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Overexpression of *ENA1* from Yeast Increases Salt Tolerance in *Arabidopsis*

Xiangqiang Kong†, Xiuhua Gao†, Weihuan Li, Jiqiang Zhao, Yanxiu Zhao, and Hui Zhang[1]*
Kay Lab of Plant Stress Research, College of Life Science, Shandong Normal University, Jinan, 250014, Shandong Province, PR China
[1]Life Science College of Shandong Normal University, Wenhua East Road 88, Jinan, 250014, Shandong Province, PR China

In yeast, the plasma membrane Na^+/H^+ antiporter and Na^+-ATPase are key enzymes for salt tolerance. *Saccharomyces cerevisiae* Na^+-ATPase (Ena1p ATPase) is encoded by the *ENA1/PMR2A* gene; expression of *ENA1* is tightly regulated by Na^+ and depends on ambient pH. Although Ena1p is active mainly at alkaline pH values in *S. cerevisiae*, no Na^+-ATPase has been found in flowering plants. To test whether this yeast enzyme would improve salt tolerance in plants, we introduced *ENA1* into *Arabidopsis* (cv. Columbia) under the control of the cauliflower mosaic virus 35S promoter. Transformants were selected for their ability to grow on a medium containing kanamyin. Southern blot analyses confirmed that *ENA1* was transferred into the *Arabidopsis* genome and northern blot analyses showed that *ENA1* was expressed in the transformants. Several transgenic homozygous lines and wild-type (WT) plants were evaluated for salt tolerance. No obvious morphological or developmental differences existed between the transgenic and WT plants in the absence of stress. However, overexpression of *ENA1* in *Arabidopsis* improved seed germination rates and salt tolerance in seedlings. Under saline conditions, transgenic plants accumulated a lower amount of Na^+ than did the wild type, and fresh and dry weights of the former were higher. Other experiments revealed that expression of *ENA1* promoted salt tolerance in transgenic *Arabidopsis* under both acidic and alkaline conditions.

Key words: alkaline pH, *Arabidopsis*, Na^+-ATPase, *Saccharomyces cerevisiae*, salt tolerance

Salinity is a major constraint of crop productivity because it reduces yields and limits the expansion of agriculture onto previously uncultivated land (Flowers and Yeo, 1995). Its toxicity in plant cells is mainly caused by osmotic stress that results from nonspecific effects of high Na^+ concentrations in soil water, which leads to water deficiency in the cells. Ionic stress arises from the specific effects of sodium overaccumulation in the cytoplasm, leading to metabolic inhibition of cellular activities (Serrano, 1996; Hasegawa et al., 2000). Several agronomic approaches have been taken, such as irrigating with fresh water and improving soil drainage. However, these expensive solutions are not always practical. Therefore, the study of plant salt tolerance, with a view toward identifying and eventually manipulating the genes involved in salt perception and response, seems to be a promising strategy (Zhu, 2000).

A vacuolar Na^+/H^+ antiporter can compartmentalize Na^+ into the vacuole to deal with salt stress because that stored sodium contributes to osmotic adjustment (Flowers et al., 1977). Many such antiporters have been cloned, and their overexpression improves salt tolerance by transgenic plants (Apse et al., 1999; Fukuda et al., 1999; Hamada et al., 2001; Zhang and Blumwald, 2001; Zhang et al., 2001; Ma et al., 2004).

A salt overly sensitive pathway is responsible for Na^+ homeostasis in plants (Zhu, 2002). The *Arabidopsis thaliana* SOS1 protein resides at the plasma membrane, where it functions to extrude Na^+ from the cytoplasm coupled to H^+ influx (Qiu et al., 2002). Overexpression of *SOS1* or a constitutively activated form of SOS2 confers increased tolerance (Shi et al., 2003; Guo et al., 2004). SOS1 can interact with RCD1, functioning in the same pathway during oxidative-stress responses. By comparison, RCD1 may contribute to the ion-homeostasis pathway of salt tolerance, possibly by regulating the Na^+/H^+ antiporter activity of SOS1 (Katiyar-Agarwal et al., 2006).

Na^+ recirculation from shoots to roots via the phloem could be important for establishing salt tolerance (Munns, 2002). In *Arabidopsis thaliana*, *AtHKT1* is expressed mainly in the phloem tissues and is suggested to function in this recirculation by loading Na^+ into the shoot phloem, then unloading it from the phloem in the roots (Berthomieu et al., 2003). Functional analysis of *OsSKC1* (corresponding to *OsHKT8*) in rice has shown that it indeed assists in this unloading from the xylem (Ren et al., 2005).

