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Expression of a calcineurin gene improves salt stress tolerance in transgenic rice

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Abstract

Calcineurin is a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase and has multiple functions in animal cells including regulating ion homeostasis. We generated transgenic rice plants that not only expressed a truncated form of the catalytic subunit of mouse calcineurin, but also were able to grow and fertilize normally in the field. Notably, the expression of the mouse calcineurin gene in rice resulted in its higher salt stress tolerance than the non-transgenic rice. Physiological studies have indicated that the root growth of transgenic plants was less inhibited than the shoot growth, and that less Na^+ was accumulated in the roots of transgenic plants after a prolonged period of salt stress. These findings imply that the heterologous calcineurin plays a significant role in maintaining ion homeostasis and the integrity of plant roots when exposed to salt. In addition, the calcineurin gene expression in the stems of transgenic plants correlated with the increased expression of the *Rab16A* gene that encodes a group 2-type late-embryogenesis-abundant (LEA) protein. Altogether our findings provide the first genetic and physiological evidence that expression of the mouse calcineurin protein functionally improves the salt stress tolerance of rice partly by limiting Na^+ accumulation in the roots.

Introduction

Soil salinity deleteriously affects the growth and yield of a variety of crops. Plants have developed multiple strategies to achieve salt tolerance (Zhu, 2001; Tester and Davenport, 2003). During the process of salt adaptation, the expression of many salt-stress related genes was induced and the synthesis of a variety of proteins was increased. Among these proteins, late-embryogenesis-abundant (LEA) proteins are a group of proteins that function in damage control or repair (Zhu, 2001). Salt stress tolerance requires a complex interplay of signaling cascades (Xiong *et al.*, 2002), and is determined by multiple factors consisting of effectors and regulators (Hasegawa *et al.*, 2000). Generating transgenic plants with enhanced salt tolerance provides a means to positively impact their response to salt stress. Recently, a number of studies have shown that salt tolerance of plants can be greatly improved through regulating the

expression of effectors or regulators functioning in a re-establishment of ion homeostasis under stress conditions. Among these cases, overexpression of a vacuolar Na^+/H^+ antiporter AtNHX1 (Apse *et al*., 1999) or a plasma membrane Na^+/H^+ antiporter SOS1 (Shi *et al*., 2003) conferred salt tolerance to transgenic plants. In addition, the expression of calcineurin (Pardo *et al*., 1998) or *HAL1* (Gisbert *et al*., 2000), regulators of K^+ and Na^+ homeostasis in yeast, has greatly improved salt tolerance of transgenic plants.

In plant cells, biotic (i.e., pathogen infection and insect herbivory) and abiotic (i.e., high or low temperature, drought and salinity) stresses induce a transient free Ca^{2+} influx into cytoplasm from outside or internal compartments (Sanders *et al*., 1999; Knight, 2000; Knight and Knight, 2001; Sanders *et al*., 2002) and the subsequent Ca^{2+} signaling pathways activate adaptive responses to these stresses. Calcium signaling is often coupled with protein phosphorylation and dephosphorylation mediated by protein kinases and phosphatases, respectively. In response to osmotic and other kinds of stresses, the transcript level of those genes that encode protein kinases is increased (Mizoguchi *et al*., 2000). One of the best studied protein kinases in the Ca^{2+} signaling pathway is CDPKs. Another family of protein kinases that function in stress tolerance is mitogen activated protein kinases (MAPKs) (Zhang and Klessig, 2001). Although protein kinases are better understood than protein phosphatases, the combination of phosphorylation and dephosphorylation plays a critical role in stress tolerance. To tightly regulate the MAPK cascade, MAPKs can be inactivated by dephosphorylation of either threonine or tyrosine residue. A MAPK phosphatase was identified from *Arabidopsis*, designated as AtDSPTP1, which was able to dephosphorylate and inactivate AtMAPK4 *in vitro* (Gupta *et al*., 1998). In *Arabidopsis*, AtCDPK1 stimulated stress-inducible gene expression, but interestingly, a protein phosphatase 2C (AtPP2CA) was able to block the CDPK-activated gene expression (Sheen, 1996; Sheen, 1998). To date, investigators have tended to focus on the functions of protein phosphorylation mediated by protein kinases. However, protein dephosphorylation mediated by protein phosphatase also has key functions in cellular processes and phosphoprotein signaling.

Calcineurin, also called protein phosphatase 2B (PP2B), is a heterodimer consisting of a catalytic subunit CNA and a regulatory subunit CNB. CNA contains four domains: the catalytic, CNB binding, calmodulin binding and autoinhibitory domains. The autoinhibitory domain of CNA inhibits its phosphatase activity, so that the truncated form of CNA, without the autoinhibitory domain, encompasses a constitutive phosphatase activity. As a Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin is a key component in Ca^{2+}-dependent signal pathways and plays an important role in a number of cellular processes (Rusnak and Mertz, 2000). In yeast *Saccharomyces cerevisiae*, calcineurin is the pivotal intermediate in Ca^{2+} signal pathway that regulates cellular K^+, Na^+ and Ca^{2+} homeostasis and mediates salt stress tolerance (Matsumoto *et al*., 2002). It was recently reported that calcineurin in the fungus *Aspergillus oryzae* was able to mediate its adaptation to salt stress and alkaline pH stress (Juvvadi *et al*., 2003). In animal cells, calcineurin has numerous functions, which include T cell activation, apoptosis, cardiac hypertrophy and Na^+ homeostasis (Aperia *et al*., 1992; Clipstone and Crabtree, 1992; Marcaida *et al*., 1996; Rusnak and Mertz, 2000). To regulate Na^+ homeostasis in cerebellar neurons, calcineurin dephosphorylates and activates the Na^+/K^+-ATPase, which has a role in maintaining Na^+ homeostasis by preventing excess Na^+ influx into cells (Marcaida *et al*., 1996). To date, the calcineurin gene has not been cloned from plants, but two EF-hand Ca^{2+}-binding proteins homologous to CNB, namely AtCBL (Kudla *et al*., 1999) and SOS3 (Liu and Zhu, 1998), have been found in *Arabidopsis*, and these two proteins both play important roles in salt stress tolerance. AtCBL is a gene family with at least 10 members (Luan *et al*., 2002), one of which is AtCBL1. AtCBL1 is most similar to animal CNB in sequence and interacts *in vivo* with CNA from rat in the yeast two-hybrid system (Kudla *et al*., 1999). The *AtCBL1* gene expression was induced by stress stimuli such as drought, cold and wounding (Kudla *et al*., 1999), and overexpression of *AtCBL1* in *Arabidopsis* increased its salt and drought tolerance (Cheong *et al*., 2003). Another CNB-like protein, SOS3, has the sequence most similar to that of the CNB subunit from yeast *Saccharomyces cerevisiae* and neuronal calcium sensors from animals (Liu and Zhu, 1998). SOS3 is a component of the SOS pathway regulating ion homeostasis and salt tolerance in

