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Review

Improving salt tolerance in plant cells

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Abstract Salt stress in plant cells is mainly caused by a combination of hyperosmotic stress resulting from a high concentration of Na^+ in the environment and ionic stress resulting from the toxicity of cytosolic Na^+. Thus, salt tolerance in plants can be improved by expressing genes involved in compatible-solute biosynthesis to increase hyperosmotic tolerance, and/or by expressing vacuolar and plasma membrane ion transporters to re-establish intracellular Na^+ homeostasis under salt stress. To increase the salt tolerance of plants, we have identified and characterized genes that can confer increased hyperosmotic- and ionic-stress tolerance to plant cells.
We identified three paralogues of the *HAL3* gene in tobacco (*NtHAL3a*, *NtHAL3b*, and *NtHAL3c*) that encode putative 4′-phosphopantothenoylcysteine decarboxylases. We found that overexpression of *NtHAL3a* in tobacco BY2 cells increased the level of proline, a major compatible solute in plants, and improved the salt tolerance of these cells. We also found that tolerance to ionic stress can be improved in plants by the expression of the yeast *ENA1* gene, which encodes a sodium efflux pump that is present in fungi but not in plants. Furthermore, to understand the molecular mechanisms underlying Na^+ and K^+ homeostasis in rice (*Oryza sativa* L.), we identified two rice HKT transporters, OsHKT1 and OsHKT2, with different properties of Na^+ and K^+ transport. Finally, we investigated the role of the conserved glycine filter residue in the K^+ selectivity of the two OsHKTs.

Key words: Compatible solute, ion homeostasis, salt tolerance.

High salinity is one of the major factors limiting the growth and productivity of crop plants in agricultural fields. Salt stress in plant cells is mainly caused by the combination of hyperosmotic stress resulting from high Na^+ concentrations in the environment, which leads to water-deficient cells, and ionic stress resulting from the toxicity of high levels of cytosolic Na^+, which leads to metabolic inhibition of cellular activities (Figure 1). Therefore, to improve the salt tolerance of plants, at least two approaches should be employed. First, an increase in the biosynthesis of compatible solutes would protect cellular molecules and adjust the turgor pressure of the cells against hyperosmotic stress. Second, an enhancement of the activity of Na^+/H^+ antiporters in the plasma membrane and vacuolar membranes would maintain cytosolic Na^+ homeostasis against ionic stress (Figure 1).

Recent studies to identify and characterize genes involved in osmotic- and ionic-stress tolerance have opened the possibility of engineering crop plants with increased salt tolerance (Apse and Blumwald 2002). In plants, the expression of genes involved in the biosynthesis of compatible solutes such as mannitol (Tarczynski et al. 1993), ononitol (Sheveleva et al. 1997), proline (Kishor et al. 1995), glycinebetaine (Sakamoto and Murata 2002), trehalose (Holmström et al. 1996), ectoine (Nakayama et al. 2000), and fructan (Pilon-Smits et al. 1995) could increase the hyperosmotic tolerance of the plant cells. The expression of vacuolar and plasma membrane ion transporters in plants provides an alternative approach to re-establish intracellular Na^+ homeostasis under salt-stress conditions. Overexpression of either a vacuolar Na^+/H^+ antiporter (Apse et al. 1999; Ohta et al. 2002; Zhang and Blumwald 2001; Zhang et al. 2001), a vacuolar H^+-pyrophosphatase (Gaxiola et al. 2001), or a plasma membrane Na^+/H^+ antiporter (Shi et al. 2003) has been reported to confer salt tolerance to transgenic plants.

Here, we review our recent reports about the identification of four genes that confer increased salt tolerance to plants. First, the expression of NtHAL3a, a putative 4′-phosphopantothenoylcysteine decarboxylase involved in coenzyme A (CoA) biosynthesis, was shown to increase compatible-solute production (Yonamine et

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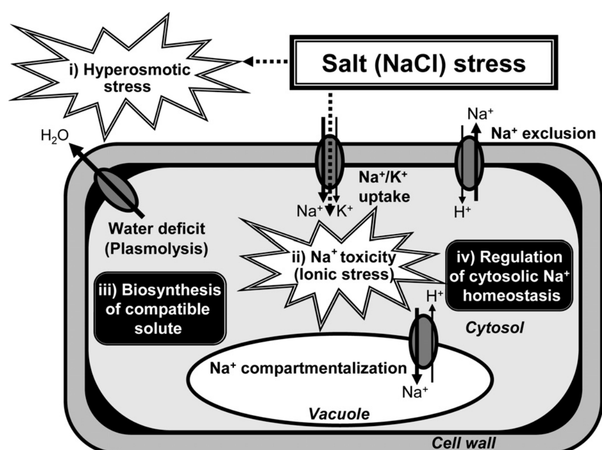


Figure 1. Two harmful effects of salt (NaCl) stress on plant cells are i) the nonspecific effect of water deficit caused by hyperosmotic stress and ii) the specific effect of ionic stress caused by cytosolic Na^+ toxicity. To improve the salt tolerance of the cells, at least two strategies are required: iii) biosynthesis of a compatible solute as an osmoprotectant and iv) regulation of cytosolic Na^+ homeostasis using Na^+-transporting proteins located in both the plasma and vacuolar membranes.

al. 2004). Second, the expression of Ena1p, a yeast sodium efflux pump, was found to export cytosolic Na^+ during salt-stress conditions (Nakayama et al. 2004). Finally, the identification and characterization of two rice OsHKTs, members of the HKT family of Na^+ and K^+ transporters, have shed light on the mechanisms of Na^+ and K^+ homeostasis in rice (Horie et al. 2001; Mäser et al. 2002a).