In contrast to plants, yeasts have not only a plasma membrane Na^+/H^+ antiporter but also Na^+-ATPase to move Na^+ out of the cytosol. *SOD2* has been identified from *Schizosaccharomyces pombe* as an Na^+/H^+ antiporter on the plasma membrane (Jia et al., 1992). Its overexpression induces a higher level of salt tolerance *in vivo* in transgenic *Arabidopsis* plants (Gao et al., 2003). In budding yeast *Saccharomyces cerevisiae*, *ENA1* also plays an important role in salt tolerance. This gene encodes a P-type Na^+-ATPase that represents the most important element for the efflux of Na^+ (Haro et al., 1991). Expression of *ENA1* in *S. cerevisiae* is tightly regulated by sodium and depends on ambient pH (Ramos, 1999). Ena1p is active mainly at an alkaline pH in *S. cerevisiae* (Banuelos and Rodríguez-Navarro, 1998). To

*Corresponding author; fax +86-531-8618-0764
e-mail zhangh@sdnu.edu.cn

†These authors contributed equally to this article.

test whether that yeast ATPase improves salt tolerance in plants, Nakayama et al. (2004) have expressed a triple hemagglutinin (HA)-tagged Ena1p in cultured cells of tobacco (*Nicotiana tabacum* L.) cv. Bright Yellow 2 (BY2). There, the Ena1p-3HA proteins are correctly localized to the plasma membrane of transgenic BY2 cells, conferring increased NaCl tolerance to those cells due to Na^+-ATPase activity. Here, we report the introduction of the yeast *ENA1* gene into *Arabidopsis*. Our objective was to evaluate its effect on seed germination and physiological responses and compare them with those of wild-type plants under stressed and non-stressed conditions, including alkaline and acidic pH values.

MATERIALS AND METHODS

Cloning of *ENA1*

RNA was isolated from *Saccharomyces cerevisiae*, using an RNeasy total RNA isolation kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from total RNA with a SuperscriptII kit (Gibco BRL, Rockville, MD, USA), and was used as template. PCR amplification was performed with ExTaq DNA polymerase (TaKaRa, Shiga, Japan) and the following two pairs of *ENA1* gene-specific primers: F1, 5′-CTCGAGCGATGGGCGAAGGAACTAC-3′ and R1, 5′-AGACA GATGACATTCGCTTCACAGT-3′; and F2, 5′-TTGCCTCACAATGCCCTTACCG-3′ and R2, 5′-CCATGGCA CTTAATAGGCCCTGCCT-3′. Here, F1 and R1 were used to amplify the 5′ fragment of *ENA1* while F2 and R2 were used to amplify the 3′ fragment. The two PCR products were separated by agarose gel electrophoresis and digested with *Apa*LI to obtain the 1.6-kb 5′ fragment and the 1.7-kb 3′ fragment of *ENA1*. These two fragments were then ligated with T4 ligase to generate the full *ENA1* gene.

Plasmid Construction and Plant Transformation

The *ENA1* gene was amplified using the above-described ligation product as template with Primers F1 and R2. *ENA1* PCR products were confirmed by sequence analysis on an Applied Biosystem 373 Automated DNA sequencer (ABI/Perkin-Elmer, Foster City, CA, USA). This *ENA1* PCR product was inserted into binary plant vector pROKII, between the cauliflower mosaic virus 35S promoter and the octopine synthase terminator. The resulting plasmid, pROKII-*ENA1*, was mobilized to *Agrobacterium tumefaciens* strain GV3101 and used to transform 5-week-old *Arabidopsis* plants by the floral-dip method (Clough and Bent, 1998). After being grown in the greenhouse, their seeds were collected and screened in an MS (Murashige and Skoog, 1962) medium supplemented with 30 $\mu g\, cm^{-3}$ kanamycin.

