Plant Biotechnology 23, 275–281 (2006)

Original Paper

Root specific expression of Na^+/H^+ antiporter gene from *Synechocystis* sp. PCC 6803 confers salt tolerance of tobacco plant

Gazi Sakir Hossain[1], Rungaroon Waditee[2], Takashi Hibino[1], Yoshito Tanaka[1], Teruhiro Takabe[2]*

[1] Graduate School of Environmental and Human Sciences, Meijo University, Nagoya 468-8502, Japan; [2] Research Institute, Meijo University, Nagoya 468-8502, Japan
*E-mail: takabe@ccmfs.meijo-u.ac.jp Tel: +81-52-838-2277 Fax: +81-52-832-1545

Received December 22, 2005; accepted February 24, 2006 (Edited by K. Yoshida)

Abstract Tobacco plants were transformed with a Nhap type Na^+/H^+ antiporter gene, *SynnhaP1* (slr1595), from a cyanobacterium *Synechocystis* sp. PCC 6803. Two kinds of promoters, *Arabidopsis* alcohol dehydrogenase gene promoter (Adh promoter) and CaMV 35S promoter (35S promoter), were used. The transgenic plants driven by Adh promoter accumulated SynNhaP1 proteins only in root whereas the transgenic plants driven by 35S promoter accumulated SynNhaP1 proteins in all tissues. Confocal imaging of SynNhaP1-GFP fusion protein suggests the intracellular localization of SynNhaP1 in plasma membrane. Transgenic plants exhibited higher germination yields, increased biomass during developmental stage, increased seed production, and decreased intracellular Na^+ content under salt-stress conditions. The transgenic plants driven by Adh promoter exhibited similar or slightly higher salt tolerance than that by 35S promoter. These results indicate the importance of expression of Na^+/H^+ antiporter in root for salt tolerance in plant.

Key words: Alcohol dehydrogenase promoter, Na^+/H^+ antiporter, SOS1, salt tolerance, SynNhaP1, Nhap type Na^+/H^+ antiporter from *Synechocystis* sp. PCC 6803.

Salinity is one of the major factors that limit the crop productivity. Organisms that thrive in hypersaline environment possess specific mechanisms to adjust their internal osmotic status or to control the ion homeostasis. To adjust the internal osmotic status, organisms usually accumulate low molecular weight compatible solutes such as glycinebetaine which have been used to improve salt- and drought-tolerance in plants by genetic engineering approaches (Sakamoto et al. 1998; Waditee et al. 2005). For ion homeostasis, the adaptation mechanism to high salinity is the internalization of Na^+ to vacuole or exclusion of Na^+ to apoplast which has been proposed as a function of Na^+/H^+ antiporter (Yamaguchi and Blumwald 2005). Vacuolar Na^+/H^+ antiporters have been cloned from several plant species, and by its overexpression, it is shown that internalizion of Na^+ into the vacuoles is an efficient mechanism for adaptation to abiotic stresses such as salt and drought (Apse et al. 1999; Yamaguchi and Blumwald 2005). In contrast, salt overly sensitive 1 Na^+/H^+ antiporter (SOS1) is the only well characterized plasma membrane Na^+/H^+ antiporter in plants (Shi et al. 2000). SOS1 has been proposed to play an important role for the control of long distance Na^+ translocation from root to shoot (Shi et al. 2002). Overexpression of *SOS1* and yeast plasma membrane Na^+/H^+ antiporter, *SOD2*, under the control of CaMV 35S promoter has been reported to increase the salt tolerance of *Arabidopsis* (Shi et al. 2003; Xiuhua et al. 2003). However, it is still not well understood how the overexpressed antiporters driven by CaMV 35S promoter contributes to salt tolerance in plants.

Here, we examined to express a cyanobacterium Na^+/H^+ antiporter from *Synechocystis* sp. PCC 6803, SynNhaP1 (slr1595), in tobacco root. SynNhaP1 is homologous to NhaP antiporter from *Pseudomonas aeruginosa* and exhibits some homology to cation/proton antiporter family 1 (CPA1) such as AtNHX1, SOS1, and mammalian NHE. To express in root, an alcohol dehydrogenase gene promoter (Adh promoter) was used. It is known that Adh gene is induced by abiotic stresses such as hypoxia, drought, and cold stresses (Dolferus et

Abbreviations: Adh, alcohol dehydrogenase; DTT, dithiothreitol; GFP, green fluorescence protein; MS, Murashige and Skoog; PMSF, phenylmethane sulfonyl fluoride; SOS1, salt overly sensitive1 Na^+/H^+ antiporter from *Arabidopsis*; SynNhaP1, Nhap type Na^+/H^+ antiporter from *Synechocystis* sp. PCC 6803.

