Introduction of a Na^+/H^+ antiporter gene from *Atriplex gmelini* confers salt tolerance to rice

Masaru Ohta[a,1], Yasuyuki Hayashi[a,*], Asae Nakashima[a], Akira Hamada[a,2], Akira Tanaka[a,3], Tatsunosuke Nakamura[b], Takahiko Hayakawa[a]

[a] *Plantech Research Institute, 1000 Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 227-0033, Japan*
[b] *Laboratory of Molecular Cell Biology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan*

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Abstract **We engineered a salt-sensitive rice cultivar (*Oryza sativa* cv. *Kinuhikari*) to express a vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelini* (*AgNHX1*). The activity of the vacuolar-type Na^+/H^+ antiporter in the transgenic rice plants was eight-fold higher than that in wild-type rice plants. Salt tolerance assays followed by non-stress treatments showed that the transgenic plants overexpressing *AgNHX1* could survive under conditions of 300 mM NaCl for 3 days while the wild-type rice plants could not. These results indicate that overexpression of the Na^+/H^+ antiporter gene in rice plants significantly improves their salt tolerance.**

Key words: Halophyte plants; Sodium/proton antiporter; Rice; Salt tolerance; Transgenic plants

1. Introduction

Rice is a major crop, especially in Asia. However, compared with other crops such as wheat and barley, the sensitivity of rice to saline soil conditions can dramatically reduce crop yields. This is particularly important in South and Southeast Asia where foods are scarce [1]. Thus, if salt tolerance can be conferred upon rice plants, the production of rice could be increased in these areas.

Na^+/H^+ antiporters exclude Na^+ from the cytosol and are localized in both plasma and vacuolar membranes [2,3]. The activity of Na^+/H^+ antiporters has been found to be dramatically increased in the tonoplast vesicles isolated from salt-tolerant plants such as sugar beet, barley, hoary plantain and ice plant in response to salt stress. Low Na^+/H^+ antiporter activity is a characteristic of salt-sensitive plants. However, even when subjected to conditions of salt stress, salt-sensitive plants such as rice displayed little or no Na^+/H^+ antiporter activity in tonoplast vesicles [4–8].

Recently, genes encoding vacuole-type Na^+/H^+ antiporters have been isolated from several plant species, including *Arabidopsis thaliana* [9,10], *Oryza sativa* [11], *Atriplex gmelini* [12], and *Mesembryanthemum crystallinum* [13]. These proteins are similar to each other, and it is unknown if higher Na^+/H^+ antiporter activities in salt-tolerant plants can be attributed to the differences in the protein itself or to differences in regulatory mechanisms. Recently it was shown that *Arabidopsis*, tomato and *Brassica* plants overexpressing the vacuole-type Na^+/H^+ antiporter gene (*AtNHX1*) displayed a significantly increased level of salt tolerance under conditions of salt stress [9,14,15].

In this work, we report the transgenic alteration of a salt-sensitive rice cultivar (*O. sativa* cv. *Kinuhikari*) by introduction of the Na^+/H^+ antiporter gene from a halophytic plant, *A. gmelini* (*AgNHX1*) [12]. The transgenic rice plants overexpressing the *AgNHX1* gene showed a strong tolerance to salt stress under the conditions of a 3-day exposure to 300 mM NaCl followed by non-stress treatments, whereas none of the wild-type rice plants survived under the same conditions.

2. Materials and methods

2.1. Transformation

The Na^+/H^+ antiporter gene from *A. gmelini* (*AgNHX1*) [12] was amplified by PCR and digested with *Sal*I and *Bam*HI restriction enzymes. The resulting fragment was inserted between the promoter region and the terminator region of the expression vector pIG221 [16] to produce p35S/I/AgNhx1, replacing the cDNA sequence of the *GUS* gene. The promoter region contains the cauliflower mosaic virus (CaMV) 35S promoter and the first intron of catalase from *Ricinus communis* L. [17], while the terminator region contains the polyadenylation signal of the nopaline synthetase gene (Nos). Subsequently, the expression cassette from p35S/I/AgNhx1 was inserted into the plasmid pHSG398 (Takara Biomedicals, Japan) to produce pHSG/AgNhx1. Transformation of rice plants was performed by electroporation using protoplasts derived from a salt-sensitive rice cultivar (*O. sativa* cv. *Kinuhikari*) as previously described [18].