Southern and Northern Analyses

Southern and northern-blotting experiments were performed using approximately 30 μg of genomic DNA and 30 μg of total RNA per track. Genomic DNA was isolated from transgenic lines and wild-type (WT) *Arabidopsis* as described by Murray and Thompson (1980), and was digested with *Bam*HI, then transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK). Southern hybridizations (65°C) were carried out as described by Sambrook et al. (1989). DNA probes were labeled with [P^{32}] dCTP using a random primer labeling kit (TaKaRa). The 5′ fragment of *ENA1* served as template for this amplification. Total RNA was extracted from transgenic lines and WT *Arabidopsis* according to the method of Chomczynski and Sacci (1987). The probes and method of hybridization were the same as for our Southern blots.

Growth Parameters and Germination Studies

Arabidopsis plants were grown at 22°C in the greenhouse under a 16-h photoperiod. To assess germination under different media conditions, WT and transgenic surface-sterilized seeds were sown in Petri dishes containing 30 mL of a standard MS medium, MS+100 mM NaCl, or MS+150 mM NaCl. The media pH was adjusted, by HCl or NaOH, to either 5.8 or 8.0. Germination was scored 6 d after sowing.

Growth Measurements

Four-day-old seedlings from vertical plates containing standard MS media were transferred to vertical agar plates with different NaCl concentrations and pH values. Each plate contained WT and transgenic seedlings, and three replicate plates were used for each treatment. Photographs were taken 30 d after this transfer. Growth was determined by measuring fresh and dry masses, the latter done after the plants were oven-dried for 48 h at 70°C.

Measurement of Proline Content

Fresh leaves (2 g) were ground in 10 mL of 5% (v/v) acetic acid, and the homogenate was diluted to 50 mL with distilled water. Proline concentration was determined as described by Troll and Lindsley (1995).

Determination of Total Na^+ and K^+ Contents

Transgenic T_4 plants from lines with a single or multiple-copy insertion of *ENA1* were evaluated for salt tolerance. Their seeds were sown in 9-cm plastic pots filled with a 2:1:1 (v:v:v) mixture of soil:perlite:vermiculite. The resultant plants were grown under greenhouse conditions of a 16-h photoperiod, day/night temperature of 25°C/20°C, and a relative humidity of 60%/80% (day/night). Four-week-old seedlings were subjected to salt stress by applying 60 mL of Hoagland solution to each pot on alternate days over the 16-d watering period. Our control group received no NaCl supplement, while the other Hoagland solutions were mixed with different concentrations of NaCl that were increased stepwise by 50 mM every 4 d. Roots and shoots were harvested at the end of each stress period, and dry weights were measured after the tissues were dried for 48 h at 70°C. All samples were digested with HNO_3, and Na^+ and K^+ contents were determined on an atomic absorption spectrophotometer (Z-8000; Hitachi, Tokyo, Japan) according to the method of Wang and Zhao (Wang and Zhao, 1995). Data were evaluated for means ± SE (n = 3).

RESULTS

Genetic Transformation

Five-week-old Arabidopsis plants were infected with *Agrobacterium tumefaciens* carrying *ENA1* that was preceded by the cauliflower mosaic virus 35S promoter and the *nptII* genes in plasmid pROK-*ENA1*. We obtained 85 kanamycin-resistant plants from 24,000 seeds. The initial transformants were named T1, while their progeny, via self-crossing, were named T2. Several transgenic homozygous lines (T3), all tolerant of kanamycin (30 μg mL^{-1}), were selected: E01-13, E03-7, E06-9, E10-1, E17-9, E21-5, and E82-2. When two lines of E06-9 and E10-1 were compared by molecular and physiological analyses, no obvious morphological or developmental differences were observed between them and the WT plants.

Molecular Characterization of Transgenic Plants

T3 kanamycin-tolerant plants were checked by PCR using primers of *ENA1*. An intense 3.3-kb band corresponded in size to the *ENA1* product whereas no such band was found from the WT. To characterize the copy number of this integrated gene, genomic DNA of T3 plants digested by *Bam*HI was hybridized with the *ENA1* probe. Southern-blot analysis showed that all transgenic plants had hybridization signals, but no signal was detected from the WT. Line E06-9 had a single inserted copy while E10-1 carried more than one copy of *ENA1* (Fig. 1A).