Results

Expression of NtHAL3a *to increase compatible-solute production*

The *HAL3*/*SIS2* gene of *Saccharomyces cerevisiae* was identified by its ability to confer salt tolerance to wild-type cells presented with toxic concentrations of sodium chloride. The first plant homologues of *HAL3* to be characterized were isolated from *Arabidopsis thaliana* (*AtHAL3a* and *AtHAL3b*; Espinoza-Ruiz et al. 1999). Interestingly, the function of the HAL3 proteins differs in plant and yeast cells. In yeast, HAL3 enhances salt tolerance by inhibiting Ppz1 phosphatase activity (de Nadal et al. 1998). On the other hand, although overexpression of *AtHAL3a* in plants also confers salt tolerance, there is no evidence that AtHAL3 possesses phosphatase inhibition activity (Espinoza-Ruiz et al. 1999). Subsequently, Kupke et al. (2001) suggested that the AtHAL3a protein functions in CoA biosynthesis. CoA is thought to be a key substrate for the biosynthesis of many amino acids including proline, a major compatible solute in plants. To investigate the possibility that plant HAL3 could confer salt tolerance by increasing the production of proline, we used a probe

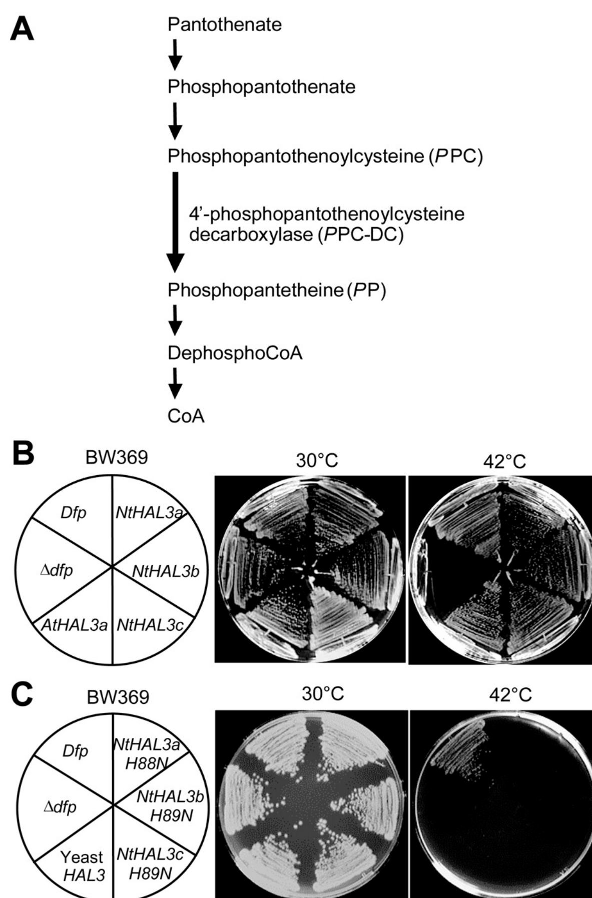


Figure 2. Complementation analysis of the *NtHAL3* genes with the temperature-sensitive *dfp* mutant of *E. coli*. (A) The coenzyme A biosynthetic pathway in *E. coli* (Begley et al. 2001). (B) Growth tests using *E. coli* strain BW369 (*dfp*-707) transformed with an expression vector containing one of the *NtHAL3* or *AtHAL3* genes. (C) Growth tests using *E. coli* strain BW369 transformed with an expression vector containing the active-site mutants of the *NtHAL3* or yeast *HAL3* genes. *E. coli* BW369 cells transformed with the *dpf* gene and an empty vector were used as a positive control (Dfp) and a negative control (*Δ*dfp), respectively. Transformants were grown on LB plates containing ampicillin at 30°C for 24 h or 42°C for 12 h as indicated.

corresponding to the AtHAL3 cDNA to isolate HAL3 homologues from tobacco (*Nicotiana tabacum* L.). The function of the three identified genes, named *NtHAL3a*, *NtHAL3b*, and *NtHAL3c*, was characterized using *E. coli* mutants and transgenic BY2 cells.

The NtHAL3 *genes complement the* E. coli dfp *mutation*

The predicted amino-acid sequences of the three NtHAL3 proteins contain four highly conserved motifs (Yonamine et al. 2004) that form a domain containing a 4′-phosphopantothenoylcysteine decarboxylase (*P*PC-DC). These motifs are also found in the AtHAL3a protein, and the *P*PC-DC activity of AtHAL3a was confirmed biochemically in an *in vitro* study (Kupke et al. 2001). The *dfp* gene encodes a *P*PC-DC in *E. coli* where it plays an important role in the coenzyme A

(CoA) biosynthetic pathway (Figure 2A; Kupke et al. 2000; Kupke 2001; Strauss et al. 2001). To investigate whether the NtHAL3 proteins display *P*PC-DC activity, we examined the ability of the *NtHAL3* genes to complement a temperature-sensitive *dfp* mutation that produces a lethal phenotype in *E. coli* at 42°C (Spitzer et al. 1985). The three *NtHAL3* genes were expressed in the *E. coli dfp* mutant using the expression vector pKC7. Each *NtHAL3* gene was able to rescue the *dfp* mutant strain at 42°C (Figure 2B). Moreover, previous studies carried out *in vitro* demonstrated that a His-90 to Asn substitution in the *P*PC-DC active site of AtHAL3a resulted in complete lost of *P*PC-DC activity (Kupke et al. 2001). Therefore, we examined the *P*PC-DC activity of the NtHAL3 active-site mutants in the same way. NtHAL3a-H88N, NtHAL3b-H89N, and NtHAL3c-H89N were used in complementation tests with the *E. coli dfp* mutant. We found that the three mutant *NtHAL3* genes did not complement the *dfp* mutation in *E. coli* (Figure 2C). The functional difference between the yeast and the plant HAL3 proteins has been previously discussed (Kupke et al. 2001). Therefore, we examined whether yeast HAL3 protein displayed *P*PC-DC activity with a complementation test using the *E. coli dfp* mutant. The results showed that yeast *HAL3* was not able to complement the *E. coli dfp* mutant (Figure 2C). These results strongly suggest that plant HAL3 proteins have *P*PC-DC activity, whereas the yeast HAL3 protein, a known regulator of protein phosphatase activity, does not.