2.2. RNA and protein blot analyses

Total RNA was extracted from leaves as described previously [19]. *AgNHX1* mRNA was detected using full-length cDNA as a probe. Protein blot analysis was carried out with a polyclonal antibody raised against the C-terminal region of AgNHX1 (amino acids 540–555) [12]. Crude microsomal fractions were prepared from leaves of the rice plants and separated on 10% SDS–PAGE followed by transfer to Immobilon membranes (Millipore, Bedford, MA, USA).

*Corresponding author. Fax: (81)-45-962 7492.
E-mail address: pri0004@cc.m-kagaku.co.jp (Y. Hayashi).

[1] Present address: Department of Plant Sciences, University of Arizona, Tucson, AZ, 85721, USA.
[2] Present address: Research Institute, Meijo University, Tenpaku-ku, Nagoya, Aichi 468-8502, Japan.
[3] Present address: Life Science Business Initiative Department, Mitsubishi Chemical Corporation, 2-5-2 Marunouchi, Chiyodaku, Tokyo 100-0005, Japan.

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2.3. Isolation of tonoplast-enriched vesicles

Suspension-cultured rice cells of the wild-type *Kinuhikari* cultivar and the transgenic line 132 were grown at 28°C for 5 days. Approximately 20 g of cells were used for isolation of tonoplast-enriched vesicles as previously described [20], except that the grinding medium was supplemented with 10 mM β-glycerophosphate and 0.45 mM butylated hydroxytoluene. In addition, a 15% (w/v) sucrose cushion was used instead of the 10% sucrose described in the original method.

2.4. Fluorescence assays of Na^+/H^+ antiport activity

The Na^+/H^+ antiport activity was assayed basically as described [21], except a buffer containing 0.25 M sorbitol, 10 mM MES/Tris (pH 7.8), 50 mM tetramethylammonium chloride, 3 mM ATP, and 2 μM acridine orange was used for the assay. Fluorescence was monitored at excitation and emission wavelengths of 492 and 525 nm, respectively, with a RF-5300PC fluorometer (Shimadzu, Kyoto, Japan). The Na^+/H^+ antiport activity was expressed as the initial rate of change in fluorescence intensities observed after the addition of Na^+ minus the rate of spontaneous H^+ leakage observed after the addition of hexokinase and glucose. All rates were expressed as the percentage change in acridine orange fluorescence per minute.

2.5. Plant growth and salt stress treatment

Both the wild-type and transgenic rice plants were germinated on soil and grown for 10–14 days. Rice plants with three or four true leaves were transferred to a hydroponic culture system in a growth chamber and grown for 1 week in the hydroponic culture medium [22] containing 100 μg/ml claforan at 28°C (60% humidity, 35 000 lux light intensity, 16 h-day/8 h-dark cycle). To improve root growth, the medium was continuously bubbled with air. The hydroponic culture medium was changed weekly.

Twelve each of the wild-type and transgenic rice plants with six or seven leaves were treated with the same hydroponic culture media supplemented with 300 mM NaCl for 3 days and then transferred to the hydroponic culture media without NaCl. After 2 weeks, the surviving plants that developed new leaves were counted.

2.6. Measurement of Na^+ content in leaves

Leaves were cut from the rice plants before and after 3 days of salt treatment and washed with deionized water. The leaves were dried in an oven at 120°C and their dry weights were measured. To extract the salts, the leaves were burned to ashes at 450°C in the presence of HNO_3. The ashes from the leaves were then dissolved in 0.1 N HCl, and the sodium content was determined by atomic absorption spectrophotometry.