Arabidopsis, and the *sos3* mutant was hypersensitive to Na^+ and Li^+ (Liu and Zhu, 1998). It has been reported that the expression of the yeast CNA and CNB in tobacco increased its salt tolerance and that calcineurin functioned mainly in the tobacco roots (Pardo *et al*., 1998), suggesting that calcineurin can function in plants and plays a significant role in plant stress tolerance. It is recently reported that a calcineurin from yeast can regulate ion homeostasis in plants (Marin-Manzano MC *et al*., 2004). The calcineurin from yeast was introduced into tomato cells and the expression of the heterologous calcineurin can decrease the activity of plasma membrane H^+-ATPase, which is the primary energy source for uptake of many ions (Marin-Manzano MC *et al*., 2004).

We report here, for the first time, that calcineurin A subunit from mouse has been expressed in rice. The transgenic plants are able to grow normally without any growth retardation and can fertilize under normal growth conditions. Calcineurin transgenic plants exhibited a higher level of salt stress tolerance in exposure to salinity, and Na^+ content in the transgenic roots was lower than that in untransformed wild-type plants. Interestingly, the expression of *Rab16A* (Mundy and Chua, 1988) that is induced when plants are subject to water-deficit stress was upregulated in transgenic stems when transgenic plants are exposed to salt stress. In a separate experiment, calcineurin transgenic plants were also exposed to hyperosmotic stress induced by mannitol, but no improved osmotic stress tolerance was seen in transgenic plants compared with untransformed wild-type plants. These results indicate that mouse calcineurin might play an important role in transgenic rice to specifically increase its salt stress tolerance through limiting Na^+ accumulation in the roots.

Materials and methods

Expression of a truncated mouse calcineurin A gene in transgenic plants

A truncated mouse calcineurin A gene, denoted *CNAtr*, was obtained as described previously (Zhu *et al*., 1996). The truncated form of calcineurin A without C-terminal autoinhibitory domain is constitutively active. A vector expressing *CNAtr* was constructed by separately cloning both the promoter (cauliflower mosaic virus promoter 35S) and the nopaline synthase terminator (NosT) from the plasmid pIB411 into the adjacent sites of pBluescript II KS with right directions. The *Hin*dIII fragment of 35S-NosT was inserted into the *Hin*dIII site of a derivative pCAMBIA1300, which has the *Eco*RI inactivated in its CMS (multiple cloning site). This resultant plasmid, denoted pCAMBIA-PT, is an intermediate plasmid that contains the promoter and terminator for those transgenes to be expressed in plants. A 1.2 Kb of *CNAtr* fragment was digested with *Eco*RI from the plasmid pBJ5 and cloned into the *Eco*RI site between the 35S promoter and NosT terminater in pCAMBIA-PT, resulting in a new plant expression vector, pCAMBIA-PT*CNAtr*. The vector pCAMBIA-PT*CNAtr* containing *CNAtr* was introduced into *Agrobacterium tumefaciens* strain EHA105 as described (Höfgen and Willmitzer, 1988). Calli induced from mature embryos of *japonica* rice (*Oryza sativa* cv Xiushui 04) were transformed by *Agrobacterium* mediated method as described (Hiei *et al*., 1994) with a slight modification. Transgenic calli and plants were screened on medium containing 50 mg/l hygromycin.

Southern blot analysis of transgenic plants

Rice genomic DNA was isolated from leaves of untransformed wild-type (WT) plants and independent transgenic lines (T0 generation) by the cetyl-trimethyl-ammonium bromide (CTAB) method as described (McCouch *et al*., 1988). Ten micrograms of genomic DNA were digested overnight with *Eco*RI and separated by electrophoresis on a 0.8% agarose gel. The denatured and fractionated DNA was transferred to Hybond-N^+ membrane. This blot was hybridized overnight at 42 °C in an ultra sensitive hybridization buffer (Ambion) with an α-^{32}P-labeled 1–Kb *CNAtr* fragment as the probe.

Northern blot analysis of transgenic plants

Transgenic (T1) and untransformed wild-type (WT) rice seedlings were grown on 1/2MS medium. For salt treatment, 10-day-old seedlings were transferred into pots filled with sand and

then were allowed to grow for another week with water irrigated using a day cycle (12 h light, 12 h dark) at 26 °C. After the transplantation into sand for a week, the rice seedlings were treated with 150 mM NaCl for zero, one and eight days. Total RNA was extracted using Trizol reagent (Invitrogen) and 15 μg of RNA was fractionated by electrophoresis through denaturing 1.2% agarose gels. Blots were hybridized with the same method as the Southern blot. A 1-Kb DNA fragment of *CNAtr* was used as the probe. Probes for the *Rab16A* and *OsCDPK7* were obtained using PCR from cDNA prepared from salt-treated rice seedlings. In order to determine the mRNA level of *Rab16A* in the stems, band intensities were measured by using software Image-Pro Plus 3.0 system. IOD (integral optical density) value shows the relative mRNA level of *Rab16A*.