Overexpression of NtHAL3a *improved salt, LiCl, and sorbitol stress tolerance and increased proline production in BY2 cells*

Coenzyme A and its thioesters are essential cofactors for many enzymatic and energy-yielding reactions including the TCA cycle, fatty-acid metabolism, and amino-acid metabolism (Begley et al. 2001). If the *P*PC-DC activity of NtHAL3a protein is required in the metabolic pathway that synthesizes coenzyme A from pantothenate, the intracellular concentration of some of the downstream metabolites of this pathway may be increased in *NtHAL3a*-overexpressing cells. Therefore, to investigate the molecular roles of plant *HAL3* genes, *NtHAL3a* was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and introduced into BY2 cells by *Agrobacterium tumefaciens*-mediated transformation. The expression levels of NtHAL3a varied widely, and three clones with high NtHAL3a expression levels were selected for further analysis (SA3, SA4, and SA5; Figure 3A). Growth inhibition of transgenic BY2 clones by salt stress (100 or 140 mM of NaCl) was observed. Although the growth of control cells (transformed with EGFP) was markedly inhibited by NaCl stress, overexpression of NtHAL3a suppressed the growth inhibition with a direct relationship between the level of NtHAL3a expression in transgenic BY2 cells and the level of inhibition (Figure 3B). High salt concentrations can negatively affect cell growth through hyperosmotic stress or ionic stress caused by Na^+ toxicity (Figure 1). In order to identify which aspect of salt stress was affected by NtHAL3 overexpression, growth inhibition experiments were carried out under hyperosmotic conditions using sorbitol, and under ionic-stress conditions using LiCl as a more toxic analog of NaCl (Serrano 1996). Suppression of growth inhibition by LiCl and sorbitol stress was observed in *NtHAL3a*-expressing BY2 cells (Figure 3B). These results relate NtHAL3a function to both hyperosmotic stress and sodium-ion toxicity in tobacco cells, and suggest that the cells can increase the levels of amino acids, such as proline, that function as compatible solutes (Delauney AJ and Verma DP 1993b; Yoshiba et al. 1997). Therefore, we determined the intracellular free amino-acid concentrations in *NtHAL3a*-overexpressing BY2 cells. Table 1 shows the concentrations of free amino acids in the SA3 clone of transgenic BY2 cells under control conditions. The percentage of the total amino acid that was found to be proline in SA3 cells was 4.4-fold higher in comparison with that in control cells under non-stress conditions. Moreover, the fraction of total amino acid that was proline was approximately three times higher than that in control cells under salt-stress conditions (SA3, 4.1%; EGFP, 1.4%).

Expression of ENA1 *to export cytosolic* Na^+ *during salt stress*

In contrast to plants, fungi employ a plasma membrane Na^+-ATPase in addition to the plasma membrane Na^+/H^+ antiporter as a major component of Na^+ efflux under salt stress. A Na^+ and Li^+ efflux P-type ATPase (Ena1p) encoded by the *ENA1* gene was initially found in budding yeast (Haro et al. 1991). To investigate whether expression of yeast Ena1p improved the salt tolerance of plant cells, we expressed HA-tagged Ena1p (Ena1p-3HA) in tobacco BY2 cells.

Functional expression of yeast ENA1 *gene in tobacco BY2 cells*

High salinity inhibits plant growth by increasing the cytosolic levels of toxic ions, such as Na^+ and Cl^-. This is observed even in callus tissue, indicating that ion homeostasis during salt stress in plants is primarily regulated at the cellular level. Therefore, to investigate the effects of plasma membrane Na^+-ATPases on ion homeostasis during salt stress, tobacco BY2 suspension-cultured cells were chosen as model plant cells. The chimeric *ENA1-3HA* gene was placed under the control of the CaMV 35S promoter in the pMS2 binary vector and introduced into tobacco BY2 cells by *A. tumefaciens*-mediated transformation. Although the