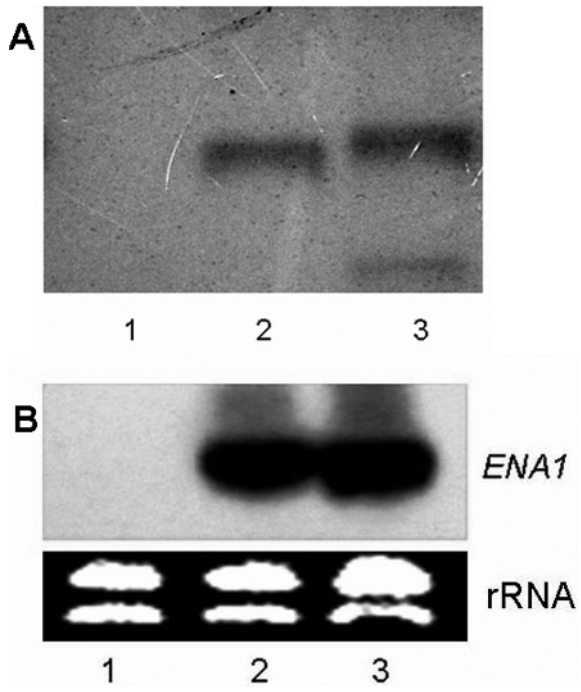

Figure 1. Southern (A) and northern (B) hybridization analysis of WT and transgenic plants. **A**, DNA (30 μg) was digested with *Bam*HI and resulting fragments were resolved by gel-electrophoresis and transferred to nylon membrane. **B**, Thirty micrograms of total RNA was analyzed by RNA gel-blotting. Membrane and blot were hybridized (65°C) with gene-specific DNA probe for *ENA1*, then exposed to x-ray film for 7 d. Ethidium bromide-stained rRNA band in agarose gel served as loading control. Lane 1, WT plants; 2, E06-9; 3, E10-1.

Northern-blot analysis revealed the presence of *ENA1* mRNA in T3 plants of several homozygous transgenic lines. Lines E06-9 and E10-1 had strong positive signals, but no signal was present in the wild-type plants (Fig. 1B).

Overexpression of ENA1 Improves Seed Germination

Arabidopsis is most sensitive to salt at the seed germination and seedling stages. To test germinability under stress conditions, seeds from the WT and several homozygous lines were surface-sterilized and placed on MS media containing different levels of NaCl (0, 100, or 150 mM NaCl) and a pH value of either 5.8 or 8.0. At 0 mM NaCl, germination rates did not differ among genotypes; at that concentration, the increase in pH caused a slight reduction in germination (Fig. 2A, B). On the medium with 150 mM NaCl, WT rates were 50% at pH 5.8 (Fig. 2A) and 48% at pH 8.0 (Fig. 2B). In contrast, the respective germination rates for T3 seed were approx. 65% (Fig. 2A) and 63% (Fig. 2B). These results indicate that overexpression of *ENA1* improves seed germination under both acidic and alkaline conditions.