Protein extraction and phosphatase activity assay

Six shoots from T1 transgenic (A5 and A10) and untransformed wild-type (WT) plants were separately ground into a fine powder with liquid N_2 and added to the extraction buffer which contained 50 mM Tris, 250 mM sucrose, 0.5 mM DTT, 5 mM ascorbate acid, 0.2 mM PMSF, 10 μg/ml Soybean trypsin inhibitor and 10 μg/ml Leupeptin. The homogenate was centrifuged at 100,000 *g* for 45 min. The supernatant obtained after centrifugation was passed through a Sephadex G-25 column to remove the free phosphate. The solution eluted from the column was used in the calcineurin phosphatase activity assay. Calcineurin protein phosphatase activity was assayed using the Calcineurin Cellular Activity Assay Kit (Calbiochem) according to the manufacturer's instruction. The assay was carried out in 50 μl volumes in a microtiter plate using RII phosphopeptide, the most efficient and commonly used peptide substrate for known calcineurin. The reaction was started by adding the extracts, followed by incubation at 30 °C for 30 min, and terminated by adding the malachite green reagent according to the manufacturer's instruction. The activity of Calcineurin protein phosphatase was determined by the amount of free phosphate released. Significance of the values was determined with *F* test ($p < 0.05$).

Salt stress tolerance test

Two independent lines (T1) of transgenic (A5 and A10) plants and untransformed wild-type (WT) plants were used for the salt stress tolerance test. Transgenic seeds were germinated on 1/2 MS medium with hygromycin and untransformed WT seeds were germinated on 1/2 MS without hygromycin at 26 °C using a day cycle (12 h light, 12 h dark) for 12 days. For salt stress test, the WT plants and hygromycin-resistant transgenic plants were transferred into pots three at a time, filled with sand, and then allowed to grow for another 2 days in the greenhouse. After growing in sand for 2 days, the two-week old plants were exposed to 120 mM NaCl for 12 days and photographed. Eight roots of each line were separately collected for fresh and dry weight determination. For the dry weight determination, the roots were dried at 70 °C for 24 h and weighed.

Hyperosmotic stress tolerance test

Two independent lines (T1) of transgenic (A5 and A10) and untransformed wild-type (WT) plants were used for hyperosmotic stress tolerance test. Transgenic (A5 and A10) and untransformed wild-type rice seeds were grown on 1/2 MS medium for 12 days. For hyperosmotic stress test, the 12-day old transgenic and WT seedlings were subject to 200 mM mannitol for 3 days. Subsequently, four roots of each line were separately collected for Na^+ and K^+ content determination.

Determination of Na^+ and K^+ content

In the assays of salt and hyperosmotic stress tolerance, Na^+ and K^+ ion contents in the roots of transgenic and untransformed control plants were measured after plants are subject to 120 mM NaCl for 12 days and 200 mM mannitol for 3 days, respectively. For the salt stress test, eight dried roots of each line were separately digested in HNO_3 and Na^+ and K^+ contents were determined by atomic absorption spectrophotometry. The statistical significance of the values was determined with *F* test ($p < 0.001$). For the hyperosmotic stress test, four roots of each line were separately collected and root Na^+ and K^+ contents were

determined by atomic absorption spectrophotometry. The statistical significance of the values was determined with LSD test ($p < 0.05$).

Measurement of protein and chlorophyll content

Total protein and chlorophyll were extracted from shoots of T1 transgenic (A5 and A10) and untransformed wild-type (WT) plants treated with 120 mM NaCl for 12 days, respectively. Protein content was determined with a Bio-Rad protein assay kit. Total chlorophyll content, which included chlorophyll *a* and *b*, was extracted with 80% acetone and determined according to its absorbency at 663 and 645 nm.

Results

Generation of transgenic rice lines

Calli induced from mature embryos of *Oryza sativa* (*japonica*) seeds were transformed with a vector containing the *CNAtr* gene driven by a cauliflower mosaic virus (CaMV) 35S promoter and a selectable antibiotic resistance gene (hygromycin phosphotransferase, *HPT*II) (Figure 1A).

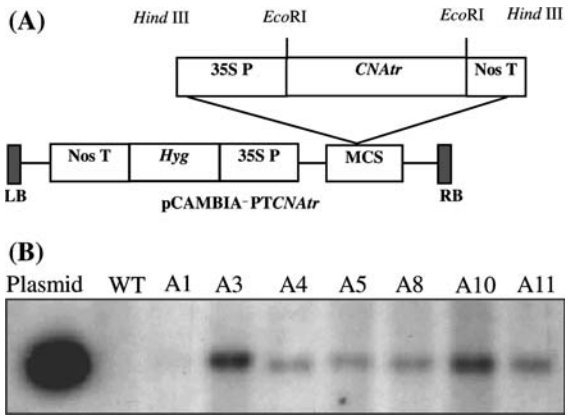

Figure 1. Schematic representation of the expression vector for rice transformation and Southern blot analysis. (A) The construct expressing *CNAtr* for rice transformation. *CNAtr* expression cassette was cloned into MCS site of plasmid pCAMBIA1300 with *Eco*RI deletion. (B) Southern blot analysis of DNA from untransformed wild-type plants (WT) and seven independent *CNAtr* transgenic plants (A1, A3, A4, A5, A8, A10, A11). Ten microgram of rice genomic DNA was digested with *Eco*RI (two sites in the plasmid pCAMBIA-PT*CNAtr*) and Southern blot analysis was performed with the [^{32}P] dCTP-labeled 1.0 kb *CNAtr* fragment as the probe.