This article can be found at http://www.jspcmb.jp/

al. 1994). As for reference, the transgenic plants driven by 35S promoter were also constructed. Salt tolerance of wild-type and transgenic plants expressing Adh-SynNhaP1 and35S-SynNhaP1 was examined. The results show the importance of root specific expression of SynNhaP1 for salt tolerance.

Materials and methods

Construction of vectors and transformation of tobacco plants

The β-Glucuronidase (GUS) gene in a binary vector, pBI121, was removed by digestion with *Xba*I and *Sac*I to which a fragment containing *Xba*I, *BamH*I, *Sma*I, *Kpn*I and *Sac*I restriction sites of a cloning vector, pUC118, was introduced (Stratagene, CA). The resulting vector was designated as pBI121H35S. The *SynNhaP1* gene was amplified from pTrcSynNhaP1 plasmid (Hamada et al. 2001) by using NhaP-F (5′-CACCATGGATACA-GCGGTCAACG-3′) and His-R (5′-GTGGTACCTCA-ATGATGATGATGATG-3′) primers which contains *Xba*I and *Kpn*I restriction sites, respectively. The amplified fragment was ligated into the *EcoR*V site of $pBSK^+$ vector (Stratagene, CA), and then subcloned into the *Xba*I/*Kpn*I sites of pBI121H35S. The resulting vector was designated as p35S:SynNhaP1. Alcohol dehydrogenase (Adh) promoter was amplified from genomic DNA of *Arabidopsis* using AdhPromF (5′-GAAAGCTTAAGAGTTGTTCTTGAGG-3′) and AdhPromR (5′-ATTCTAGACAGTGAAGAACTTGCT-TTT-3′) primers containing *Hind*III and *Xba*I restriction sites, respectively. The amplified fragment was cloned into the *EcoR*V restriction site of $pBSK^+$, and then ligated into the *Hind*III/*Xba*I sites of p35S:SynNhaP1. The plasmids, pAdh:SynNhaP1and p35S:SynNhaP1, were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Tobacco plants (*Nicotiana tabacum* cv Petit Havana SR1) were transformed by using leaf disc method (Miyagawa et al. 2000). Thirty-nine plants (T_0) were grown aseptically on Murashige and Skoog (MS) (Murashige and Skoog 1962) agar medium supplemented with 3% sucrose containing 50 mg l^{-1} kanamycin. Kanamycin-resistant transformants were selected and grown at 25°C in the growth chambers with a 16-h light (200 μE m^{-2} s^{-1})/6-h dark cycle at 60% relative humidity. Twenty independent plants (T_1) were allowed to flower and set seeds. Cross-pollination was prevented by placing a paper bag on flower prior opening. Expression level of SynNhaP1 in the transgenic plants was checked by RT-PCR (Figure 1A, B). Total RNA was extracted by SDS-phenol method. The NhaP1-F1 and His-R1 primers were used to amplify *SynNhaP1* gene. Four 35S:NhaP1 transgenic plants (T_2) and three Adh:NhaP1 transgenic plants (T_2) showing high level of SynNhaP1 expression were germinated on medium

