μM ABA.

Using a phenol extraction method (Ausubel *et al*. 1989), total RNA was isolated from untreated or treated (0.3 M NaCl) *Salicornia* shoot tissues. The mRNA differential display was performed with the RNAimage kit (*GenHunter*, Nashville, TN, USA) according to the manufacturer's instructions. The PCR products were separated on 6 % denaturing polyacrylamide gels, excised, re-amplified and ligated into a *pGEM-T-easy* vector (*Promega*, Madison, WI, USA). A cDNA library was constructed using Poly $(A)^+$ RNA extracted from the 0.3 M NaCl-treated shoots of *Salicornia*. Poly $(A)^+$ RNA was isolated using an oligo(dT)-cellulose chromatography method (Sambrook and Russell 2001) and was used to generate a cDNA library in the Uni-ZAP XR vector (*Stratagene*, La Jolla, CA, USA). Approximately 2.8×10^5 recombinant plaques were screened by plaque hybridization using the cDNA fragments obtained from mRNA differential display as probes. Sequencing was performed using the dideoxy chain terminator method and an *ABI prism*^{TM} *3100* DNA sequencer (*Perkin Elmer*, Foster City, CA, USA).

The *Arabidopsis* cDNA template was amplified to obtain a DNA probe that was specific for *SITIP* (Pih *et al*. 1999) by polymerase chain reaction (PCR) using Taq-polymerase (*Promega*) and gene-specific primers (forward, 5'-GCGGCCTTGGCTGAGTTCATTTCA-3'; reverse, 5'- GTAGTCGGTGGTTGGGAGCTGCTC-3'). The resultant PCR fragment (753 bp) was then cloned into a *pGEM-T Easy* vector (*Promega*) and sequenced.

Northern blot analysis was performed by fractionating 15 μg of total RNA on a denaturing formaldehyde agarose gel and subsequently transferring the RNA to a nylon membrane (*Hybond-N*$^+$, *Amersham Biosciences*, Buckinghamshire, UK). DNA probes that were used to detect the *TIP* genes were prepared from the cloned cDNA inserts using the multiple labeling systems (*Amersham Biosciences*) and ^{32}P-dCTP. After hybridization, the intensity of the signal was determined by using a Bio-imaging analyzer (*BAS-2000*; Fuji Photo Film, Japan).

The full-length *ShTIP* was subcloned into pYES2 (*Invitrogen*, CA, USA), which is an expression vector containing the *GAL1* promoter. The constructed plasmid was transformed into the calcineurin-null (*cnbΔ*) yeast (*Saccharomyces cerevisiae*) cells using the PEG lithium acetate method (Elble 1992). As a control, the empty vector was also introduced into the same yeast strain. The transformed cells were grown overnight at 30 °C in standard synthetic medium lacking uracil. Cells were harvested by centrifugation at 3000 *g* for 1 min at room temperature and resuspended in sterilized water. Cell density was adjusted to $A_{600} \sim 0.5$. Aliquots (2 mm^3) of 10-fold serial dilutions of cells were spotted onto YPG (2 % galactose, 1 % yeast extract, and 2 % peptone) plates containing 0.8 M NaCl, 1.5 M sorbitol, or 1.5 M mannitol.

Results

Characterization of *ShTIP*: We used an mRNA differential display approach to identify genes that are differentially regulated in *Salicornia* during salt stress. We found a DNA fragment of approximately 300 bp in length that encodes a putative tonoplast intrinsic protein (TIP). Using this differential display-derived fragment as a probe, we isolated a cDNA clone from the *Salicornia* cDNA library and designated it as *ShTIP*.

The full length of the *ShTIP* gene was 1014 bp and it contained an open reading frame of 254 amino acids with a predicted molecular mass of 26 kDa. The hydrophobicity profile of ShTIP calculated using the *ProtScale* program at *Expasy* server (Geneva, Switzerland) showed six highly hydrophobic regions (data not shown), which corresponded to membrane-spanning α-helices that are typical of aquaporins. Moreover, the signature motif Asn-Pro-Ala (NPA) was also found in the protein sequence. Computer analysis using the *ClustalW* program at *EMBL-EBI* (http://www.ebi.ac.uk/) revealed that the amino acid sequence of ShTIP shared a high level of identity with tonoplast intrinsic proteins of *Arabidopsis thaliana* (SITIP 68 %), *Mesembryanthemum crystallinum* (McTIP 78 %), *Zea mays* (ZmTIP 70 %) and *Oryza sativa* (OsTIP 71 %) (Fig. 1).