A total of forty T0 transgenic *CNAtr* plants were generated.

Seven independent transgenic lines (T0 generation) were chosen for Southern-blot analysis. Southern analysis of these transgenic lines confirmed that the *CNAtr* transgene had integrated into the rice genome (Figure 1B). Three independent transgenic lines (T1 generation), A1, A5 and A10, were chosen for the analysis of *CNAtr* expression. Northern-blot analysis showed that the *CNAtr* gene from mouse expressed in the leaves, stems and roots of transgenic plants (Figure 2).

Shoot extracts of T1 transgenic (A5 and A10) and untransformed wild-type plants (WT) were utilized for a calcineurin phosphatase activity assay using the malachite green colorimetric detection system (Figure 3). A standard curve of absorbance at 655 nm was established for the assay conditions (not shown). Calcineurin phosphatase activity was

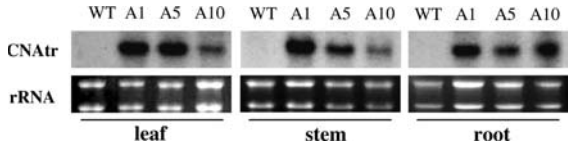

Figure 2. Northern blot analysis of mouse *CNAtr* transcript in transgenic rice plants. Total RNA was isolated from leaves (left), stems (middle) and roots (right) of untransformed wild-type plants (WT) and three independent *CNAtr* transgenic plants (A1, A5, A10). Each lane was loaded with 15μg of total RNA and Northern blot analysis was performed with the [^{32}P] dCTP-labeled probe specific for *CNAtr* gene.

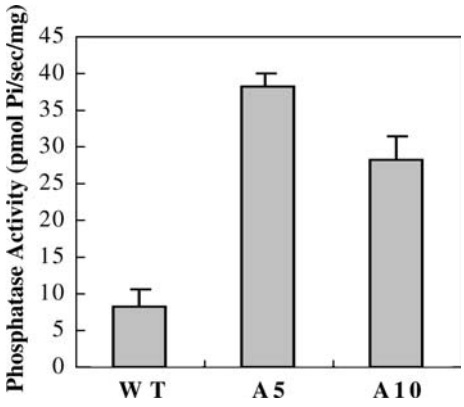

Figure 3. Calcineurin phosphatase activity in transgenic rice plants. Calcineurin activity in crude extracts from the shoots of transgenic T1 (A5 and A10) plants and untransformed wild-type plants (WT) were measured with the malachite green assay as described in experimental protocol.

measured using calcineurin effective substrate RII phosphopeptide and was represented by the amount of free phosphate released. The detected calcineurin protein phosphatase activity in transgenic lines, A5 and A10, were 4.6 and 3.4 times higher than that of the untransformed control plants, respectively. This result indicates that the activated form of calcineurin phosphatase was synthesized in *CNAtr* transgenic rice. The calcineurin phosphatase activity detected in untransformed control plants might be due to the presence of protein phosphatase similar to calcineurin (Luan *et al.*, 1993), which can interact with the substrate used in the phosphatase activity assay.

Salt stress tolerance conferred by CNAtr *expression in transgenic plants*

We performed the analysis of salt tolerance in transgenic *CNAtr* plants (T1 generation) by exposing two-week old seedlings to 120 mM NaCl for 12 days. Salt stress inhibited the growth of both transgenic and control plants, but affected the development of transgenic plants and their roots to a lesser degree. The transgenic line, A5, displayed an increased growth of shoots and roots compared with untransformed control plants after a prolonged period of salt stress. The new top leaves of A5 grew much faster (Figure 4A) and the roots of A5 were longer and more vigorous than those of the untransformed control plants (Figure 4B) under salt stress. Our results are consistent with the experimental result of Pardo and colleagues (Pardo *et al.*, 1998). In contrast to

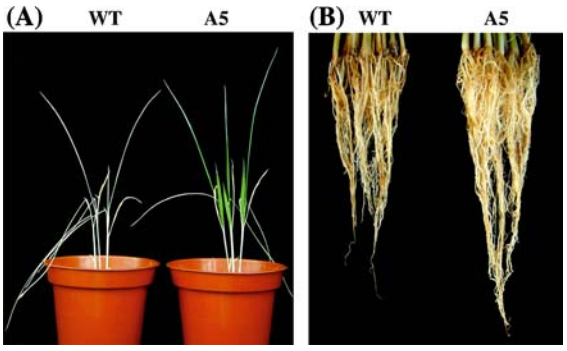

Figure 4. Salt stress tolerance of transgenic rice plants expressing *CNAtr* gene from mouse. (A) Rice plants grown in the presence of 120 mM NaCl for 12 days. (B) Root growth of rice plants grown in the presence of 120 mM NaCl for 12 days.

untransformed control plants, the salt tolerance of transgenic plants also manifested in the faster growth, increased fresh and dry weight of transgenic roots (Table 1, A, B, C).

The protein and chlorophyll content in plant shoots can indicate the growth and metabolism of rice plants when they are exposed to salt stress. In our experiment, protein and chlorophyll content of shoots from transgenic and untransformed control plants were measured. The results indicate that total protein content and total chlorophyll content were both higher in transgenic plant shoots (with statistical significance in A5) than in control plants when the rice plants were subjected to salt stress (Table 1, D, E). The statistical significance of the values was determined with F test ($p < 0.05$).