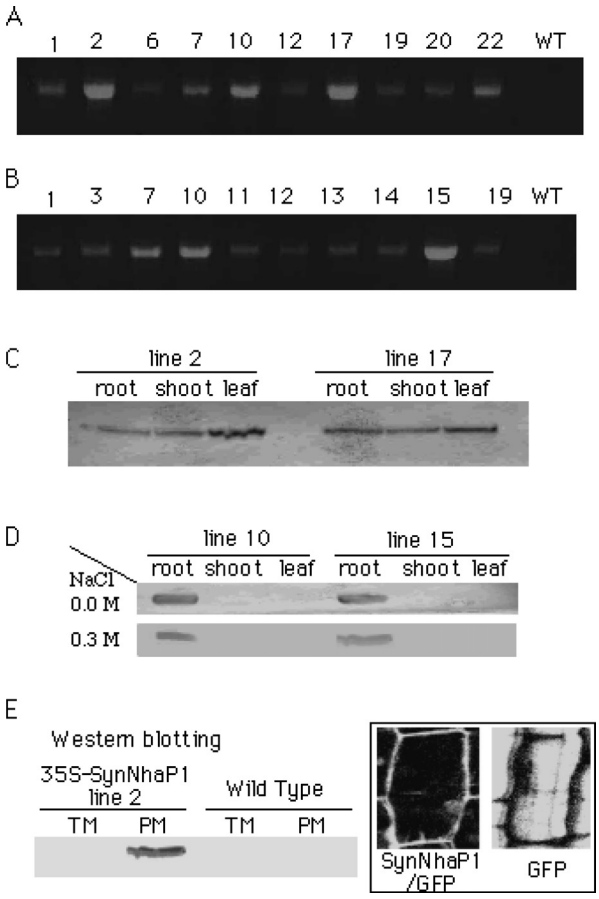

Figure 1. Expression of SynNhaP1 in tobacco. (A) RT-PCR analysis of SynNhaP1 in 35S-SynNhaP1 indicated lines and in wild type (WT). (B) RT-PCR analysis of SynNhaP1 in the Adh-SynNhaP1 indicated lines and in wild type (WT). (C) Western blot analysis of SynNhaP1 in the 35S-SynNhaP1 indicated lines and in wild type (WT). (D) Western blot analysis of SynNhaP1 in the Adh-SynNhaP1 indicated lines and in wild type (WT). The plants were subjected with salt stress with 300 mM NaCl for 1 week Then, leaf, stem, and root fractions were used for Western blot analysis as described in Materials and methods. (E) Western blotting and confocal imaging. Left, the microsomal membrane fractions were separated by sucrose density gradient as described in Materials and methods, then western blotting experiments were carried out. Right, confocal imaging of SynNhaP1::GFP fusion protein and GFP protein.

containing kanamycin and set seeds. All selected transformants exhibited similar phenotypes. Two transformants were used for the detail analysis of stress tolerance.

A pBluescript vector containing modified *GFP* gene (S65-*GFP*) was a kind gift from Prof. Niwa (Shizuoka University). The *GFP* gene was amplified by using gfp-F (5′-ATGTCGACGATCCATGGTGAGCAA-3′) and gfp-R (5′-CGGGTACCTTACTTGTACA-GCTCGTC-3′) primers containing *Sal*I and *Kpn*I restriction sites, respectively. The amplified fragment was cloned into the $pBSK^+$ and replaced with the His-tag sequence in p35S:SynNhaP1. The resulting vector, p35S:SynNhaP1::GFP, was transferred into *Agrobacterium tumefaciens* strain LBA4404

by electroporation. Transgenic plants containing SynNhaP1::GFP were made by leaf disc transformation method (Miyagawa et al. 2000). GFP fluorescence was measured by a confocal laser-scanning microscope (Olympus Inc., Japan) using a long pass 505-nm emission filter upon the excitation at 488-nm.

Salt stress treatments

For the germination test, surface-sterilized seeds of transgenic- and wild type-plants were soaked and plated on MS media containing various concentrations of NaCl. The number of germinated seed was scored after dincubation for 12 days. For salt stress experiment at the vegetative stage, eight week-old wild type and transgenic plants were subjected to 100 mM NaCl stress. The concentration of NaCl in the growth medium was increased stepwise to 200 mM after 2 days and then to 250 mM or 300 mM or 350 mM after 4 days. The plants were kept for additional 2 or 3 weeks at the same final salinity. After that, photograph was taken, and plant height, fresh weight, and ion contents were measured. To investigate the yield production, the same stepwise salt stress was applied to ten week-old wild type and transgenic plants. The salt stress (350 mM NaCl) was continued until the seed production.

Immunoblotting

To check the expression of SynNhaP1 in different tissues, microsomal membranes from four weeks old transgenic plants expressing SynNhaP1 were prepared as described (Venema et al. 2003). Briefly, roots, shoots, and leaves of plants were homogenized with mortar and pestle using a homogenization buffer containing 50 mM Tris-HCl (pH 8.2), 20% glycerol, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail set II (Calbiochem, EMD Bioscience, Inc, LaJolla, USA). After filtration through two layers of miracloth (Calbiochem), the homogenate was centrifuged at 12,000 *g* for 10 min to remove the intact organelles and cell walls. The supernatant was centrifuged at 100,000 *g* for 1 hr. Membrane pellets were suspended in a buffer containing 25 mM Tris-HCl (pH 7.5), 10% (w/v) sucrose. SDS-PAGE with 12.5% polyacrylamide and immunobloting analysis were carried out as previously described (Waditee et al. 2002). An anti-6X-His antibody was used (RD system, Minneapolis, MN, USA). Protein concentration was measured by Lowry method (Lowry et al. 1951).