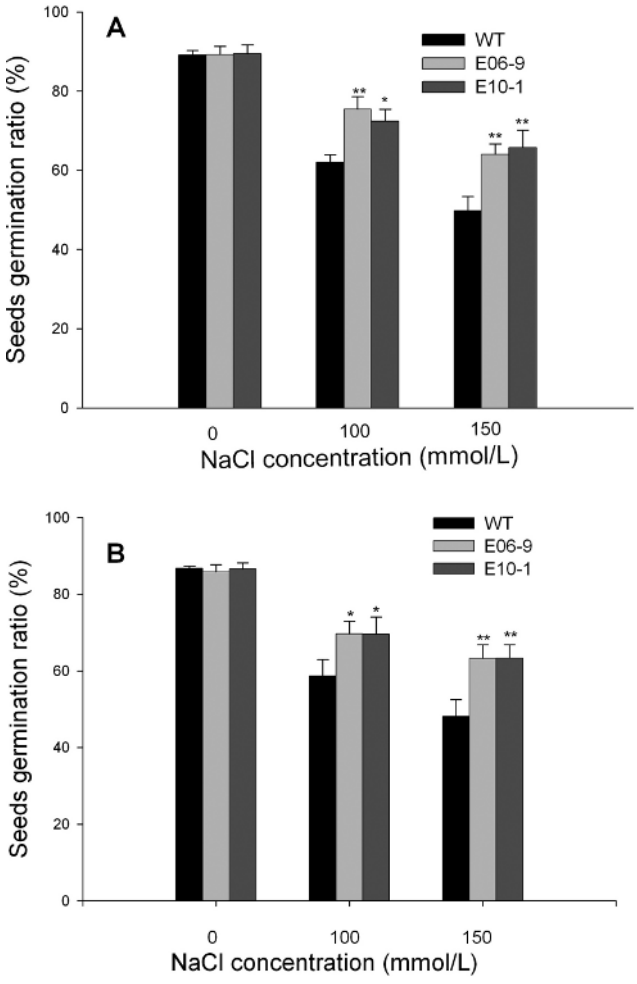

Figure 2. Germination of WT and transgenic plants after 6 d on MS media with different NaCl concentrations (0, 100, or 150 mM) and pH values (**A**, 5.8; **B**, 8.0). Results are presented as means ± SE (n = 3). * and **, indicate significant difference from WT at $P < 0.05$ and 0.01, respectively, by Student's t-test.

Overexpression of *ENA1* Enhances Seedling Tolerance under Salt Stress

Expression of *ENA1* in *Schizosaccharomyces pombe sod2* mutants restores the ability of those cells to export the sodium ion, and greatly increases their resistance to Na^+ (Banuelos et al., 1995). Overexpression of *ENA1* also increases salt tolerance by cultured tobacco cells (Nakayama et al., 2004). Therefore, to determine whether *ENA1*-overexpression could elevate salt tolerance in *Arabidopsis*, seeds from the WT and homozygous *ENA1*-expressing Lines E06-9 and E10-1 were germinated on MS media containing 150 mM NaCl but having different pH values (Fig. 3A). All plants first grew normally without additional NaCl (Fig. 3Aa, Ab). However, after 10 d of cultivation, the WT seedlings turned yellow and ceased growth while the transgenic plants grew only slowly. WT roots also were shorter than those from the transgenic lines (Fig. 3Ac-Af).

To obtain further evidence that overexpression of *ENA1* confers resistance to salt stress, seeds of the WT and Lines E06-9 and E10-1 were placed on a standard MS medium for 7 d, then transferred to MS plates with 150 mM NaCl. Seedlings from both transgenic lines continued to grow slowly while the wild types became chlorotic and stopped developing (Fig. 3B). These results indicate that overexpression of *ENA1* indeed enhances salt tolerance in *Arabidopsis*.

Effect of Constitutive ENA1 Expression on Fresh and Dry Weights and Proline Content

Wild-type plants and progeny of the T3 homozygous kanamycin-tolerant plants (T4 generation) from Lines E06-9 and E10-1 were cultivated under salt or no-salt conditions. The WT plants displayed gradual chlorosis, reduced leaf size, and general growth inhibition when watered with an NaCl solution. These inhibitory effects increased progressively with higher NaCl concentrations, and those plants stopped flowering and eventually died (Fig. 4A). In contrast, the transgenic plants from Line E06-9 were only slightly affected, surviving even when exposed to up to 200 mM NaCl (Fig. 4B). Progeny of Line E10-1 showed a similar response (data not shown).

When the salt treatment ended, plants were harvested.