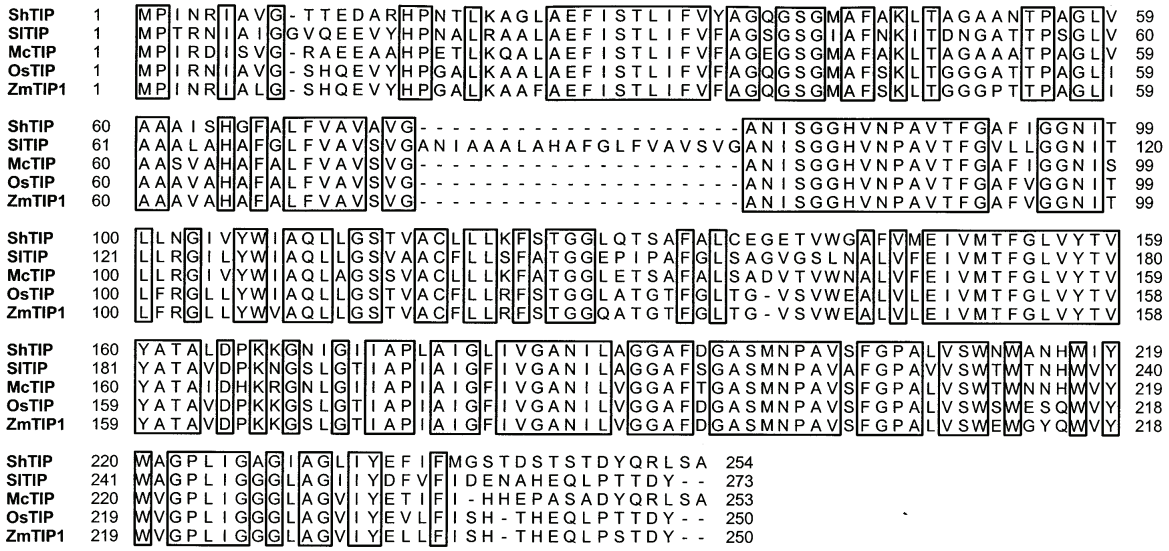

Fig. 1. The alignment of the deduced amino acid sequence of *Salicornia* TIP (ShTIP) with TIP homologs from other species of plant. The nucleotide sequence of the *ShTIP* cDNA was deposited in *GenBank* under the accession No. AY639596. The sequences of the TIP homologs were obtained from a public database NCBI, accession numbers: McTIP (common ice TIP, AAB17284), ZmTIP1 (maize TIP, AAC09245), OsTIP (rice TIP, AAK98737), SITIP (*Arabidopsis* salt inducible TIP, AAB62692). Gaps represented by dashes were introduced to maximize alignment. Identical amino acids are boxed.

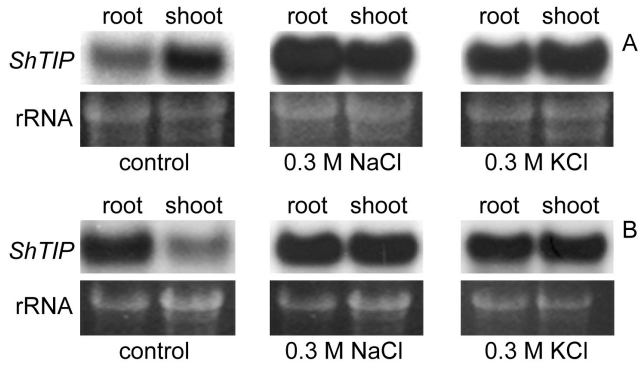

Fig. 2. The expression patterns of *ShTIP* (*A*) and *SITIP* (*B*) in the roots and the shoots or leaves of *Salicornia* and *Arabidopsis*, respectively. Four week-old *Salicornia* seedlings were treated with 0.3 M NaCl for 15 d or 0.3 M KCl for 7 d. *Arabidopsis* seedlings grown for 2 weeks in liquid medium were treated with 0.3 M of NaCl or 0.3 M KCl for 3 d. Aliquots (15 µg) of total RNA were subjected to Northern blot analyses and detected with either the *ShTIP* or *SITIP* probes. The lower panel shows ethidium bromide stained rRNA to determine the loading of equal amounts of RNA.