Determination of Na^+ and K^+ contents

About 1.0 g of leaves, roots and shoots of stressed and non-stressed plants were homogenized with 1.0 m*l* H_2O and filtrated with millipore filter. Intracellular ion contents were measured by a Shimadzu personal Ion Analyzer PIA-1000.

Results

Expression profiles of SynNhaP1driven by Adh- and 35S promoters

Tobacco plants were transformed with a construct containing the *SynnhaP1* gene driven by root-specific *Arabidopsis* Adh promoter and constitutive 35S promoter. Western blot analysis data showed that in the 35S-SynNhaP1 transgenic plants, SynNhaP1 was detected in the whole part of the plants (Figure 1C), whereas in the Adh-SynNhaP1 transgenic plants, SynNhaP1 was detected only in the root both in non-stressed and stressed tissues (Figure 1D). Western blotting analysis of microsomal membranes and confocal imaging of SynNhaP1::GFP fusion protein suggest the intracellular localization of SynNhaP1 in plasma membrane (Figure 1E). Only GFP expression was found in the whole cytosol of the plant cell (Figure 1E).

Expression of SynNhaP1 improved the germination and growth at the vegetative stages under salt stress

We previously showed that the expression of *SynNhaP1* in the *E. coli* strain (TO114) lacking three Na^+/H^+ antiporter (*ΔnhaA*, *ΔnhaB*, *ΔchaA*) (Ohyama et al. 1995) restored the ability of these cells to efflux sodium and greatly increased their resistance to both Na^+ and Li^+ in the growth media (Hamada et al. 2001). Here, we examined the germination yield of tobacco seeds. Under normal condition, the transgenic plants expressing SynNhaP1 did not show any significant difference from the wild type plants during germination and early developmental stage (Figure 2). However, at a higher concentration of NaCl (200 mM) in the medium, germination yield of transgenic plants, Adh-SynNhaP1 and 35S-SynNhaP1, was higher than that of wild-type plants (Figure 2). Germination yield of Adh-SynNhaP1 and 35S-SynNhaP1 transgenic plants was similar (Figure 2).

To study the effects of SynNhaP1-expression on the growth at the vegetative stage, two month-old plants were subjected to salt stress. After the salt treatment, the leaves of the wild-type plants become yellowish and more severely damaged than the transgenic plants (Figure 3C). The fresh weight and height of the wild-type and transgenic plants were decreased upon the increase of salinity. However, their values of wild-type plants decreased more significantly than the transgenic plants (Figure 3A, B). The fresh weight and height of Adh-SynNhaP1 and 35S-SynNhaP1 were similar, but slightly larger in the former.

Reduced accumulation of sodium in Adh-SynNhaP1 plants

Next, the ion contents were examined. Under normal

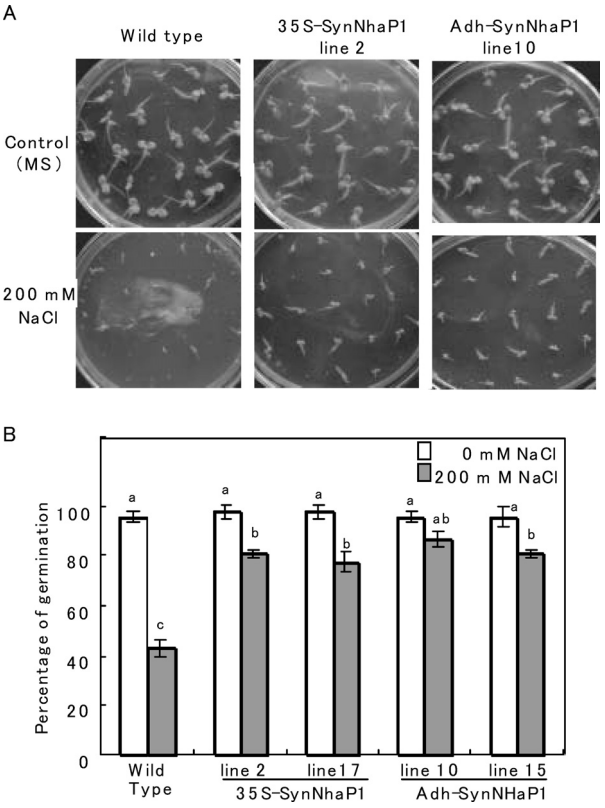

Figure 2. Enhance salt tolerance of SynNhaP1 expressing transgenic plants during germination. (A) Photograph of seedlings in MS media with- or without 200 mM NaCl. (B) Percentage of seed germination in MS media with- or without 200 mM NaCl. Seeds were surface-sterilized and placed on the media. Vertical bars represent the S.D. (n=3). Different letters (a, b, and c) upper the bar graph denote significant differences ($P<0.05$) from wild-type plants.

