

# Exclusion of Na<sup>+</sup> via Sodium ATPase (PpENA1) Ensures Normal Growth of *Physcomitrella patens* under Moderate Salt Stress<sup>1</sup>

Christina Lunde\*, Damian P. Drew, Andrew K. Jacobs, and Mark Tester

Plant Biochemistry Laboratory, Department of Plant Biology, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg C, Copenhagen, Denmark (C.L.); and Australian Centre for Plant Functional Genomics, University of Adelaide, Waite Campus, Glen Osmond, South Australia 5064, Australia (D.P.D., A.K.J., M.T.)

The bryophyte *Physcomitrella patens* is unlike any other plant identified to date in that it possesses a gene that encodes an ENA-type Na<sup>+</sup>-ATPase. To complement previous work in yeast (*Saccharomyces cerevisiae*), we determined the importance of having a Na<sup>+</sup>-ATPase in planta by conducting physiological analyses of PpENA1 in *Physcomitrella*. Expression studies showed that PpENA1 is up-regulated by NaCl and, to a lesser degree, by osmotic stress. Maximal induction is obtained after 8 h at 60 mM NaCl or above. No other abiotic stress tested led to significant increases in PpENA1 expression. In the gametophyte, strong expression was confined to the rhizoids, stem, and the basal part of the leaf. In the protonemata, expression was ubiquitous with a few filaments showing stronger expression. At 100 mM NaCl, wild-type plants were able to maintain a higher K<sup>+</sup>-to-Na<sup>+</sup> ratio than the PpENA1 (*ena1*) knockout gene, but at higher NaCl concentrations no difference was observed. Although no difference in chlorophyll content was observed between *ena1* and wild type at 100 mM NaCl, the impaired Na<sup>+</sup> exclusion in *ena1* plants led to an approximately 40% decrease in growth.

Soil salinity represents a growing problem in agriculture because high sodium concentrations are toxic to most plants, causing reduced growth and productivity (Munns et al., 1999). With more than 20% of irrigated land worldwide affected by increasing salinity, it is important to understand the basis for the different levels of salinity tolerance that are commonly observed in plants and to discover how certain plants are able to maintain growth in saline conditions. Plants are sessile and have to adapt and thrive in many different types of soils with varying ionic compositions. This is in contrast to animal cells, which are more or less exposed to a constant ionic environment. The membrane potential across the plasma membrane is approximately -150 mV for most plant cells (Benito et al., 2002). When this negative potential is combined with a difference in pH, the resulting electrochemical potential difference for H<sup>+</sup> energizes H<sup>+</sup>-coupled transport catalyzed by the many symporters and antiporters in the plasma membrane and hence allows the

uptake of essential nutrients, such as K<sup>+</sup>, and efflux of toxic solutes, such as Na<sup>+</sup>.

Na<sup>+</sup> is the main toxic ion in saline soils for most plants, including the major cereals (Tester and Davenport, 2003). Many cytosolic enzymes are inactivated by Na<sup>+</sup> and high Na<sup>+</sup> concentrations are detrimental to cellular metabolism. In addition, excess Na<sup>+</sup> creates hyperosmotic stress, causing a range of effects, such as secondary oxidative damage (Mittler, 2002). To survive in a saline environment, plants have evolved a range of protective mechanisms. Recent studies, which have identified and characterized key enzymes in these protective pathways, especially in salt-tolerant species, have allowed the engineering of crop plants with increased salt tolerance. Several strategies for improvement of salt tolerance have been employed (Chinnusamy et al., 2005). The initial strategies were to increase the production of small osmolytes like mannitol, Glybetaine, Pro, and trehalose (Tarczynski et al., 1993; Kishor et al., 1995; Xu et al., 1996; Sakamoto and Murata, 2002). A more recent strategy has involved the overexpression of vacuolar and plasma membrane ion transporters, increasing Na<sup>+</sup> exclusion from the cytosol. Overexpression of two Na<sup>+</sup>/H<sup>+</sup> antiporters, NHX1 and SOS1 (for salt overly sensitive), has conferred salt tolerance to transgenic plants by sequestering Na<sup>+</sup> in the vacuole or transporting Na<sup>+</sup> across the plasma membrane out of the cell, respectively (Apse et al., 1999; Shi et al., 2002, 2003). AtNHX1 was identified in Arabidopsis (*Arabidopsis thaliana*) based on homology to the yeast (*Saccharomyces cerevisiae*) NHX1 transporter and was able to complement the