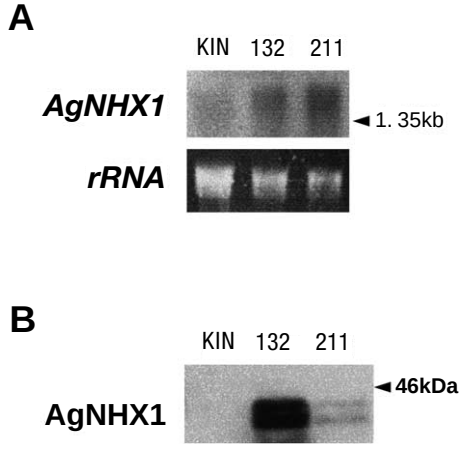

Fig. 1. Expression of the *AgNHX1* gene in rice plants (*O. sativa* cv. *Kinuhikari*). A: Northern blot analysis of total RNA from leaves of wild-type (KIN) and transgenic rice plants (lines 132 and 211). Samples of 20 μg of total RNA were used for the experiments. To assess the relative quantities, the RNAs were stained with ethidium bromide (rRNA). B: Immunoblot analysis of AgNHX1 in wild-type (KIN) and transgenic rice plants (lines 132 and 211). Microsomal proteins (40 μg) from young leaves were loaded into each lane.

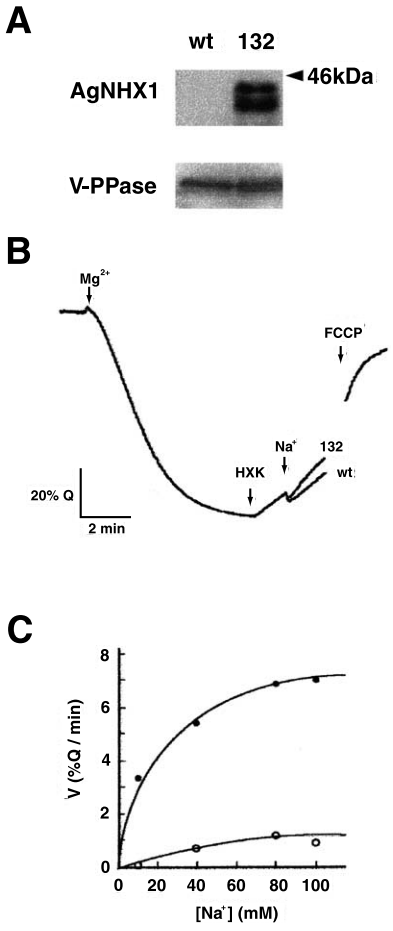

Fig. 2. Na^+/H^+ antiport activities in the suspension-cultured cells from wild-type and transgenic rice plants. A: AgNHX1 is co-fractionated with vacuolar H^+-pyrophosphatase. Tonoplast vesicles containing 30 μg of proteins from the wild-type (wt) and transgenic rice plants (line 132) were applied to 10% SDS–PAGE and subjected to immunoblot analysis. B: ATP-dependent fluorescence quenching of acridine orange fluorescence and its recovery by Na^+. Tonoplast vesicles containing 100 μg of protein from wild-type (wt) and transgenic rice plants (line 132) were assayed. Formation of the pH gradient was initiated by adding 3 mM $MgCl_2$ (Mg^{2+}) and terminated by the addition of hexokinase and glucose. Next, Na_2SO_4 (Na^+) was added and the change in recovery of fluorescence was determined. The addition of FCCP completely abolished the pH gradient. A represent trace recording is shown in this figure. C: Kinetic analysis of Na^+/H^+ antiport activities in tonoplast fractions from wild-type (open circles) and transgenic (solid circles) suspension-cultured cells.