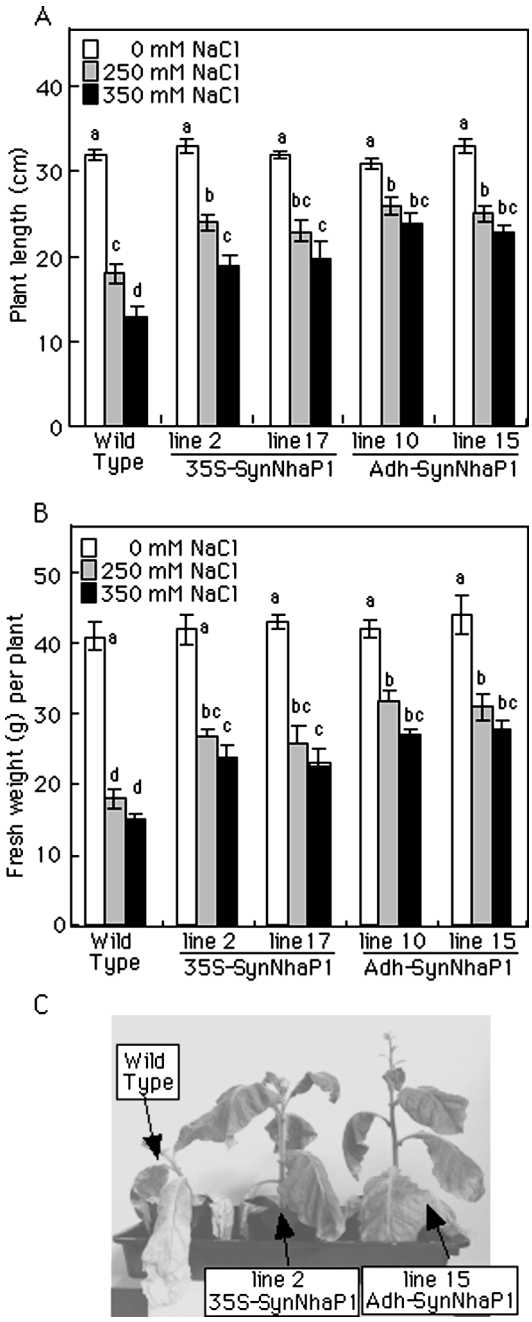

Figure 3. Increased resistance to NaCl stress of transgenic plants during developmental stage. (A) Plant height. (B) Plant fresh weight. The plants were treated with 0, 250, and 350 mM NaCl for 3 weeks as described in Materials and methods. Then, plant height (A) and fresh weight (B) were measured. Vertical bars represent the S.D. (n=3). (C) Photograph. The plants were subjected with salt stress with 350 mM NaCl for 3 weeks. Then, photograph was taken. Different letters upper the bar graph in Figure 3A, B denote significant differences ($P<0.05$) from wild-type plants.

condition, the content of Na^+ was very low in both wild-type and transgenic plants (Figure 4A–C). In response to 300 mM NaCl treatment for 2 weeks, the Na^+ contents of both wild-type and transgenic plants significantly increased. But, its contents in leaves, shoots, and roots of transgenic plants were considerably lower than those of wild-type plants. In this case, the Na^+ content in Adh-SynNhaP1 transgenic plants was lower than that in 35S-SynNhaP1 plants. Under normal growth condition, the content of K^+ in the wild-type and transgenic plants were similar. The levels of K^+ decreased upon the salt stress in both wild-type and transgenic plants, but more decreased in wild type plants (Figure 4D–F). The levels of K^+ in leaf, shoot, and root of Adh-SynNhaP1 and 35S-SynNhaP1 plants were almost the same (Figure 4D–F).

Increased yield in the transgenic plant under salt-stress conditions

Salt stress was applied to the ten week-old plants, just before the bud appearances, until the complete seed production. The number of ovaries, seed number, and weight per seed were similar among the wild-type and transgenic plants under normal growth conditions (Figure 5A–C). Upon NaCl stress treatment, the above values were decreased, but more severely decreased in wild-type plants (Figure 5A–C). In this case, the number of ovaries and seeds were higher in Adh-SynNhaP1 transgenic plants that those in 35S plants.