<sup>1</sup> This work was supported by the Australian Research Council and the State Government of South Australia (grants to D.P.D., A.K.J., M.T.) and by the Danish Research Council (grant to C.L.).

\* Corresponding author; e-mail chlu@life.ku.dk; fax 45-35-33-33-33.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Christina Lunde (chlu@life.ku.dk).

[www.plantphysiol.org/cgi/doi/10.1104/pp.106.094946](http://www.plantphysiol.org/cgi/doi/10.1104/pp.106.094946)

salt-sensitive nhx1 yeast strain (Gaxiola et al., 1999). *AtNHX1* is ubiquitously expressed in the plant and expression is induced by NaCl and abscisic acid (ABA; Shi and Zhu, 2002). *AtSOS1* was initially identified as a locus required for salt tolerance in Arabidopsis and loss-of-function mutations in *AtSOS1* caused plants to be extremely salt sensitive and to accumulate more Na<sup>+</sup> than wild type in the shoots under salt stress (Wu et al., 1996; Shi et al., 2000). *AtSOS1* transcription is specifically up-regulated upon NaCl stress and expression is mainly found in parenchyma cells bordering the xylem and in the epidermal cells surrounding the root tip (Shi et al., 2002).

Soil fungi are like plants in that they are exposed to similar challenges arising from close contact to a changing ionic environment. Phylogenetic analyses have shown that plant and fungal cells share many ion transporters; however, one major difference is the apparent absence of the ENA-type Na<sup>+</sup>-ATPases in vascular plants. In plants, maintaining nontoxic levels of Na<sup>+</sup> appears to rely solely upon Na<sup>+</sup>/H<sup>+</sup> antiporters. A surprising exception was the recent identification of two Na<sup>+</sup>-ATPases (PpENA1 and PpENA2) in the bryophyte, *Physcomitrella patens* (Benito and Rodríguez-Navarro, 2003). PpENA1 was shown to act as a Na<sup>+</sup> pump when expressed heterologously in yeast and complemented a salt-sensitive yeast strain deficient in Na<sup>+</sup> and K<sup>+</sup> efflux (Benito and Rodríguez-Navarro, 2003). This implies that *Physcomitrella* has either gained the gene or the gene has been lost during the evolution of higher plants. In contrast, PpENA2 was unable to complement the salt-sensitive yeast strain.