Determination of Na^+ *and* K^+ *content*

Pardo's work indicated that calcineurin from yeast functioned mainly in roots and resulted in less inhibition of root growth in exposure to salinity (Pardo *et al.*, 1998). Our work demonstrates that the growth of transgenic roots was also less disturbed by NaCl treatment (Figure 5A). It might be possible that Na^+ accumulation was inhibited in these root cells. We examined the concentration of Na^+ and K^+ in the roots of transgenic and untransformed control plants after 12 days of 120 mM NaCl treatment. Salt stress inhibited the growth of roots (Figure 5A), increased root Na^+ content, but decreased root K^+ content in both transgenic and untransformed control plants. Under the control growth condition, the Na^+ content in the roots of transgenic and untransformed control plants was not apparently different. Whereas under the saline condition, Na^+ content in the roots of transgenic lines A5 and A10 were 22.6% and 23.3% lower than that in untransformed control plants, respectively (Figure 5B). Root Na^+ content in A5 was significantly lower than that in untransformed control plants. The changes of the K^+ content in transgenic (A5 and A10) and untransformed control plant (WT) roots were consistent under the saline condition. Root K^+ content in both transgenic and untransformed control plants were approximately four to five times lower than they were under the control growth condition (Figure 5C). This result is consistent with earlier findings that K^+ content in plant roots is decreased in high

Table 1. CNAtr expression improves root growth, fresh weight and dry weight, and shoot chlorophyll and protein content of transgenic plants.

A. Root growth (cm)			
	0 mM NaCl	120 mM NaCl	Relative growth (%)
A5	8.22	7.30*	88.8
A10	8.08	7.88*	97.5
WT	8.07	6.07	75.2
B. Root fresh weight (mg)			
	0 mM NaCl	120 mM NaCl	Reduction by (%)
A5	97.2	83.27*	14.3
A10	103.92*	78.17*	24.8
WT	88.78	60.73	31.6
C. Root dry weight (mg)			
	0 mM NaCl	120 mM NaCl	Reduction by (%)
A5	10.69	6.61*	38.2
A10	12.53*	7.24*	42.2
WT	10.44	5.29	49.3
D. Shoot chlorophyll content (μg/ml)			
	0 mM NaCl	120 mM NaCl	
A5	0.8666	0.2569*	
A10	0.9330	0.1985	
WT	0.9168	0.1388	
E. Shoot protein content (mg/gFW)			
	0 mM NaCl	120 mM NaCl	Reduction by (%)
A5	3.1494	1.1072	64.8*
A10	3.1433	0.7106	77.4
WT	3.1870	0.5406	83.0

Roots and shoots of transgenic and untransformed wild-type rice plants were harvested 12 days after salt treatment. Each value is the mean (n = 7–12 individual plants). Statistical significance was determined by F test (*$p < 0.05$) compared with the value of WT plants. FW, fresh weight.

salinity (Epstein, 1973). These results suggest that the expression of *CNAtr* in transgenic plants may limit Na^+ accumulation in roots, but has no obvious effect on K^+ uptake.

For hyperosmotic stress tolerance test, transgenic and untransformed wild-type plants were subject to 200 mM mannitol for 3 days. After the treatment of hyperosmotic stress, shoot growth of transgenic and untransformed rice seedlings was obviously inhibited; root growth of transgenic and untransformed rice seedlings was affected in certain extent (Figure 6A). Nevertheless, the transgenic plants displayed no higher hyperosmotic stress tolerance than control wild-type plants. Root Na^+ and K^+ contents in transgenic and untransformed rice seedlings were measured. The flux of Na^+ in the rice roots was affected by hyperosmotic (200 mM mannitol) stress. Hyperosmotic (200 mM mannitol) stress decreased root Na^+ content in transgenic and wild-type rice seedlings, but root Na^+ content in transgenic and untransformed wild-type seedlings was not statistically different after 200 mM mannitol treatment for 3 days (Figure 6B). After 200 mM mannitol treatment for 3 days, root K^+ content of transgenic rice was slightly lower than that of untransformed wild-type plants, but there is no statistical difference between them (Figure 6C). In summary, after 200 mM mannitol treatment for 3 days, Na^+ and K^+ contents in the roots of transgenic and untransformed wild-type seedlings were not statistically different, separately.

Salt-stress related gene expression in the transgenic plants

OsCDPK7 is a member of CDPKs and recently reported to be induced by salt stress. Overexpression of this gene in rice had improved its cold and salt/drought tolerance (Saijo *et al.*, 2000). Rab16A, a group 2-type LEA protein expressed in vegetative tissues, was considered as the compatible solute to function in cytoplasm and to play a role in maintaining the cell structure under

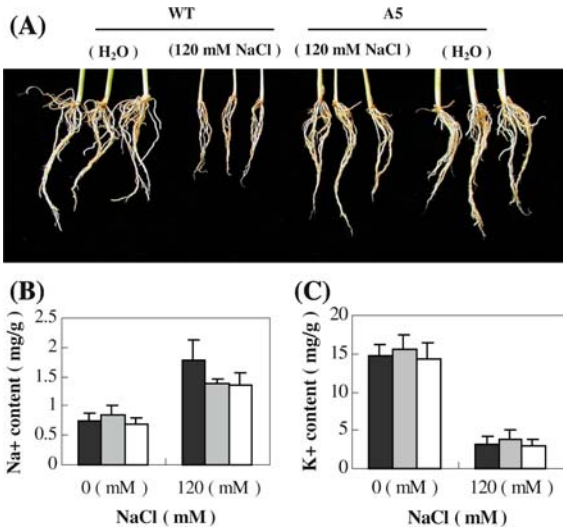

Figure 5. Root growth and root Na^+/K^+ content in *CNAtr* transgenic and untransformed wild-type plants under salt stress. (A) Root growth of two-week old T1 transgenic (A5) and wild-type (WT) seedlings under control (H_2O) or salt stress (120 mM NaCl) conditions for 12 days. (B) Na^+ content in the roots grown under salt stress and no stress conditions. Black solid bar, nontransformant; gray solid bar, A5 transgenic line; open bar, A10 transgenic line. (C) K^+ content in the roots grown under salt stress and no stress conditions. Black solid bar, nontransformant; gray solid bar, A5 transgenic line; open bar, A10 transgenic line. Error bars represent standard deviations ($n = 8$.).