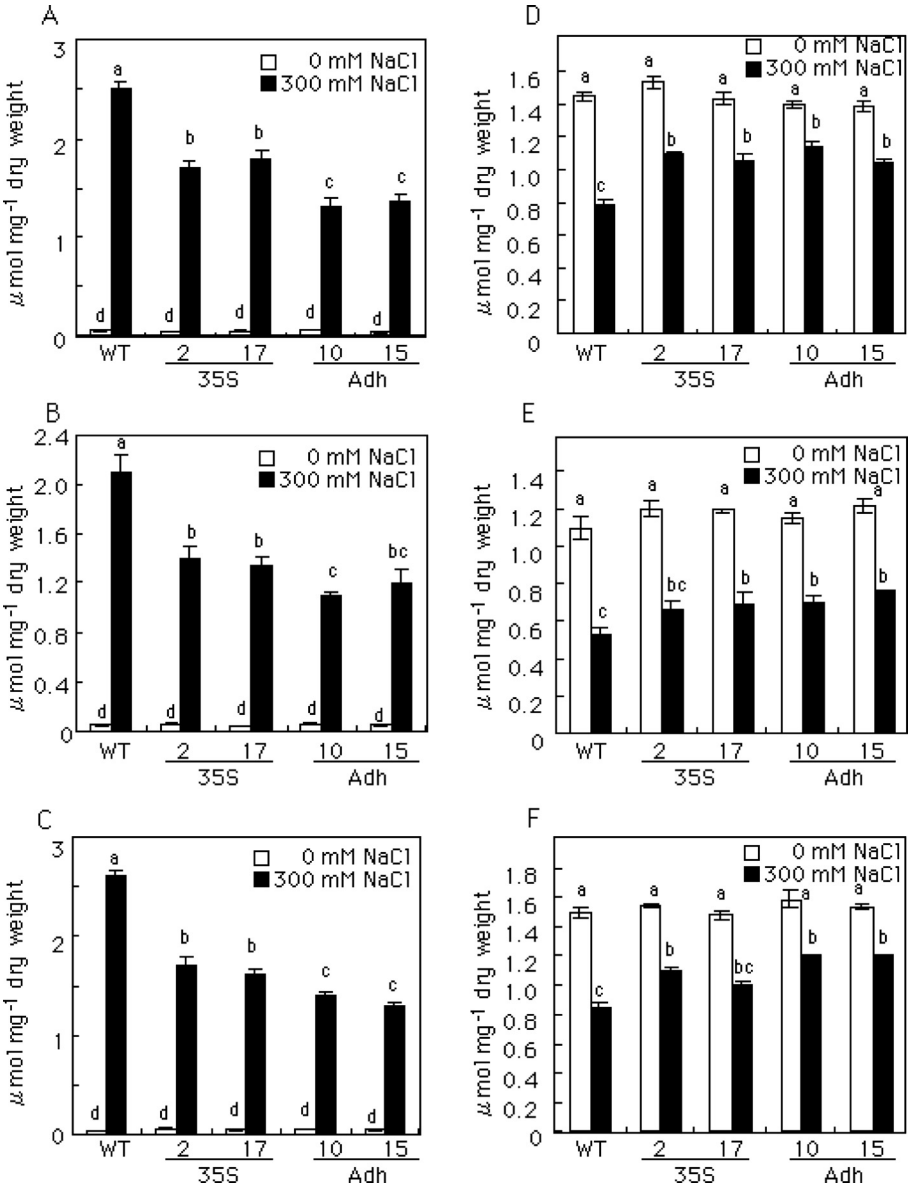

Figure 4. The Na^+ and K^+ contents in the wild-type and transgenic plants. The plants were treated with 300 mM NaCl for 2 weeks as described in Materials and methods. Then, ion contents were measured by a Shimadzu ion analyzer PIA-1000. Vertical bars represent the S.D. (n=3). Different letters upper the bar graph in Figure 4A–F denote significant differences ($P<0.05$) from wild-type plants.