The expression of *ShTIP* and *SITIP* under salt and osmotic stress: Tonoplast intrinsic protein (*SITIP*) from *Arabidopsis* is known to be a salt-inducible protein. Based on the similarity of the protein sequence between SITIP and ShTIP, we performed Northern blot analyses to examine the expression pattern of *ShTIP* and *SITIP* genes in the shoots and roots of plants subjected to salt stress. Total RNA was extracted from the shoots and roots of *Salicornia* and *Arabidopsis* that had been treated with either 0.3 M NaCl or 0.3 M KCl. *ShTIP* and *SITIP* were expressed in both the root and shoot tissues under control and salt stress conditions. Interestingly, under control conditions, *ShTIP* expression was higher in the shoots than in the roots (Fig. 2*A*), whereas *SITIP* expression was higher in the roots than in the leaves (Fig. 2*B*). After treatment with NaCl or KCl, *ShTIP* expression was dramatically increased in the roots compared with that in the shoots. Treatment of *Salicornia* with 0.3 M NaCl increased *ShTIP* transcript levels 5-fold and 2-fold in the roots and shoots, respectively. The expression pattern of *ShTIP* after KCl treatment was similar to that after treatment with NaCl. Under treatment with both NaCl and KCl, *SITIP* expression was increased rapidly in the leaves but remained comparatively constant in the roots (Fig. 2*B*). These results indicated that the induction of *ShTIP* in *Salicornia* and *SITIP* in *Arabidopsis* can be modulated by both NaCl and KCl.

The expression levels of *ShTIP* and *SITIP* were also investigated following osmotic stress (15 % PEG 6000) and treatment with ABA (100 μM) that may act as a signal in response to both salt and osmotic stress. *ShTIP* expression in both the shoots and roots of *Salicornia* reached a high level at 2 h after NaCl treatment and then decreased gradually over 8 h. In the roots of *Salicornia*, the expression pattern of *ShTIP* in response to PEG was similar to that observed after NaCl treatment. However, *ShTIP* transcripts showed constant expression in shoots after 2 h of PEG treatment (Fig. 3*A*). On the other hand, the expression of *SITIP* in the roots was rapidly decreased in response to NaCl, whereas the expression of *SITIP* in the leaves was increased after 4 h of NaCl treatment (Fig. 3*B*). *ShTIP* transcript contents did not change appreciably when the plants were subjected to ABA. The expression of *ShTIP* in the shoots was slightly increased by ABA treatment, while its expression in the roots was increased significantly after 8 h of treatment. These results suggested that *ShTIP* is rapidly up-regulated in response to salt stress; however, the expression of *ShTIP* in response to NaCl is unlikely to be mediated by ABA. In contrast, *SITIP* was rapidly expressed in response to ABA in the leaves and roots of *Arabidopsis*. This data suggests that osmotic responsiveness is related in ABA signaling.