water-deficit conditions (Ingram and Bartels, 1996). The *Rab16A* gene was markedly induced by salt and ABA treatment (Mundy and Chua, 1988). Thus, both *OsCDPK7* and *Rab16A* play important roles in rice salt stress tolerance. We analyzed the expression of these two genes in leaves, stems and roots of transgenic (A1 and A5) and untransformed control rice plants treated with 150 mM NaCl for 0, 1 and 8 days. *OsCDPK7* was expressed in both shoots and roots of transgenic (A1 and A5) and untransformed control plants (WT). The mRNA level of *OsCDPK7* in leaves and roots of transgenic and untransformed control plants was slightly increased after exposure to salt for 1 day, though it remained similar between the transgenic and WT plants (Figure 7). But when rice plants are subject to salt stress for 8 days, OsCDPK7 mRNA abundance in transgenic leaves was decreased compared with untransformed control plants. The expression pattern of *Rab16A* was different from that of *OsCDPK7*. In the leaves and stems of both transgenic and untransformed control plants, no induction of *Rab16A* transcript was observed after salt treatment for 1 day; however, it was markedly induced after salt treatment for 8 days (Figure 7A, 7B). The mRNA levels of *Rab16A* in the leaves of transgenic and untransformed control plants were nearly the same (Figure 7A). Interestingly, the mRNA level of *Rab16A* in the stems of *CNAtr* transgenic plants was higher than untransformed control plants after salt treatment for 8 days (Figure 7B). In roots of both transgenic and untransformed control plants, the mRNA of *Rab16A* was nearly undetectable after salt treatment for 0, 1 and 8 days (Figure 7C), implying that some other genes might be involved in transgenic roots to contribute to its enhanced salt tolerance. These results indicate that the heterologous calcineurin can increase the expression of *Rab16A* gene in the stems of transgenic rice. It is quite obvious that *Rab16A* in stems of the transgenic lines, especially in A5 line, was strongly induced after salt treatment for 8 days (Figure 8).

Discussion

Calcineurin, a key component in Ca^{2+} signaling pathway in animals, may also play an important role in plant signaling pathways (Luan *et al.*, 1993; Allen and Sanders, 1995; Bethke and Jones, 1997; Liu and Zhu, 1998; Kudla *et al.*, 1999; Marin-Manzano *et al.*, 2004). At present, calcineurin regulatory components such as calmodulin and CNB-like proteins, AtCBL (Kudla *et al.*, 1999) and SOS3 (Liu and Zhu, 1998), have been found in plants. In plant guard cells, calcineurin from bovine was found to regulate the opening and closure of the stomatal pore by inhibiting inward K^+-channel activity (Luan *et al.*, 1993). Meanwhile, a protein phosphatase similar to calcineurin was found in the *V. faba* guard cells and mediated Ca^{2+}-dependent inward K^+-channel inactivation (Luan *et al.*, 1993). Heterologous calcineurin also can modulate the slow-vacuolar ion channel of stomatal guard cells (Allen and Sanders, 1995) and aleurone cells (Bethke and Jones, 1997). In addition, the expression of yeast calcineurin in tobacco was able to increases its salt stress tolerance (Pardo *et al.*, 1998). It is recently reported that a calcineurin from yeast can regulate ion homeostasis in tomato cells (Marin-Manzano