3. Results

3.1. Expression of the AgNHX1 gene in transgenic rice plants

The Na^+/H^+ antiporter gene (*AgNHX1*) [12] from *A. gmelini*, a halophytic plant, was introduced into a salt-sensitive rice cultivar (*O. sativa* cv. *Kinuhikari*) under control of the cauliflower mosaic virus (CaMV) 35S promoter. Ten independent transgenic plants were generated. Two of these lines, line 132 and line 211, harbored a single copy of the *AgNhx1* gene in their genome (data not shown). The offspring of these two lines were used for further analysis. Overexpression of the *AgNHX1* gene in the transgenic plants was confirmed by mRNA detection. Endogenous mRNA for the Na^+/H^+ antiporter gene (*OsNHX1*) was also observed (Fig. 1A). Two peptide bands of ~45 kDa were observed in the samples from

the transgenic lines by immunoblot analysis (Fig. 1B), both of which were also observed in the analysis of proteins from *A. gmelini* (data not shown). We did not detect any bands in the wild-type rice samples. Presumably, the antibody used in this study was specific to the AgNHX1.

To confirm the localization of the AgNHX1 protein, tonoplast-enriched vesicles were isolated from the rice suspension-cultured cells of line 132 by sucrose density gradient centrifugation. The AgNHX1 was co-fractionated with a vacuolar membrane marker protein, H^+-pyrophosphatase, indicating the proper localization of the heterologous antiporter in rice plants (Fig. 2A).

3.2. Na^+/H^+ antiport activity was improved in the transgenic rice plants

To assess whether the overexpression of the *AgNHX1* gene reinforces vacuolar Na^+/H^+ antiport activity in rice, fluorometric measurements were performed with tonoplast-enriched vesicles prepared from suspension-cultured cells from line 132. Although 50 mM KNO_3 strongly inhibited H^+ pumping activity, neither 100 mM Na_3VO_4 nor 2 mM NaN_3 affected H^+ pumping activity (data not shown), indicating that the tonoplast-enriched vesicles were predominantly derived from vacuoles [23]. The tonoplast-enriched vesicles from the transgenic rice plants caused more rapid fluorescence recovery by Na^+ exchange than did those from wild-type rice plants (Fig. 2B). At 100 mM Na^+ concentration, Na^+/H^+ antiport activity of the transgenic rice was eight-fold higher than that of the wild-type rice. The Na^+/H^+ antiport activity displayed Michaelis–Menten kinetics with respect to Na^+ concentration (Fig. 2C). The apparent K_m value of the exchange for Na^+ was 14 mM for the transgenic plants. This value is similar to the K_m value obtained from the salt-treated *A. gmelini* leaves [24].

3.3. Salt tolerance in the transgenic rice plants

To examine whether the overexpression of the *AgNHX1* gene conferred resistance to salt stress, approximately 3-week-old rice plants from lines 132 and 211 with six true leaves were treated with 300 mM NaCl in the hydroponic culture medium for 3 days (Fig. 3, lane 1). Leaves of both the wild-type and transgenic rice plants could not expand in

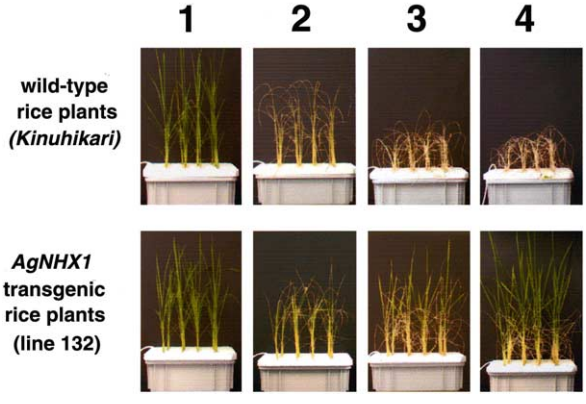

Fig. 3. Salt-treatment assay of wild-type and transgenic rice plants. The wild-type cultivar (*Kinuhikari*) and the transgenic plants (line 132) (lane 1) were treated with hydroponic culture media containing 300 mM NaCl for 3 days (lane 2). Subsequently, the rice plants were transferred to hydroponic culture media without NaCl and grown for 1 week (lane 3) and 2 weeks (lane 4). Each container has 12 (4×3) plants.