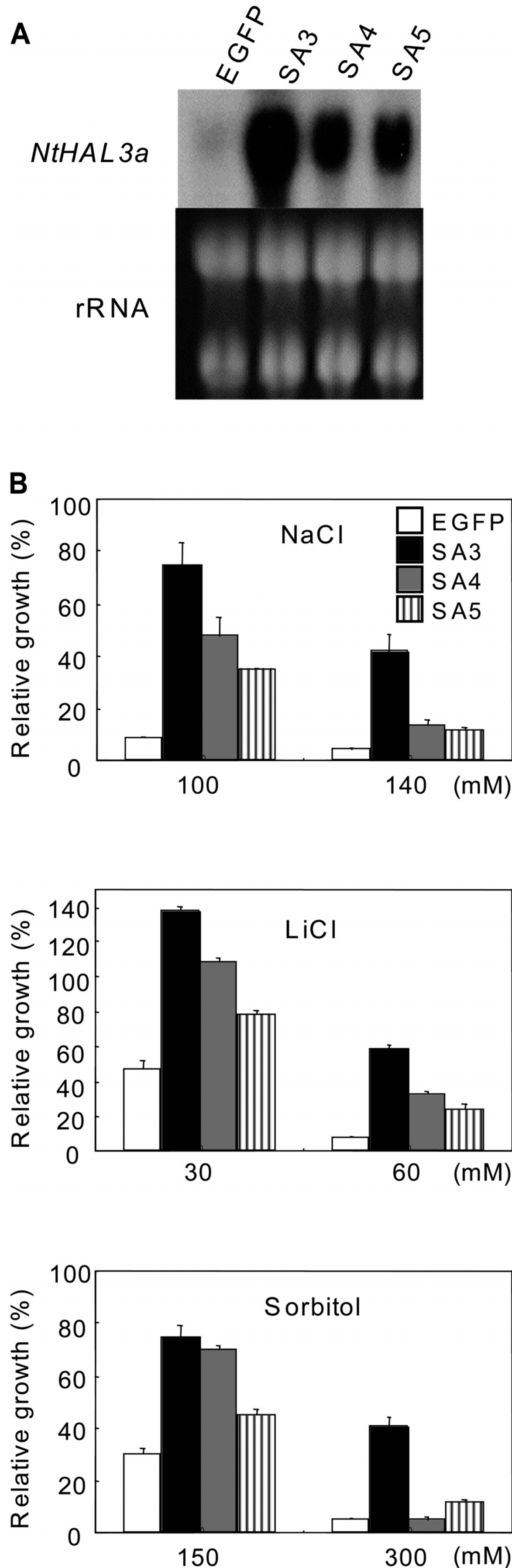
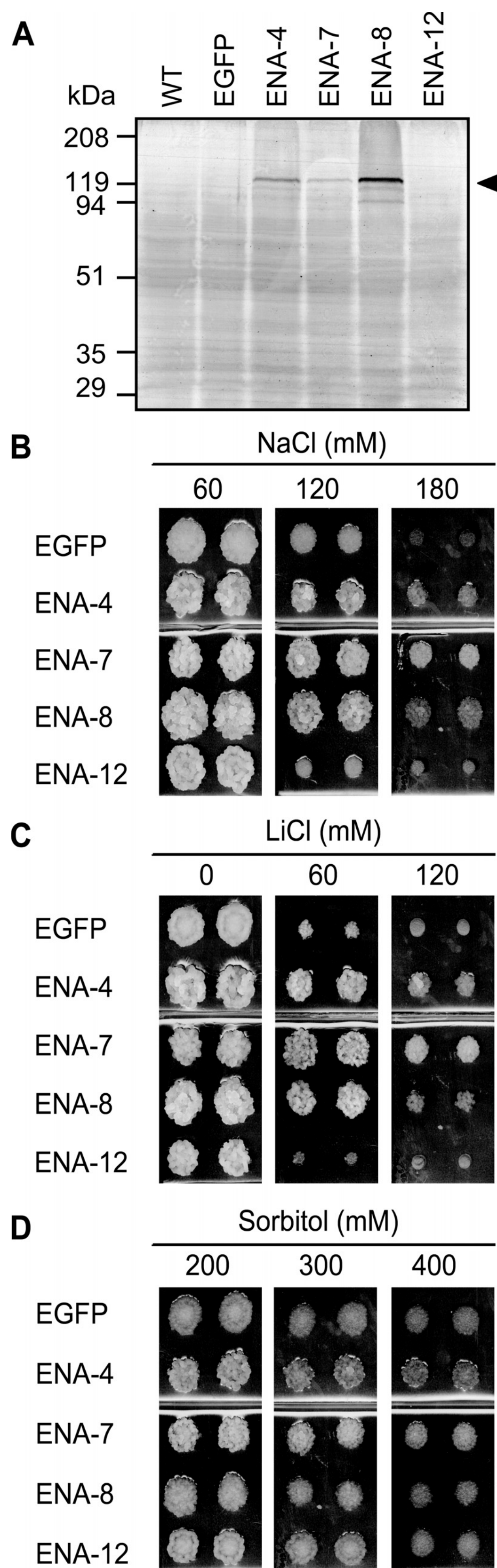


Figure 3. The effect of *NtHAL3a* overexpression on the growth of BY2 cells in medium containing NaCl, LiCl, or sorbitol. (A) The Expression level of *NtHAL3a* in the transgenic BY2 cells was detected by Northern blot analysis. RNA was isolated from 5-day-old transgenic BY2 cells harboring the *EGFP* gene (control) and three independent clones harboring the *NtHAL3a* gene (SA3, SA4, and SA5). Each lane contains 20 μg of total RNA, and the membrane was probed with a ^{32}P-labelled probe for *NtHAL3a*. rRNA stained with ethidium bromide was used as a control for the amount of total RNA that was loaded. (B) Transgenic BY2 clones were cultured for 5 days in liquid medium containing the indicated concentrations of NaCl, LiCl, or sorbitol. Cell growth was measured by the fresh weight of the cells, and the growth of each clone under the indicated stress conditions was calculated relative to the growth under non-stress conditions. Error bars represent ± standard deviation (n=3).

expression level of Enalp-3HA was quite low in these cells (Figure 4A), the HA-tag allowed us to efficiently screen transgenic BY2 clones expressing Ena1p-3HA using anti-HA antibodies. Wild-type BY2 cells (WT) and kanamycin-resistant BY2 cells transformed with *EGFP* driven by the CaMV 35S promoter in the pMS2 binary vector (EGFP) were used as controls. On growth media without salt or with various salt concentrations, WT and EGFP clones were phenotypically indistinguishable from each other (data not shown). Western blot analysis of yeast and plant microsomal membranes using anti-HA antibodies showed that Ena1p-3HA was correctly translated as a 124-kDa membrane protein in tobacco BY2 cells (Figure 4A). We identified four ENA clones (ENA-4, ENA-7, ENA-8, and ENA-12) with different expression levels of Ena1p-3HA (Figure 4A). Ena1p-3HA was strongly expressed in the ENA-8 clone, moderately expressed in the ENA-4 clone, weakly expressed in the ENA-7 clone, and no expression was detected in the ENA-12 clone, EGFP cells, or WT cells (Figure 4A).