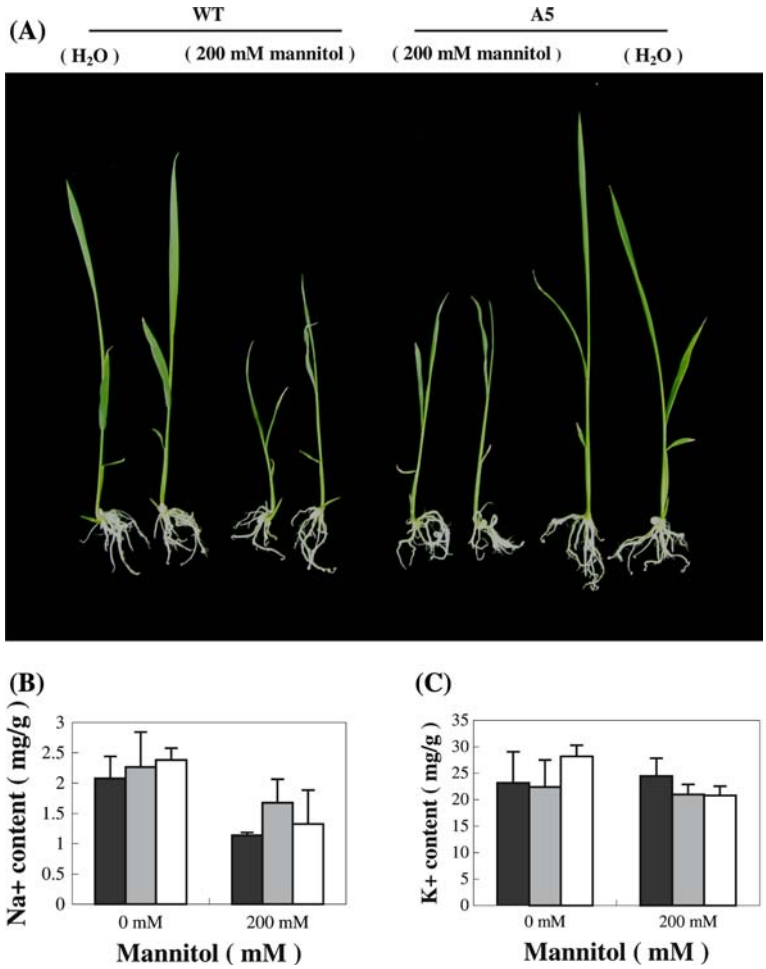

Figure 6. The growth and root Na^+/K^+ content in *CNAtr* transgenic and untransformed wild-type plants under hyperosmotic stress. (A) Growth of 12-day old T1 transgenic (A5) and wild-type (WT) seedlings under control (H_2O) or hyperosmotic stress (200 mM mannitol) conditions for 3 days. (B) Na^+ content in the roots grown under hyperosmotic (200 mM mannitol) stress and no stress conditions. Black solid bar, nontransformant; gray solid bar, A5 transgenic line; open bar, A10 transgenic line. (C) K^+ content in the roots grown under hyperosmotic stress (200 mM mannitol) and no stress conditions. Black solid bar, nontransformant; gray solid bar, A5 transgenic line; open bar, A10 transgenic line. Error bars represent standard deviations ($n = 4$).

et al., 2004). These reports provide evidence to show that heterologous calcineurin is able to function in different types of plant cells to regulate their cellular processes.

Recently, great progress has been made in target and signaling pathway research of calcineurin B-like proteins. A new family of serine-threonine protein kinase, designated as CIPKs (for CBL-interacting protein kinases), has been found in *Arabidopsis* and act as common targets for AtCBL proteins (Shi *et al*., 1999). CIPKs, unlike most serine-threonine kinases, are putative Mn_{2+}-binding proteins and interact with AtCBL proteins to form an AtCBL-kinase complex in a Ca^{2+}-dependent manner (Shi *et al*., 1999). This interaction of AtCBL-CIPK sheds light on the function of AtCBLs and also provides a mechanism for Ca^{2+} signaling in plant cells. In a yeast two-hybrid system, however, AtCBL1 was also able to interact with CNA from rats (Kudla *et al*., 1999) implying that another Ca^{2+} signaling protein(s) might exist in plants, which might possibly be a calcineurin-like protein. The proteins in the SOS pathway in plants have been well characterized (Zhu, 2002). The SOS pathway initiates from SOS3, a new Ca^{2+} sensor, which senses the calcium signals and then transmits the signals to the following targets. SOS3 binds to the regulatory

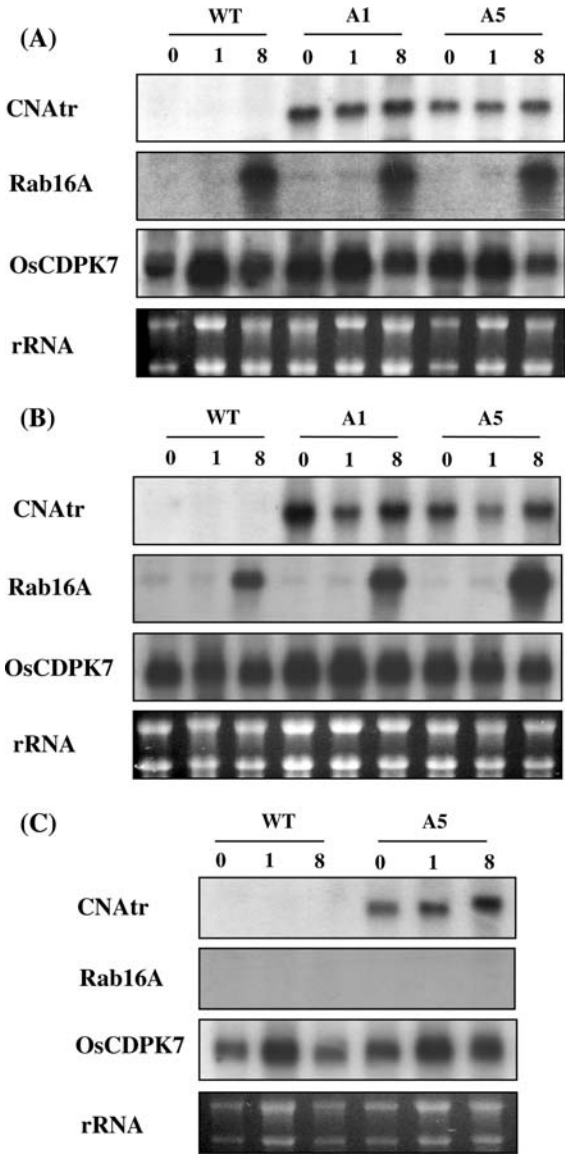

Figure 7. The mRNA level of *OsCDPK7* and *Rab16A* in transgenic and untransformed wild-type rice plants in response to salt stress. Two-week old seedlings were subjected to 150 mM NaCl for 0, 1 and 8 days. (A) Northern-blot analyses of leaf RNAs from untransformed wild-type plants (WT) and transgenic T1 (A1 and A5) plants. (B) Northern-blot analyses of stem RNAs from untransformed wild-type plants (WT) and transgenic T1 (A1 and A5) plants. (C) Northern-blot analyses of root RNAs from untransformed wild-type plants (WT) and transgenic T1 (A5) plants.