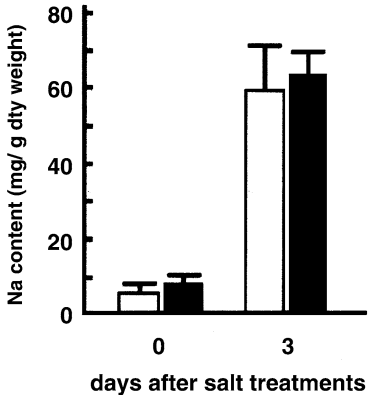

Fig. 4. Comparison of sodium content in leaves. Wild-type (open bars) and transgenic (solid bars) rice plants were treated with hydroponic culture media containing 300 mM NaCl for 3 days. Na^+ was extracted from the leaves before (0) and after (3) salt treatments, and Na^+ content was determined by atomic absorption spectrometry. Values are the mean ± S.D. ($n = 5$).

size during the salt treatment, indicating that 300 mM NaCl completely inhibited their growth. After 3 days of the salt treatment, most of the lower leaves appeared to be bleached and were senescent in both the wild-type and the transgenic rice (Fig. 3, lane 2). However, 3–5 days after the plants were transferred to the fresh hydroponic culture media without NaCl, new leaves and roots appeared only on the transgenic plants (Fig. 3, lane 3). After 2 weeks of growth under normal conditions following NaCl exposure, 81–100% of the plants from line 132 (Fig. 3, lane 4) and 50% of the plants from line 211 survived, while none of wild-type plants survived under the same stress conditions. Triplicate experiments for the plants from line 132 plants and a duplicate experiment for the plants from line 211 produced similar results.

The sodium content in the leaves of both the wild-type and transgenic rice plants was increased 10-fold after 3 days of 300 mM NaCl treatment, indicating that the transgenic plants absorbed and translocated Na^+ into leaves to the same extent as the wild-type (Fig. 4). After salt-stress treatments, the surviving transgenic rice plants were transferred to soil conditions without salt stress and grown in the green house. Although the number of tillers was reduced compared to untreated transgenic rice plants, the transgenic rice plants grew until the flowering stage and set seeds after 3.5 months, demonstrating that the salt shock did not completely damage the fertility of the transgenic rice plants (data not shown).

4. Discussion

Previously, overexpression of choline oxidase (*COD*), Ca^{2+}-dependent protein kinase (*OsCDPK7*) and a gene encoding a protein abundant in late embryogenesis from barley (*HVA1*) improved growth performance of rice plants in the presence of 150–200 mM NaCl [25–27]. These experiments were conducted under conditions whereby wild-type rice plants also survived following the salt treatments. In contrast, we have shown that transgenic rice plants overexpressing a vacuolar-type Na^+/H^+ antiport gene from a halophytic plant, *A. gmelini* (*AgNHX1*), survive under conditions that generally kill wild-type rice plants (300 mM NaCl for 3 days). These results demonstrate that transgenic rice plants containing the *AgNHX1* gene are suitable for practical applications and are

capable of producing crops even if they are exposed for a short period to a high concentration of saline.

Reduction of Na^+ uptake and translocation of Na^+ into shoots are two of the strategies identified in plants for the acquisition of salt tolerance [28]. In our experiments, the sodium content in the leaves of the *AgNHX1* transgenic rice plants was almost the same as that in wild-type rice plants after salt treatments, excluding the possibility that reduced Na^+ uptake resulted in salt tolerance of the transgenic plants. Thus, increased Na^+/H^+ antiport activity in the transgenic plants caused larger amounts of Na^+ to be excluded into vacuoles in individual cells, thus rendering the transgenic rice plants more tolerant to salinity.