To specifically investigate the function of the plasma membrane Na^+-ATPase in plant cells, cells should only be exposed to ionic stress. To reduce the effects of osmotic stress on the growth of the plant cells, we used low calcium medium (0.3 mM $CaCl_2$) because plant cells show an increased sensitivity to salts when the calcium concentration in the growth medium is reduced (LaHaye and Epstein 1969). Moreover, we used LiCl instead of NaCl to induce ionic stress because Li^+ is a more toxic analog of Na^+. Thus, LiCl can be used at a lower concentration than Na^+ to inhibit growth (Serrano 1996). As a control, sorbitol was used to induce general osmotic stress without ionic stress. To determine the optimal conditions for testing the effects of ionic and osmotic stress on plant cells, we grew wild-type tobacco BY2 cells on various media and found that the growth of these cells was significantly inhibited on the low calcium medium (0.3 mM $CaCl_2$) containing 120 mM NaCl, 60 mM LiCl, or 300 mM sorbitol (data not shown). Next, to investigate whether Ena1p-3HA functions as a plasma



membrane $Na^+(Li^+)$-ATPase in plant cells, transgenic tobacco BY2 cells expressing Ena1p-3HA (the ENA-4, ENA-7, and ENA-8 clones) and non-expressing cells (the ENA-12 and EGFP clones) were grown on the low calcium medium with or without NaCl, LiCl, or sorbitol as indicated (Figure 4). Transgenic tobacco BY2 cells expressing Ena1p-3HA showed increased salt-stress tolerance on the low calcium media containing at least 120 mM NaCl, whereas these cells showed the same growth rate as non-expressing cells on the media containing 60 mM NaCl (Figure 4B). Although the osmolarities of the NaCl and LiCl solutions were the same, the growth of the non-expressing cells was significantly inhibited on the medium containing 60 mM LiCl compared to the medium containing 60 mM NaCl because of the higher toxicity of Li^+ (Figure 4B and 4C). Under LiCl stress conditions, Ena1p-3HA-expressing tobacco BY2 cells showed increased ionic-stress tolerance on low calcium medium containing more than 60 mM LiCl (Figure 4C). The difference in growth between Ena1p-3HA expressing cells and non-expressing cells was more obvious with LiCl than with NaCl (Figure 4B and 4C). At high sorbitol concentrations (more than 300 mM), the growth of transgenic BY2 cells was inhibited by osmotic stress, and the growth rates of Ena1p-3HA-expressing cells and non-expressing cells were indistinguishable (Figure 4D). These results show that Ena1p-3HA confers increased tolerance to salt stress, especially ionic stress generated by Na^+ or Li^+, and that Ena1p-3HA likely functions as a plasma membrane $Na^+(Li^+)$-ATPase in plant cells. We observed that the highest Ena1p-3HA-expressing ENA-8 clone, however, showed the lowest tolerance to severe ionic stress generated by 120 mM LiCl (Figure 4C). This result indicates that an excess amount of Ena1p-3HA may have some negative effects on cell growth during

Figure 4. The effect of Ena1p expression on the growth of tobacco BY2 cells under ionic- and osmotic-stress conditions. (A) Expression of HA-tagged Ena1p (Ena1p-3HA) in transgenic tobacco BY2 cells was detected by Western blot analysis. Wild-type BY2 cells (WT), control BY2 cells transformed with *EGFP* gene (EGFP), and BY2 cells transformed with *ENA1-3HA* (ENA-4, ENA-7, ENA-8, and ENA-12) were freshly subcultured and grown for 5 days at 27°C in liquid growth medium. Microsomal membrane proteins extracted from the clones (120 μg each) were electrophoresed in a 7.5% (w/w) polyacrylamide-SDS gel, blotted onto a PVDF membrane, and probed with anti-HA antibodies. Molecular mass markers are shown on the left. The arrowhead points to the position of the 124-kDa band corresponding to Ena1p-3HA. (B–D) Growth tests of transgenic tobacco BY2 cells expressing Ena1p-3HA (ENA-4, ENA-7, and ENA-8) or control non-expressing cells (ENA-12 and EGFP). The cells were pre-cultured in liquid growth medium containing low calcium (0.3 mM $CaCl_2$) for 1 week, and were subcultured in the same medium. Five-day-old subcultured cells were diluted to 50% (v/v) cell density with the low calcium medium, and duplicate drops (50 μL each) of the dilutions were deposited on solid low calcium medium with various concentrations of NaCl (B), LiCl (C), or sorbitol (D) as indicated. The photograph shows growth of the tobacco BY2 cells after 4 weeks of incubation at 27°C in the dark.

salt-stress conditions.

Identification and characterization of OsHKTs, proteins involved in Na^+ and K^+ homeostasis in rice

K^+ and Na^+ homeostasis is important for salt tolerance in plants. Moreover, the availability of K^+ is crucial for inducing Na^+ uptake (Horie and Schroeder 2004). TaHKT1, a Na^+/K^+ co-transporter from wheat, was the initial member of the HKT family of Na^+ and K^+ transporters to be isolated (Schachtman and Schroeder 1994). An HKT homologue from *Arabidopsis*, AtHKT1, was later identified and characterized (Uozumi et al. 2000). AtHKT1, a major Na^+ entry site for *Arabidopsis* cells, was shown to be a Na^+ transporter that lacks K^+ transport activity (Rus et al. 2001; Mäser et al. 2002b). To understand Na^+ and K^+ homeostasis and salt tolerance in rice, we isolated and functionally characterized HKT homologues from a salt-sensitive japonica rice (cv. Nipponbare) and from a salt-tolerant indica rice (cv. Pokkali).

Functional analysis of rice HKT *genes using yeast mutants*

Two *HKT* genes, *OsHKT1* and *OsHKT2*, were identified in rice. Although *OsHKT1* was a singleton in the Nipponbare cultivar (Ni-*OsHKT1*), the cultivar Pokkali contained two closely related genes, Po-*OsHKT1* and Po-*OsHKT2*.

Previous studies showed that plant K^+ transporters could rescue the high-affinity K^+ uptake-deficient *trk1* and *trk2* mutants of the *S. cerevisiae* strain CY162 (Schachtman and Schroeder 1994). The *TaHKT1* cDNA was isolated by functional complementation in the CY162 strain (Schachtman and Schroeder 1994). *AtHKT1*, however, was not able to complement the *trk* mutations (Uozumi et al. 2000). We tried the same complementation assay using our isolated *OsHKT* cDNAs in CY162 cells and found a significant difference between the two *OsHKT* genes in their ability to complement the *trk* mutations. Whereas the expression of *OsHKT1* was incapable of rescuing the *trk* mutations, the mutant host expressing *OsHKT*2 grew on medium containing 0.1 mM KCl (Figure 5A). Even though the amino-acid sequences of the OsHKT1 and OsHKT2 proteins shared 91% identity with each other and the proteins show similar hydrophobic profiles, the K^+ uptake properties of the transporters seem to be different.