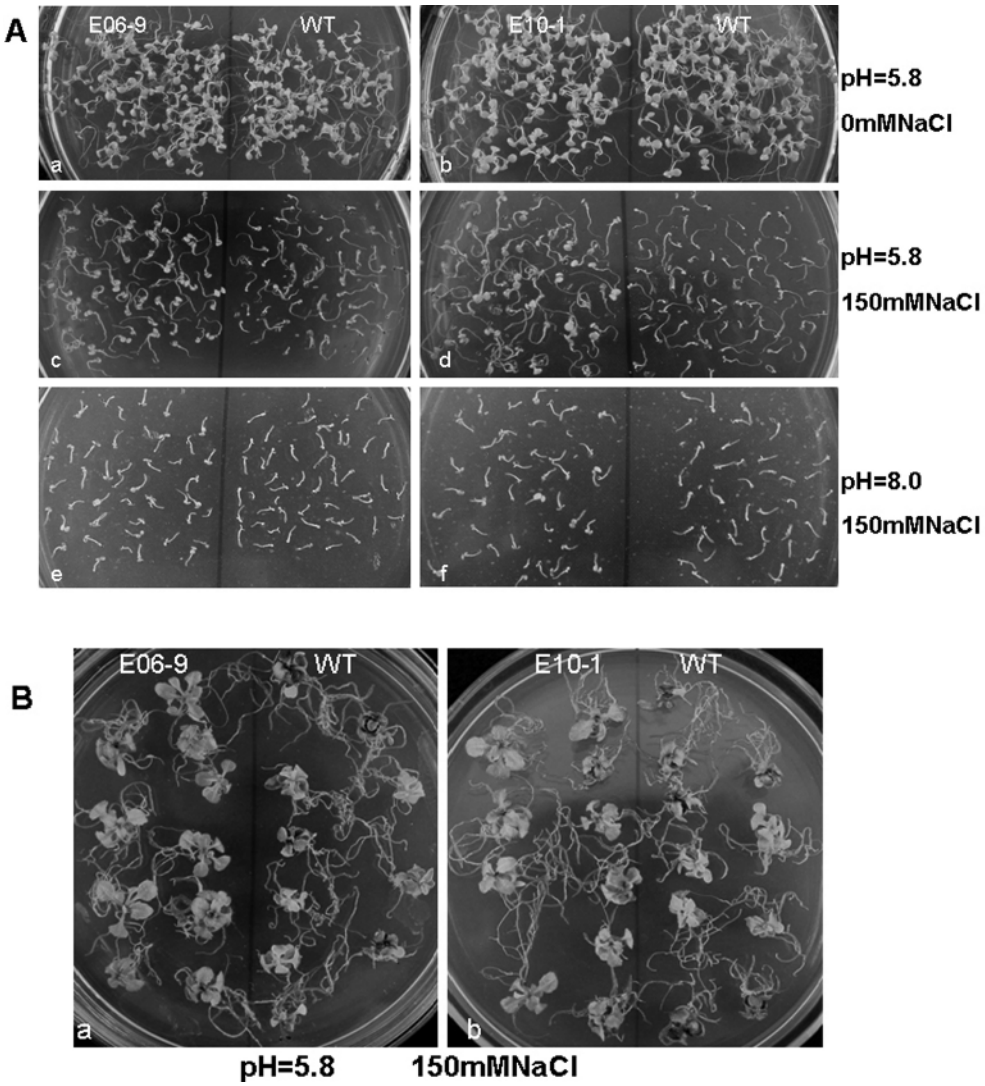

Figure 3. A, Wild-type and transgenic plants of Lines E06-9 and E10-1 after 10 d of cultivation on: MS + 0 mM NaCl (**a** and **b**; pH 5.8) or MS + 150 mM NaCl medium (**c**-**f**; pH 5.8 or 8.0, from top to bottom). **B**, Seeds of E06-9, E10-1, and WT were grown on MS media for 7 d, then transferred to MS plates with 150 mM NaCl (**a** and **b**; pH 5.8).