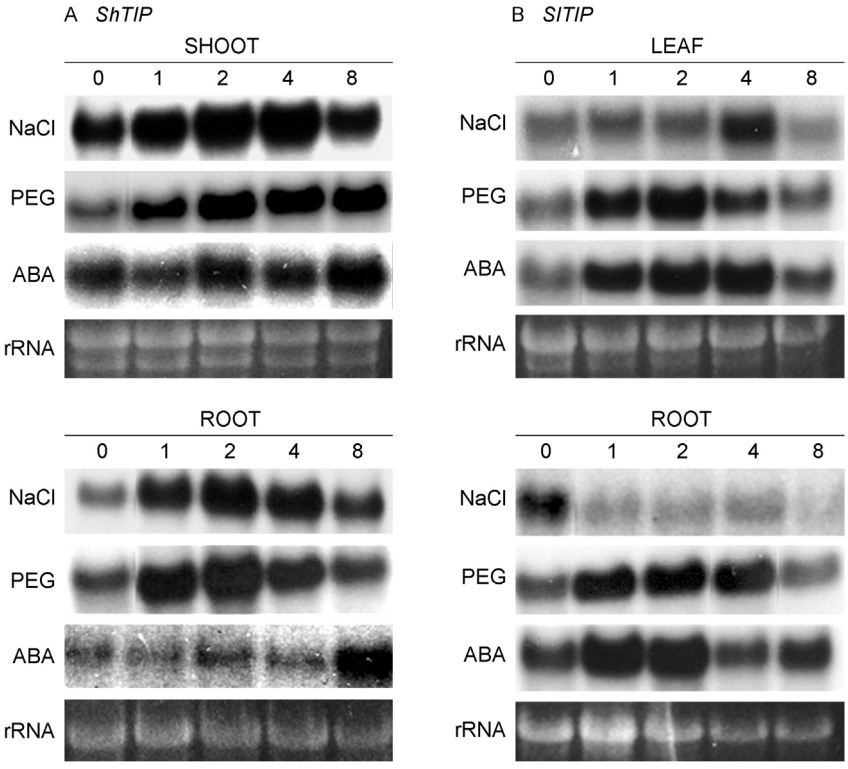

Fig. 3. The effects of NaCl, PEG or ABA on the expression of *ShTIP* (*A*) and *SITIP* (*B*) in the roots and shoots or leaves of *Salicornia* and *Arabidopsis*. Four-week-old seedlings of *Salicornia* and *Arabidopsis* were treated with 0.3 M NaCl, 15 % PEG or 100 μM ABA for the 0 to 8 d. Total RNA was extracted from the roots and shoots of *Salicornia* and the roots and leaves of *Arabidopsis*. Aliquots (15 μg) of total RNA were subjected to Northern blot analyses. The lower panel shows ethidium bromide stained rRNA to determine the loading of equal amounts of RNA.

The expression of ShTIP in *S. cerevisiae* confers salt tolerance: In order to study the biological function of *ShTIP*, the coding region of *ShTIP* was expressed in the salt-sensitive calcineurin (CaN)-deficient *S. cerevisiae*. The expression of *ShTIP*, which was inserted into pYES2, was transcriptionally regulated by the *GAL1* promoter and induced by galactose. The growth of ShTIP transformed yeast cells was examined on YPG plates supplemented with NaCl, sorbitol, or mannitol.

ShTIP transformed cells showed significant tolerance to high concentrations of NaCl (Fig. 4), compared with the control yeast containing the empty pYES2 vector. In contrast, transformants that were grown in sorbitol or mannitol had a growth rate similar to that of the control. These results suggest that ShTIP is able to selectively suppress the Na^+ sensitivity of calcineurin deficient yeast.

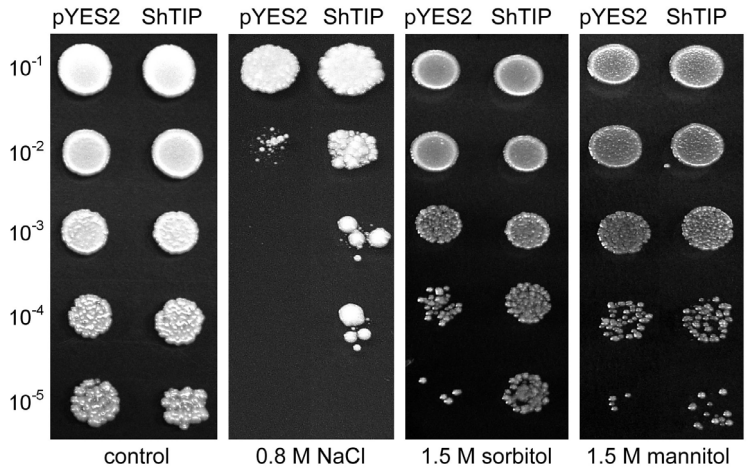

Fig. 4. Complementation of the calcineurin yeast mutant with ShTIP. The pYES2 vector containing the *ShTIP* gene or the empty vector (pYES2) was introduced into the calcineurin mutant (*cnbΔ*), and the complementation of the transformed cells was examined. Aliquots (2 mm^3) of 10-fold serial dilutions of the transformed cells were spotted on YPG plates containing 0.8 M NaCl, 1.5 M sorbitol, or 1.5 M mannitol. Plates were photographed after incubation for 2 d at 30 °C.