Overexpression of *TaHKT1* or *AtHKT1* in yeast cells caused Na^+ hypersensitivity due to increased Na^+ uptake (Rubio et al. 1995, 1999; Uozumi et al. 2000). The Na^+ uptake properties of OsHKT1 and OsHKT2 were studied by a growth inhibition test using *S. cerevisiae* strain G19, which displays increased Na^+ sensitivity due to disruption of the *ENA* genes (*ena1*-4), which encode

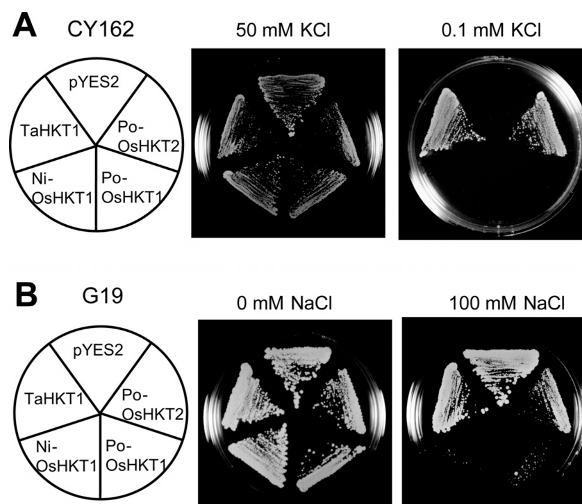


Figure 5. Expression of OsHKTs in yeast. (A) Complementation analysis of high-affinity K^+ uptake deficient mutant yeast cells (strain CY162) with *OsHKT1*, *OsHKT2*, *TaHKT1*, or control vector pYES2. The yeast cells were grown on arginine phosphate medium (Rodriguez-Navarro and Ramos, 1984) containing the indicated concentrations of KCl. (B) Na^+-induced growth inhibition of salt-sensitive mutant yeast cells (strain G19) expressing *OsHKT1*, *OsHKT2*, *TaHKT1*, or control vector pYES2. The yeast cells were grown on SC medium containing the indicated concentrations of NaCl.

Na^+-extruding ATPases. The G19 transformants expressing *OsHKT1* or *OsHKT2* displayed more sensitivity to Na^+ than *TaHKT1*-expressing cells (Figure 5B). Growth inhibition of *OsHKT*-expressing cells was observed at 50 mM NaCl, and the growth was completely inhibited at 150 mM NaCl (data not shown). Interestingly, at 100 mM NaCl, the expression of OsHKT1 seemed to make G19 cells more sensitive to Na^+ than the expression of OsHKT2 did (Figure 5B).

A putative glycine filter residue that confers K^+ selectivity is present in OsHKT2 *but not in* OsHKT1

Although the presumed P-loops of the HKT/Trk/KtrB proteins are highly conserved, they only contain three or four amino-acid residues that are homologous to the complete K^+ channel P-loops (Figure 6A). Alignment of the presumed P-loops of the HKT/Trk/KtrB proteins with known P-loops from K^+ channels revealed a glycine residue in the HKT/Trk/KtrB transporters at the position that corresponds to the first glycine residue of the GYG motif (Figure 6A). HKT, Trk, and KtrB have four putative P-loops (Durell and Guy 1999; Durell et al. 1999; Kato et al. 2001). The four glycines, one at the end of each of the four P-loops, are invariably present in bacterial TrkH and KtrB proteins as well as fungal Trk transporters. The same holds true for TaHKT1, where the four glycines are found at amino-acid positions 91, 246, 370, and 477. The predicted amino-acid sequences of OsHKT1 from Pokkali and Nipponbare are identical and

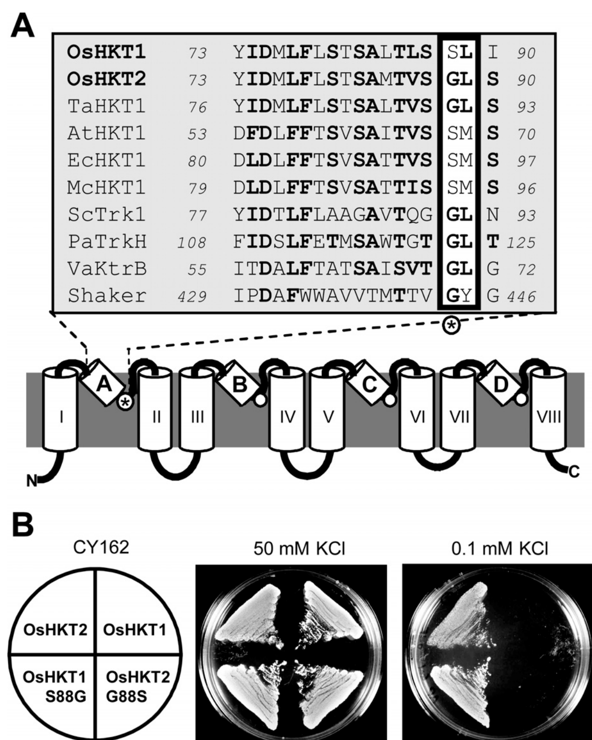


Figure 6. Point mutation of OsHKTs. (A) A schematic model of the OsHKT proteins. A model of HKT/Trk/KtrB transporters, based on the known structure of K^+ channels (Mäser et al. 2002a). The predicted P-loops are labeled A–D, whereas transmembrane domains are labeled I–VIII. The sequence alignment shows P-loop A of various plant HKTs compared with Trk1 from *S. cerevisiae* (M21328), TrkH from *Pseudomonas aeruginosa* (AAG06598), KtrB from *Vibrio alginolyticus* (BAA32063), and the P-loop of the *Drosophila* Shaker channel (S00479). The residue corresponding to the first glycine of the K^+ channel GYG motif is marked with an asterisk. (B) Site-directed mutagenesis of OsHKT1 and OsHKT2. Expression of OsHKT2 enabled K^+-uptake-deficient yeast cells (CY162) to grow on 0.1 mM KCl, but OsHKT1 did not. Mutation of Ser-88 to a glycine residue in OsHKT1 (*OsHKT1*-S88G) restores K^+-uptake complementation, whereas mutation of Gly-88 to a serine residue in OsHKT2 (OsHKT2-G88S) abrogates K^+-uptake complementation.