Discussion

The above data clearly indicate that ectopic expression of a plasma membrane-localized cyanobacterium Na^+/H^+ antiporter, SynNhaP1, increased the salt tolerance of tobacco plants. Hitherto, SOS1 is the only well characterized plasma membrane Na^+/H^+ antiporter in plants (Shi et al. 2000). Very few examples are known on the ectopic expression of Na^+/H^+ antiporter in plasma membrane. Two functions of SOSI have been proposed, extrude Na^+ at the root epidermal cells and long distance transport of Na^+ between root and shoot (Shi et al. 2000). The ectopic expression of Ena1p ATPase showed that ATPase was correctly localized to the plasma membrane of transgenic tobacco cultured cells and conferred increased salt tolerance to the cells (Nakayama et al. 2004). The present data demonstrate the importance of root specific expression of Na^+/H^+ antiporter. Moreover, the success of use of Na^+/H^+ antiporter from prokaryotic origin suggests the possibility of use of many other antiporter genes to improve the salt tolerance in plants.

In this work, the transgenic tobacco plants showed a tendency to accumulate less Na^+ under saline condition than the wild-type plants (Figure 4). As shown in Figure 4C, the Na^+ content in root of wild-type plants was significantly higher than that in the transgenic plants. Moreover, the Na^+ content in root of Adh-SynNhaP1 plants was lower than that of 35S-SyNhaP1 plants. Similar results were obtained for the Na^+ content in leaf

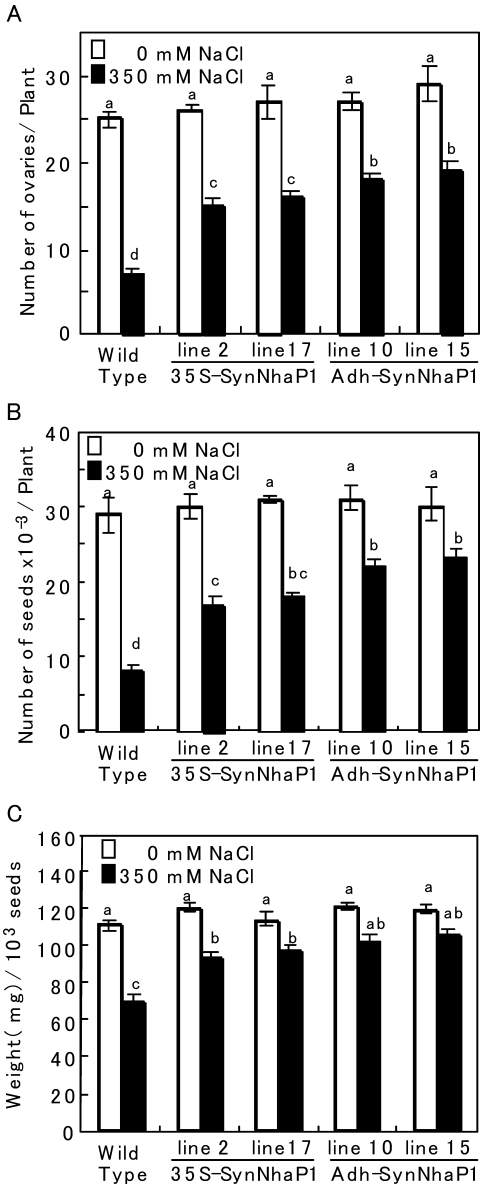

Figure 5. Increased seed yield of transgenic plants expressing SynNhaP1 under high salinity. (A) Number of ovaries per plant. (B) Number of seeds per plant. (C) Seed weight per 10^3 seed. Just before the bud formation, plants were transferred to the growth medium containing 350 mM NaCl. Vertical bars represent the S.D. (n=3). Different letters upper the bar graph in Figure 3A, B denote significant differences ($P<0.05$) from wild-type plants. Different letters upper the bar graph in Figure 5A–C denote significant differences ($P<0.05$) from wild-type plants.

and shoot (Figure 4A, B). The Na^+ content in whole plant was most high in wild-type plant, then 35S-SynNhaP1 and Adh-SynNhaP1 plants followed. It is worthwhile to mention that the Na^+ content in leaf and shoot of Adh-SynNhaP1plants was similar or slightly lower than that of 35S-SynNhaP1 plants although SynNhaP1 was not expressed in leaf and shoot of Adh-SynNhaP1 plants (Figure 4A, B). This suggests that the ectopic expression of SynNhaP1 in leaf and shoot might play minor effects on the salt tolerance of plant. High level expression of Na^+ efflux carrier in all cells may not be beneficial to plants because Na^+ extruded by one cell would become toxic to its neighboring cells. Although the precise location of SynNhaP1 expression in root is unknown at the moment, the present study suggests that SynNhaP1 contributes to extrude Na^+ at the root epidermal cells.