Discussion

Salicornia grows in a high saline environment, indicating that an investigation of the expression of aquaporins in this plant could broaden our understanding about salt stress adaptation mechanisms. In this study, we isolated a gene that encoded a membrane intrinsic protein from a *Salicornia* cDNA library. The deduced amino acid sequence of the *ShTIP* gene showed a high level of similarity to the tonoplast intrinsic protein, SITIP, expressed in *Arabidopsis* (Fig. 1). However, the expression patterns of the two genes were different under salt and osmotic stress conditions (Figs. 2 and 3). These data indicate that the response to salt stress in halophyte plants is different from that in glycophyte plants. In glycophytes, the influx of ions into the root is controlled, resulting in a limitation on the subsequent movement of ions into the shoot (Adams *et al*. 1992). On the other hand, halophytes are more readily take up Na^+ and coordinate the distribution of this ion into differentiated organs (Yeo 1998). The rapid increase in *ShTIP* transcript levels following salt stress suggests that *ShTIP* is involved in the regulation of Na^+ distribution in the halophyte *Salicornia* (Figs. 2,3).

Intracellular K^+ and Na^+ homeostasis is important for maintaining the activity of many cytosolic enzymes and for maintaining both the membrane potential and the appropriate osmoticum for regulating cell volume (Niu *et al*. 1995). Under salt stress, Na^+ competes with K^+ for uptake into the roots. However, in halophyte plants, both NaCl and KCl can be absorbed by roots at the same time, whereupon they stimulate the action of GA_3 to induce cell elongation (Kawasaki *et al*. 1978). This function presumably depends on the capacity of the plant to maintain K^+ uptake under salt stress conditions. In the *Salicornia*, the transcription level of *ShTIP* was not only triggered by NaCl but also by KCl (Fig. 2*A*). This may indicate that ShTIP has a dual Na^+ and K^+ specificity, allowing it transport either Na^+ or K^+ into vacuoles. However, increasing the concentration of KCl to 0.3 M caused the *Salicornia* plant to quickly wilt.

Plants that are tolerant of high NaCl concentrations maintain high sodium concentrations within their cells, and especially show considerable efficiency in sequestering Na^+ into vacuoles (Parks *et al*. 2002). The increased expression of *ShTIP* in the shoots and roots of the halophyte *Salicornia* was related to tolerance to salt stress. This tolerance may reflect by an adjustment of the osmotic gradient or by an increase in the transport of Na^+ into the vacuoles *via* the tonoplast water channels. In *Arabidopsis*, sensitivity to salt has been associated with an inability to effectively remove sodium ions from the cytoplasm or vacuoles (Parks *et al*. 2002). Apparently, down-regulation of *SITIP* during the early response to osmotic stress is a mechanism that restricts water loss

from, or limits the influx of sodium into vacuoles.

Many genes are induced by ABA during periods of water deficiency in a variety of plants. However, several genes induced by various stresses do not respond to ABA (Yamaguchi-Shinozaki and Shinozaki 1993). The differences in the time-dependent induction of *ShTIP* by salt stress and ABA suggested that the rapid induction of *ShTIP* transcription caused by the presence of salt was not mediated by exogenous ABA. In contrast, ABA induced a more rapid expression of the *SITIP* gene compared to that induced by NaCl and dehydration stresses, which suggests that the expression of *SITIP* is dependent on ABA biosynthesis.

Yeast cells have been particularly useful for studying membrane transporter proteins and the mechanisms that underlie the tolerance to salt stress in higher plants (Dreyer *et al*. 1999). To examine the function of *ShTIP*, we performed complementation analysis using a salt-sensitive calcineurin (CaN)-deficient yeast mutant, *cnbΔ*. CaN encodes a Ca^{2+} calmodulin-dependent type 2B protein phosphatase that regulates the activity of Na^+ influx and efflux transporter protein in yeast (Bressan *et al*. 1998, Pardo *et al*. 1998, Mendoza *et al*. 1996). The *cnbΔ* mutants accumulated a high level of Na^+ ions because of reduced Na^+ efflux and failure to take up K^+. Therefore, the mutant cells could not grow on medium containing Na^+ (Mendoza *et al*. 1996). In this study, we showed that the yeast mutants that expressed *ShTIP* were able to grow on medium containing Na^+. This result suggests that the expression of *ShTIP* suppresses the NaCl sensitivity of the *cnbΔ* mutants. However, the growth of the control or transformed mutant cells was not affected by high osmotic stress induced by either mannitol or sorbitol. Therefore, the function of ShTIP in yeast cells is involved in Na^+ or ion-specific transport, suggesting that this gene is required for ionic rather than osmotic stress adaptation.

In future studies, we will investigate the development of transgenic plants that express *ShTIP* in the hope of acquiring a broader understanding of the function of *ShTIP* in regulating salt tolerance in plants.

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