have a serine at the predicted filter position in P-loop A (Ser-88, Figure 6A), whereas a glycine residue (Gly-88) is preserved in OsHKT2 from Pokkali. Apparent replacement of the predicted filter glycine in P-loop A by a serine residue also occurs in HKT proteins identified from Arabidopsis, eucalyptus, and ice plant (Figure 6A).

Only OsHKT2 was able to completely suppress the *Δtrk1,2* phenotype of strain CY162; OsHKT1 did not allow growth under low K^+ conditions (Figure 5A). To further test the hypothesis that the terminal glycine residues in the P-loop-like domains of HKT/Trk/KtrB transporters are related to the K^+-selective P-loop filters in K^+ channels, we mutated Ser-88 of OsHKT1 to a glycine (OsHKT1-S88G) and Gly-88 of OsHKT2 to a serine (OsHKT2-G88S). Mutants and wild-type transporters were expressed in mutant CY162 yeast cells. CY162 cells expressing OsHKT1 did not grow on medium containing 0.1 mM KCl, whereas OsHKT1-S88G-expressing cells did (Figure 6B). OsHKT2-G88S, in contrast to OsHKT2, did not permit growth at low KCl concentrations (Figure 6B). Thus, as with AtHKT1 and TaHKT1, the switch from a serine to a glycine residue in P-loop A was sufficient to confer complementation.

Discussion

Expression of NtHAL3a *to increase compatible-solute production*

Our results support the hypothesis based on the results of *in vitro* experiments by Kupke et al. (2001) that the plant HAL3 proteins function in the CoA biosynthetic pathway *in vivo*. Acetyl-CoA is involved in amino-acid synthesis at several points, including providing the carbon skeleton for leucine and a series of acetylated intermediates for the synthesis of ornithine and arginine (Ireland 1997; Yokota et al. 2002). We therefore determined the intracellular free amino-acid concentrations of transgenic BY2 cells overexpressing NtHAL3a. We found that the percentage of total amino acid that was proline, a major compatible solute in plants, increased by more than four-fold in *NtHAL3a*-overexpressing cells (Table 1). In plant cells, proline is synthesized from both the glutamate and ornithine pathways (Delauney and Verma 1993). The pathway used depends on the developmental stage of the plant and environmental stress (Delauney et al. 1993; Roosens et al. 1998). Acetyl-CoA is used as the first metabolic substrate in the ornithine pathway (Ireland 1997; Yokota et al. 2002). In the *NtHAL3a*-overexpressing cells, the percentage of total amino acid that was arginine, which is also synthesized by the ornithine pathway, increased by approximately two-fold (Table 1). These results suggest that the increased amount of proline and arginine in *NtHAL3a*-overexpressing cells was a consequence of an increased use of the ornithine biosynthesis pathway by way of enhanced *P*PC-DC activity. It will be important to determine whether the production of CoA and acetyl-CoA actually increases due to the overexpression of *NtHAL3a*. Based on our study, plant *HAL3* genes should be useful for the production of salt-tolerant plants. Moreover, in plant cells, these genes are important for the metabolic regulation of CoA biosynthesis from pantothenate and its effects on the biosynthesis of several amino acids through the ornithine pathway.

In BY2 cells, *NtHAL3a* overexpression abolished the growth inhibition caused by LiCl or NaCl stress (Figure 3B). Previous reports proposed that a compatible solute, such as proline, can function as a free radical scavenger under salt-stress conditions (Smirnoff and Cumbes 1989; Kishor et al. 1995). The improved salt tolerance of transgenic BY2 cells could therefore result from the

Table 1. Amino acid contents in transgenic BY2 cells.

Amino acids	EGFP		SA3	
	Amino acid content		Amino acid content	
	nmol g^{-1} FW	%	nmol g^{-1} FW	%
Asp	650±240	11	900±270	13
Thr	440±170	7.3	300±120	4.2
Ser	330±150	5.5	280±90	3.9
Glu	820±440	14	940±520	13
Gln	310±160	5.2	870±400	12
Gly	71±36	1.2	80±35	1.1
Ala	2100±780	35	2200±530	30
Val	820±170	14	860±110	12
Cys	ND		ND	
Met	ND		ND	
Ile	81±18	1.4	130±46	1.8
Leu	170±69	2.8	190±46	2.7
Tyr	27±11	0.45	19±5	0.26
Phe	77±13	1.3	110±17	1.5
Lys	13±7	0.21	19±11	0.27
His	8.6±5	0.14	16±6	0.22
Arg	16±4	0.26	41±9	0.56
Pro	63±37	1.0	320±65	4.4
Total amino acids	6000±2300	100	7200±2200	100

Amino acids were extracted from transgenic BY2 cells after cultivation in modified LS medium. $n=3$. ND, Not detected.

increased level of proline caused by *NtHAL3* overexpression as a free radical scavenger. In future experiments, the free-radical scavenging ability of *NtHAL3a*-expressing cells should be compared to that of control cells.