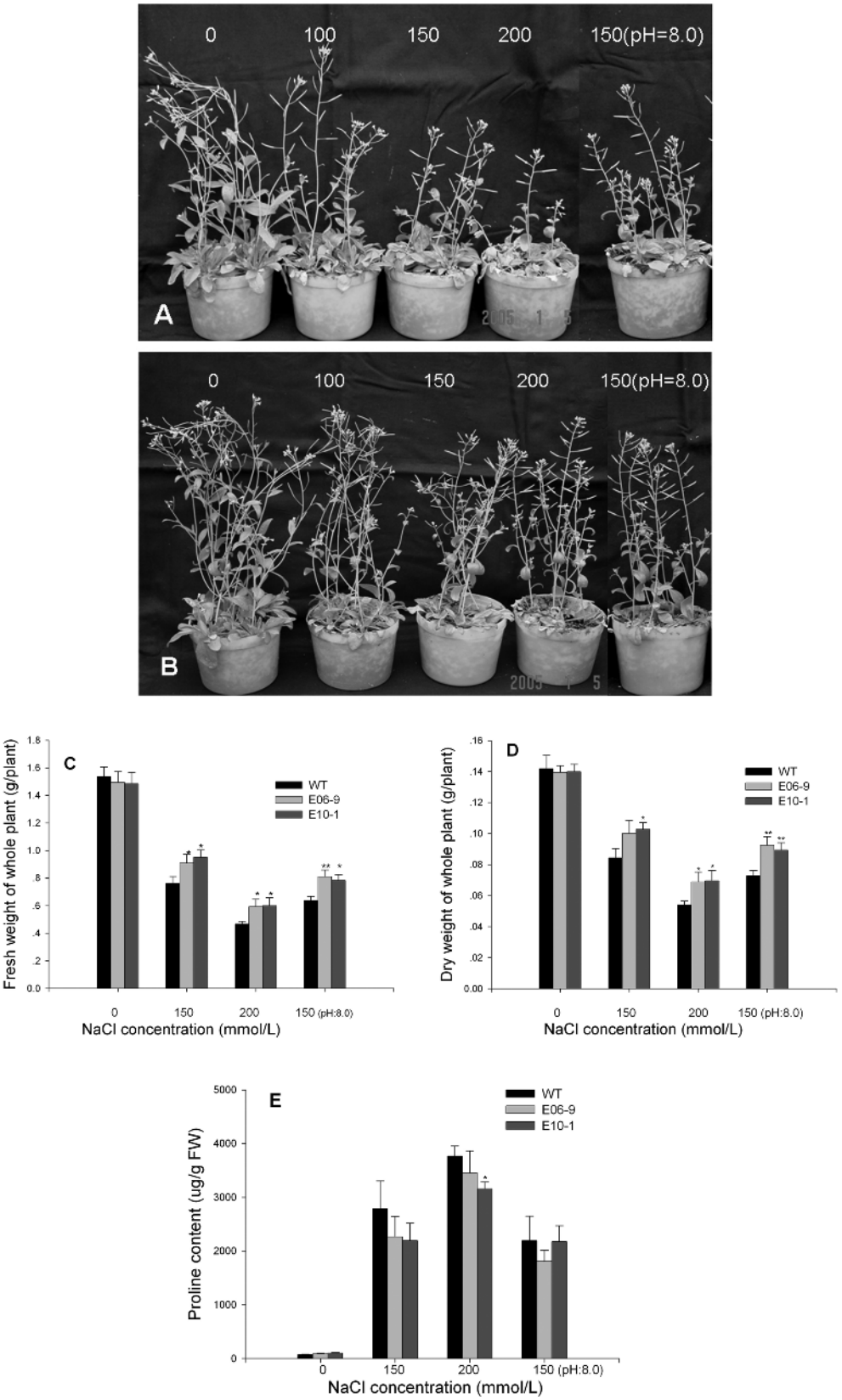

Figure 4. Increased resistance to NaCl stress by transgenic plants over-expressing *ENA1*. Phenotypes of WT (**A**) and T4 transgenic plants of Line E06-9 (**B**) after treatment with different NaCl concentrations. Seedlings were grown in soil and watered every 3 d before treatment began. Whole-plant fresh weights (**C**), dry weights (**D**), and proline contents (**E**) were measured after salt treatment finished. Results are presented as means ± SE (n = 3). * and **, indicate significant difference from WT at $P < 0.05$ and 0.01, respectively, by Student's t-test.

Irrigation with NaCl significantly decreased the fresh and dry weights of WT plants compared with those of the transgenics (Fig. 4C, D).

No significant difference in proline content was observed between the transgenic and WT plants, except for Line E10-1 at 200 mM NaCl, although values were always slightly lower from the transgenics (Fig. 4E).

Transgenic Plants Accumulate Less Na^+ and K^+

Under severe salt stress, *ENA1* functions to export Na^+, thereby limiting its accumulation in yeast cells. To determine if overexpression of *ENA1* would lead to the same outcome in *Arabidopsis*, Na^+ contents in the transgenic and WT plants were analyzed before and after exposure to salt. Without the stress treatment, Na^+ contents were nearly the same in all genotypes. As expected, treatment increased cellular Na^+ levels in both transgenic and WT plants, although values for shoots and roots were distinctly lower in the transgenic tissues (Fig. 5A, B). Furthermore, the K^+ content did not differ significantly between transgenic and WT plants, being only slightly lower in the former (data not shown). This