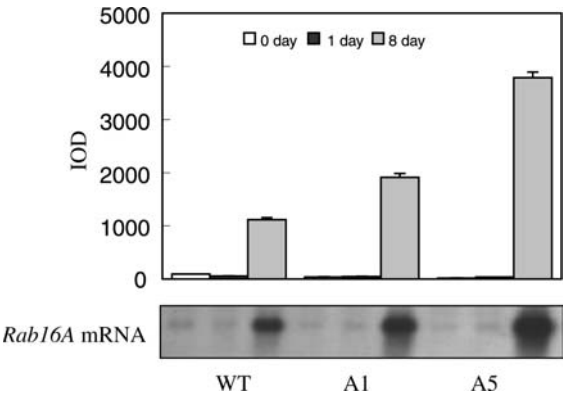

Figure 8. The mRNA level of *Rab16A* in stems of transgenic (A1 and A5) and untransformed wild-type (WT) rice plants treated with 150mM NaCl for 0, 1 and 8 days. In Northern-blot analysis, band intensity was measured three times with software Image-Pro Plus 3.0 system, respectively. IOD (integral optical density) value shows different accumulation of *Rab16A* mRNA in rice stems. Values are the mean ± standard deviation. Error bars represent standard deviations (s.d.).

domain of SOS2, a serine/threonine kinase and activates it. SOS2, or together with SOS3 regulates the expression level of *SOS1*, a gene encoding a plasma membrane Na^+/H^+ antiporter. Na^+/H^+ antiporter is a salt tolerance effector controlling long-distance Na^+ transport (Shi *et al*., 2002). Similar to the SOS signaling pathway, the heterologous calcineurin in plants might play a role in regulating ion transport systems, but the adaptive mechanism in guard cells and root cells might be different. In guard cells, heterologous calcineurin may regulate the closure of stomatal pore by limiting K^+ influx into cells (Marcaida *et al*., 1996). However, in root cells, heterologous calcineurin may presumably regulate ion transport systems to control Na^+ accumulation in cells (Pardo *et al*., 1998; Trewavas, 1999). These two regulation mechanisms remain to be investigated.

In the work of Pardo and his colleagues, calcineurin from yeast was introduced into tobacco, which allowed it to improve its salt stress tolerance (Pardo *et al*., 1998). Root Na^+ content had not been measured in their experiment, but it was speculated that calcineurin might function to block Na^+ influx into root cells. In our experiment, Na^+ content in rice roots was determined and Na^+ content in transgenic roots was lower than that in untransformed control plants after a prolonged period of salt treatment. It suggests that the expression of the heterologous calcineurin in transgenic plants is able to inhibit Na^+ accumulation in root cells. And the reduced Na^+ accumulation in root cells can limit Na^+ entering

into the root xylem and then being further transported into the shoots (Adams *et al.*, 1992; Niu *et al.*, 1995). Salt stress imposes ionic and osmotic stresses on plants. In order to understand whether the heterologous calcineurin plays a role in Na^+-specific pathways to limit root Na^+ accumulation in response to salt stress, the calcineurin transgenic plants were also exposed to hyperosmotic stress induced by 200 mM mannitol, and root Na^+ and K^+ content was measured. The growth of transgenic and untransformed wild-type plants was inhibited and the osmotic stress tolerance of transgenic plants was not improved compared with untransformed wild-type plants. After osmotic stress treatment for 3 days, root Na^+ content in transgenic and untransformed wild-type plants was decreased but was not statistically different. These findings suggest that mouse calcineurin appears to limit Na^+ accumulation in transgenic roots in a Na^+ stress specific manner. But the precise function of calcineurin in this process remains to be investigated. It is recently reported that the expression of a yeast calcineurin in tomato cells can decrease the activity of plasma membrane H^+-ATPase, which can modify the membrane potential and facilitate the uptake of many ions through secondary transport systems (Marin-Manzano MC *et al.*, 2004). In the transgenic rice, it is plausible that the heterologous calcineurin may also regulate the activity of plasma membrane H^+-ATPases or Na^+ transport systems to control Na^+ accumulation in the cells (Marcaida *et al.*, 1996).

Rice OsCDPK7 is a component of calcium-dependent signal pathway and rice Rab16A is markedly induced by ABA and salt. They were both detected in the same vascular tissues upon salt stress and *Rab16A* was considered to be the target gene of OsCDPK7 (Saijo *et al.*, 2001). It is interesting to see the expression pattern of these two genes in *CNAtr* transgenic rice. We analyzed the expression of these two genes in transgenic rice. When rice seedlings were treated with 150 mM NaCl for 8 days, leaf *OsCDPK7* mRNA in transgenic plants was slightly decreased compared with untransformed control plants. Nevertheless, stem *OsCDPK7* mRNA in transgenic rice was nearly the same as that of untransformed control plants. The expression pattern of *Rab16A* appears to be different from that of *OsCDPK7*. When rice seedlings were treated with 150 mM NaCl for 8 days, leaf *Rab16A* mRNA in transgenic plants was nearly the same as that in untransformed control plants. Interestingly, stem *Rab16A* in transgenic plants was upregulated compared with that in untransformed control plants. These findings indicate that the heterologous calcineurin is able to regulate the expression of *Rab16A* gene in a tissue specific manner when rice plants are subject to salt stress. In plants, several protein phosphatases have been characterized (Gosti *et al.*, 1999; Monroy *et al.*, 1998). Serine-threonine protein phosphatases such as the PP1/PP2A family and the PP2C family, are present in plants as well as in animals with significant sequence identities. These serine/threonine phosphatases function as positive or negative regulators in plant signaling pathways. PP2C is a negative regulator of ABA signaling (Gosti *et al.*, 1999; Merlot *et al.*, 2001) in *Arabidopsis*. For example, ABI-1 is a member of PP2C and its mutant *abi-1*, loss of PP2C phosphatase activity, was hypersensitive to ABA, and exhibited enhanced drought tolerance compared with wild type plants (Gosti *et al.*, 1999). It was reported by Sheen that PP1, PP2A, or PP2B was not the negative regulator of ABA signaling in maize leaf cells (Sheen, 1998). The work of Pardo and colleagues (Pardo *et al.*, 1998) provided the experimental evidence that calcineurin is a positive regulator of salt stress tolerance in higher plants. In our experiment, calcineurin expressing in rice was able to increase its salt stress tolerance. Thus, it would be intriguing to examine the function of the heterologous calcineurin in plant signaling pathways in response to salt stress.

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