Expression of ENA1 *to export cytosolic Na⁺ during salt stress*

When faced with salinity stress, plants and fungi use similar ion transport systems to maintain ion homeostasis (Serrano and Rodríguez-Navarro 2001). Plants, however, do not contain a plasma membrane Na^+-ATPase, which evolved only in fungi as they adapted to high-salinity environments (Benito et al. 2002; Garciadeblas et al. 2001). We have demonstrated that the expression of plasma membrane Na^+-ATPases confers increased salt tolerance to plant cells. Our data show for the first time that the plasma membrane Na^+-ATPase encoded by the yeast *ENA1* gene increases salt tolerance in plants.

Growth tests using the transgenic BY2 cells demonstrated that Ena1p-3HA functions as a plasma membrane Na^+(Li^+)-ATPase in plant cells, and that expression of Ena1p-3HA confers increased tolerance to salinity stress, especially ionic stress generated by increased levels of Na^+ or Li^+ (Figures 4B and 4C).

In plants, three mechanisms function cooperatively to prevent the accumulation of Na^+ in the cytoplasm: restriction of Na^+ influx, compartmentalization of Na^+ into vacuoles, and an active Na^+ efflux (Niu et al. 1995). Recent progress in molecular genetic studies of Arabidopsis revealed key Na^+-transporting proteins that contribute to salt tolerance in plants. The influx of Na^+ through the plant plasma membrane is mediated by high- and low-affinity K^+-transporting proteins and other non-specific cation-transporting proteins (Maathuis and Amtmann 1999). AtHKT1 was identified as a salt-tolerance determinant that regulates Na^+ influx into roots (Rus et al. 2001) and Na^+ recirculation from shoots to roots (Berthomieu et al. 2003). Disruption of *AtHKT1* resulted in reduced Na^+ entry during salt stress (Mäser et al. 2002; Rus et al. 2001).

The compartmentalization of Na^+ into vacuoles is mediated by a tonoplast Na^+/H^+ antiporter, which is driven by the electrochemical gradient of protons generated by a H^+-pyrophosphatase and a H^+-ATPase (Blumwald 1987). The overexpression of AtNHX1, which encodes the tonoplast Na^+/H^+ antiporter of Arabidopsis, conferred increased salt tolerance to transgenic Arabidopsis plants (Apse et al. 1999). Transgenic plants overexpressing the Arabidopsis tonoplast H^+-pyrophosphatase AVP1 displayed enhanced salt tolerance due to the additional driving force for Na^+ compartmentalization into vacuoles (Gaxiola et al. 2001).

The extrusion of Na^+ through the plant plasma membrane is mediated by the plasma membrane Na^+/H^+ antiporter, and is driven by the electrochemical gradient of protons generated by plasma membrane H^+-ATPases (Blumwald 2000). Arabidopsis *SOS1* was recently identified as the plasma membrane Na^+/H^+ antiporter that exports Na^+ from the cytosol to prevent the rapid accumulation of Na^+ in the cytoplasm. Transgenic Arabidopsis plants overexpressing *SOS1* showed increased salt tolerance underlining the importance of Na^+ extrusion during salt stress (Shi et al. 2003). In addition to these ion transport systems, we have established a novel system to improve salt tolerance. By expressing Ena1p, a yeast plasma membrane Na^+-ATPase, in plant cells, we were able to reduce the accumulation of Na^+ during salt stress. Because Ena1p is a self-powered Na^+ pump that does not need the force generated by a proton gradient, it should remain functional even under high pH conditions, a property that would desirable for salt-tolerant plants.

Identification and characterization of OsHKTs, which are involved in Na⁺ and K⁺ homeostasis in rice

We isolated three different cDNAs that encoded HKT proteins from *O. sativa* cv. Nipponbare (Ni-*OsHKT1*) and cv. Pokkali (Po-*OsHKT1* and Po-*OsHKT2*), the salt-sensitive and salt-tolerant cultivar of rice, respectively. Complementation assays in CY162 cells showed that OsHKT2 could rescue the *trk* mutations under low-K^+

conditions, whereas OsHKT1 could not (Figure 5A). This result suggests that, at least in the heterologous systems, OsHKT1 is a Na^+ transporter with a similar K^+ selectivity as AtHKT1, whereas OsHKT2 is a Na^+- and K^+-coupled transporter similar to TaHKT1. Although the Na^+ uptake ability of OsHKT2 is similar to that of OsHKT1, we observed that *OsHKT2*-expressing G19 cells were less sensitive to salt than *OsHKT1*-expressing cells (Figure 5B). This sensitivity might be caused by the difference in the internal Na^+/K^+ concentration ratio. This ratio might be lower in *OsHKT2*-expressing cells than in *OsHKT1*-expressing cells due to the difference in the K^+ uptake abilities of OsHKT1 and OsHKT2.

We compared the amino-acid sequences of OsHKT1, OsHKT2, TaHKT1, and AtHKT1, while focusing on the candidate sites responsible for K^+ selectivity (Figure 6A). TaHKT1 is a member of the K^+ symporters, which contain four loops that are homologous to the selectivity filter-forming P-loops of K^+ channels (Durell and Guy 1999; Durell et al. 1999). Each of the four P loops contains highly conserved glycine residues that are predicted to play an important role in K^+ selectivity. Although the 88th amino acid in OsHKT2 is conserved as a glycine, it is a serine residue in OsHKT1 and AtHKT1 (Figure 6A). The rice cultivar Pokkali contains two HKT paralogues, one of which has a glycine filter residue in P-loop A, and is more salt resistant than the cultivar Nipponbare, which only has the 88-Ser variant *OsHKT1* (Figure 6). Salt tolerance in Pokkali correlated with a decrease in the expression of the *OsHKT* genes and reduced uptake of Na^+ during salt stress (Golldack et al. 2002). Our findings support the notion that HKTs with a serine in P-loop A function mainly as Na^+ transporters, possibly contributing to Na^+ uptake and Na^+ toxicity (Horie and Schroeder 2004). One of the most important questions is how HKT-type transporters contribute to salt tolerance in rice. In the future, functional analyses of *OsHKT* genes from various rice strains should address the relationship between salt tolerance and the Na^+-transporting activity of the OsHKTs.

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