We observed that in the transgenic rice plants, only the top young leaves, but not the lower old leaves, survived and expanded after salt treatments. Under these circumstances, the increased activity of Na^+/H^+ antiport into vacuoles is likely to enlarge the capacity for Na^+ deposition in the mature leaves. Such a leaf-to-leaf compartmentalization that effectively controls the differential distribution of Na^+ may improve salt tolerance at the cellular level and in turn may protect younger leaves from salt toxicity [29].

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References

[1] Ponnamperuma, F.N. (1984) in: Salinity Tolerance in Plants: Strategies for Crop Improvement, (Staples, R.C. and Toenniessen, G.A., Eds.), pp. 255–271, John Wiley, New York.
[2] Mennen, H., Jacoby, B. and Marschner, H. (1990) J. Plant Physiol. 137, 180–183.
[3] Blumwald, E., Aharon, G.S. and Apse, M.P. (2000) Biochem. Biophys. Acta 1465, 140–151.
[4] Blumwald, E. and Poole, R.J. (1987) Plant Physiol. 83, 884–887.
[5] Garbarino, J. and DuPont, F.M. (1988) Plant Physiol. 86, 231–236.
[6] Garbarino, J. and DuPont, F.M. (1989) Plant Physiol. 89, 1–4.
[7] Fukuda, A., Yazaki, Y., Ishikawa, T., Koike, S. and Tanaka, Y. (1998) Plant Cell Physiol. 39, 196–201.
[8] Staal, M., Maathuis, F.J.M., Elzenga, J.T.M., Overbeek, J.H.M. and Prins, H.B. (1991) Physiol. Plant. 82, 179–184.
[9] Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Science 285, 1256–1258.
[10] Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L. and Fink, G.R. (1999) Proc. Natl. Acad. Sci. USA 96, 1480–1485.
[11] Fukuda, A., Nakamura, A. and Tanaka, Y. (1999) Biochem. Biophys. Acta 1446, 149–155.
[12] Hamada, A., Shono, M., Xia, T., Ohta, M., Hayashi, Y., Tanaka, A. and Hayakawa, T. (2001) Plant Mol. Biol. 46, 43–56.
[13] Chauhan, S., Forsthoefel, N., Ran, Y., Quigley, F., Nelson, D.E. and Bohnert, H.J. (2000) Plant J. 24, 511–522.
[14] Zhang, H.-X. and Blumward, E. (2001) Nat. Biotech. 19, 765–768.
[15] Zhang, H.-X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001) Proc. Natl. Acad. Sci. USA 98, 12832–12836.
[16] Ohta, S., Mita, S., Hattori, T. and Nakamura, K. (1990) Plant Cell Physiol. 31, 805–813.
[17] Tanaka, A., Mita, S., Ohta, S., Kyozuka, J., Shimamoto, K. and Nakamura, K. (1990) Nucleic Acids Res. 18, 6767–6770.
[18] Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H. (1989) Nature 338, 274–276.
[19] Fukuda, Y., Ohme, M. and Shinshi, H. (1991) Plant Mol. Biol. 16, 1–10.
[20] Yamanishi, H. and Kasamo, K. (1992) Plant Physiol. 99, 652–658.
[21] Blumwald, E., Cragoe, E.J. and Poole, R. (1987) Plant Physiol. 85, 30–33.
[22] Yoshida, S., Forno, D., Cock, J.H. and Gomez, K.I. (1976) in: Laboratory Manual for Physiological Studies of Rice, 3rd edn., pp. 61–66, The International Rice Research Institute, Los Baños.
[23] Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.
[24] Matoh, T., Ishikawa, T. and Takahashi, E. (1989) Plant Physiol. 89, 180–183.
[25] Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.-H.D. and Wu, R. (1996) Plant Physiol. 110, 249–257.
[26] Sakamoto, A., Alia and Murata, N. (1998) Plant Mol. Biol. 38, 1011–1019.
[27] Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Plant J. 23, 319–327.
[28] Matsushita, N. and Matoh, T. (1991) Physiol. Plant. 83, 170–176.
[29] Munns, R. (1993) Plant Cell Environ. 16, 15–24.