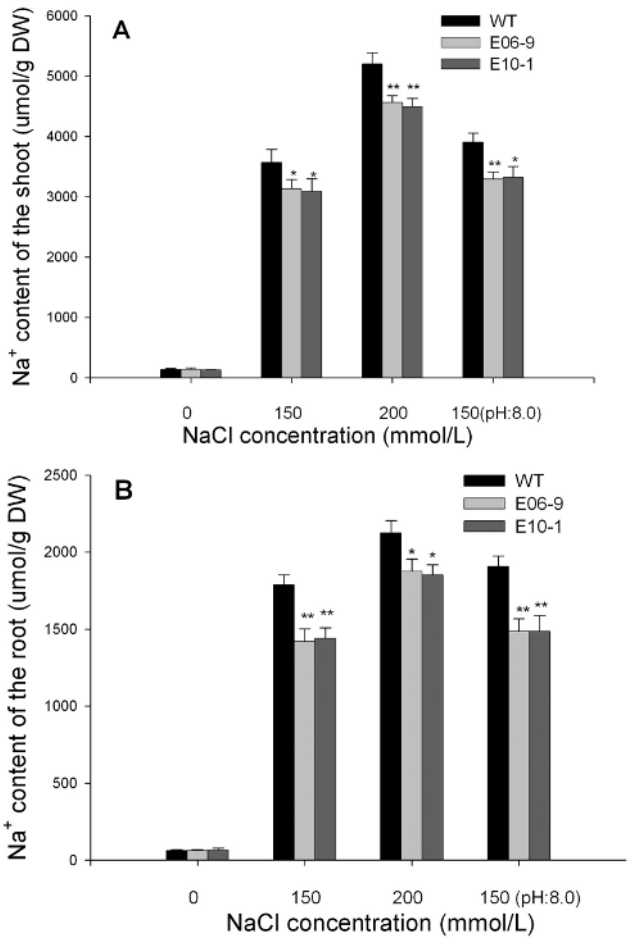

Figure 5. Na^+ contents in tissues of WT and T4 transgenic plants of Line E06-9 after treatment with 0, 150 (pH 5.8), 150 (pH 8.0), or 200 mM NaCl. Ion contents were determined by atomic absorption spectrophotometry. **A**, shoot; **B**, root. Results are presented as means ± SE (n = 3). * and **, indicate significant difference from WT at $P < 0.05$ and 0.01, respectively, by Student's t-test.

was because Ena1p can extrude not only Na^+ but also K^+ from the cytoplasm at high pH and an elevated level of potassium. Nakayama et al. (2004) also have confirmed that, even with K^+-ATPase activity, Na^+-ATPase activity of the yeast Ena1p confers increases salt tolerance to stressed plant cells. Our results also suggest that overexpression of *ENA1* decreases Na^+ accumulation and enhances tolerance by *Arabidopsis*.

DISCUSSION

Plants and fungi use similar ion transport systems to maintain ion homeostasis under salinity stress (Serrano and Rodríguez-Navarro, 2001). However, plants do not contain the plasma membrane Na^+-ATPase. In yeast, the *ENA1* gene plays an important role in salt tolerance, and its overexpression also increases such tolerance in transgenic tobacco cell cultures (Nakayama et al., 2004). Here, we demonstrated that expression of the plasma membrane Na^+-ATPase could confer increased salt tolerance in *Arabidopsis* plants under both acidic and alkaline conditions.

Salinity tolerance clearly depends on sustaining the cytosolic environment, limiting Na^+ accumulation, and maintaining an appropriate K^+ concentration. In this study, transgenic *Arabidopsis* plants tended to accumulate less Na^+ under saline and alkaline pH conditions than did WT plants (Fig. 5A, B). Under salt stress, fresh and dry weights of the transgenic plants were higher than those of the WT, illustrating the beneficial effects of *ENA1* expression. Therefore, our results show that this Na^+ export strategy into transgenic plants works to decrease the Na^+ content, thereby increasing their salt tolerance.

Soil salinity is a major factor in reducing plant growth and productivity. One means for enhancing salt tolerance is to improve the production of small osmolytes or stress proteins that protect against or reduce stress-induced damage (Zhu, 2001). Here, the transgenic lines accumulated less proline (Fig. 4E), perhaps because cytosolic Na^+ was extruded into the apoplastic space by *ENA1*. This response may have reduced cellular damnification so that the plants did not need to increase Pro levels. Alternatively, those transgenic plants may have had greater accumulations of other solutes in their vacuoles and cytosol to compensate for this rise in Na^+ export from cells.

This is the first report, to our knowledge, of a yeast plasma membrane Na^+-ATPase that is ectopically expressed in intact, higher plants. Here, Na^+ content was lower in the transgenic lines, and their salt tolerance was improved not only under acidic but also alkaline conditions. Therefore, these transgenic plants may have used the activity of Ena1p to export Na^+ back to the growth medium or to apoplastic spaces, thereby reducing sodium levels. We have now demonstrated that overexpression of *ENA1* can promote a higher degree of salt tolerance in transgenic *Arabidopsis* plants.

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