Previously, we reported that overexpression of the same NhaP-type Na^+/H^+ antiporter gene from a halotolerant cyanobacterium *Aphanothece halophytica* (*ApNhaP1*) enhanced dramatically the salt tolerance of freshwater cyanobacterium so that the freshwater cyanobacterium could grow in seawater (Waditee et al. 2002a). However, the overexpression of *ApNhaP1* in *Arabidopsis* seems to be not effective to increase the salt tolerance (data not shown). The proper reason could not explain so far. One possibility might be due to the difference of ion selectivity between SynNhaP1 and ApNhaP1. SynNhaP1 catalyzes the Na^+/H^+ and Li^+/H^+ exchange activities whereas ApNhaP1 catalyzes the Na^+/H^+ and Ca^{2+}/H^+ exchange activities (Waditee et al. 2001). Efflux of Ca^{2+} by ApNhaP1 might be toxic to plant, but not to a fresh water cyanobacterium.

Soil salinity is a major factor in reducing plant growth and productivity. Recently, we showed that direct methylation of glycine accumulated high levels of glycinebetaine and increased abiotic-stress tolerance in *Synechococcus* and *Arabidopsis* (Waditee et al. 2005). Therefore, *SynNhaP1* and methylation genes together could be used to produce the crop plants such as rice with increased stress-tolerance and increased seed-yield under saline environments.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education and Science and Culture of Japan, the High-Tech Research Center of Meijo University. We thank Mrs. Eiko Tsunekawa for her expert technical assistance.

References

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science* 285: 1256–1258

Dolferus R, Jacobs M, Peacock WJ, Dennis ES (1994) Differential interactions of promoter elements in stress responses of the *Arabidopsis* Adh gene. *Plant Physiol* 105: 1075–1087

Hamada A, Hibino T, Nakamura T, Takabe T (2001) Na^+/H^+ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity. *Plant Physiol* 125: 437–446

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265–275

Miyagawa H, Tamoi M, Shigeoka S (2000) Evaluation of defence system in chloroplast to photooxytative stress caused by

paraquat using transgenic tobacco plants expressing catalase from *E. coli*. *Plant Cell Physiol* 41: 311–320

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Nakayama H, Yoshida K, Shinmyo A (2004) Yeast plasma membrane Ena1p ATPase alters alkali-cation homeostasis and confers increased salt tolerance in tobacco cultured cells. *Biotechnol Bioeng* 85: 776–789

Ohyama T, Igarashi K, Kobayashi H (1995) Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J Bcteriol* 176: 4311–4315

Sakamoto A, Alia A, Murata N (1998) The role of glycine betaine in the protection of plants from stress: clues from transgenic plant. *Plant Cell Environment* 25: 1387–1394

Shi H, Ishitani M, Kim C, Zhu JK (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. *Proc Natl Acad Sci USA* 97: 6896–6901

Shi H, Quintero FJ, Pardo JM, Zhu JK (2002) Role of SOS1 as a plasma membrane Na^+/H^+ antiporter that controls long distance Na^+ transport in plant. *Plant Cell* 14: 465–477

Shi H, Wu SJ, Zhu JK (2003) Overexpression of a Plasma membrane Na^+/H^+ antiporter improves salt tolerance in *Arabidopsis*. *Nat Biotechnol* 21: 81–85

Venema K, Belver A, Marin-Manzano MC, Rodriguez-Rosales MP and Donaire JP (2003) A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants. *J Biol Chem* 278: 22453–22459

Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Takabe T (2001) Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. *J Biol Chem* 276: 36931–3698

Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Hayakawa S, Futsuhara, Y, Kawamitsu Y, Takabe T, Takabe T (2002) Functional characterization of betaine/proline transporters in betaine-accumulating mangrove. *J Biol Chem* 277: 18373–18382

Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Hayakawa S, Futsuhara Y, Kawamitsu Y, Takabe T, Takabe T (2002a) Functional characterization of betaine/proline transporters in betaine-accumulating mangrove. *J Biol Chem* 277: 18373–18382

Waditee R, Bhuiyan MN, Rai V, Aoki K, Tanaka Y, Hibino T, Suzuki S, Takano J, Jagendorf AT, Takabe T, Takabe T (2005) Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proc Natl Acad Sci USA* 102: 1318–1323

Xiuhua G, Zhonghai R, Yanxiu Z, Hui Z (2003) Overexpression of *SOD2* Increases Salt Tolerance of *Arabidopsis*. *Plant Physiol* 133: 1873–1881

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci* 10: 615–620