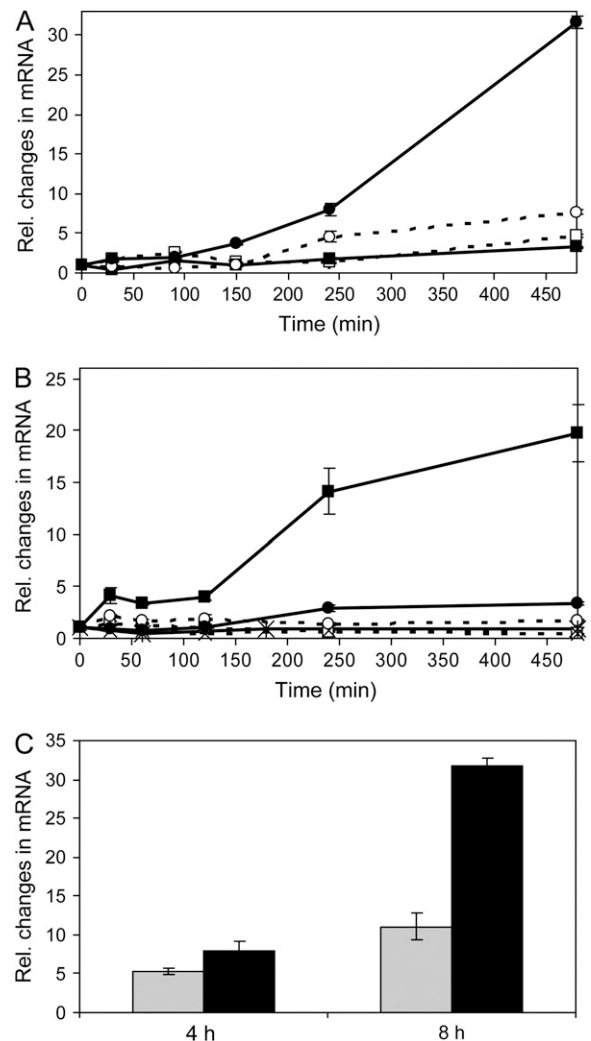
Although PpENA1 activity has been characterized in a heterologous system by Benito and Rodríguez-Navarro (2003), little is known about the functional importance of Na<sup>+</sup>-ATPases in bryophytes. Furthermore, heterologous expression of membrane transporters can alter the properties of the transporter (e.g. the ion selectivity of the transporter; Pardo et al., 2006). For this reason, study of this gene's properties in situ provides a valuable test of function. *Physcomitrella* is an excellent plant model system because it performs homologous recombination with high efficiency. This allowed us to study the physiological role of PpENA1 in planta under sodium stress. From expression analyses and by generating knockouts via homologous recombination, we determined that PpENA1 plays an essential role in *Physcomitrella* under moderate salt stress by improving K<sup>+</sup>/Na<sup>+</sup> homeostasis and allowing normal growth.

## RESULTS

### Induction of PpENA1 under NaCl Stress

To determine over what time frame and under which salt concentrations PpENA1 plays a physiologically important role, quantitative reverse transcription (qRT)-PCR was performed on protonemata exposed to various NaCl concentrations (5–400 mM)

for different times (30 min to 3 d). Exposing protonemata to low NaCl concentrations (530 mM) caused a 3- to 8-fold increase in expression. However, increasing the NaCl concentration to 60 mM caused a much higher, 30-fold, induction in PpENA1 mRNA levels after 8 h (Fig. 1A). Longer exposure to higher NaCl concentrations caused no further changes in PpENA1 expression (data not shown). The response to increasing Na<sup>+</sup> was nonlinear, suggesting the induction response is saturated by 60 mM NaCl (Fig. 1, A and



**Figure 1.** Transcription pattern of *PpENA1* under abiotic stress. A, Ten-day-old protonemata were exposed to different concentrations of NaCl. Tissue was harvested at different time points and mRNA extracted for qRT-PCR; 5 mM (white square), 10 mM (black square), 30 mM (white circle), and 60 mM (black circle). B, *PpENA1* expression in protonemata exposed to different abiotic stresses. Protonemata were either not treated (hatched, white square), or exposed to 100 mM NaCl (black square), UV-B (black, cross), 50 μM ABA (hatched, cross), cold (hatched, white circle), or 50 μM methyl viologen (solid, black circle). C, The level of *PpENA1* up-regulation after protonemata were exposed for 4 or 8 h to either 30 mM PEG (gray) or 60 mM NaCl (black), which impose a similar change in the osmotic potential.

**Table I.** mRNA copy number of *PpENA1*, *PpENA2*, and selected control genes in  $10^{-9}$  L of cDNA

Total mRNA was extracted from either 10-d-old protonemata or 5-week-old gametophytes, quantified, adjusted to the same RNA concentration for cDNA synthesis, and used for RT-PCR.

Tissue	<i>PpTub1</i>	<i>PpEF1<math>\alpha</math></i>	<i>PpHis-3</i>	<i>PpMdh2</i>	<i>PpENA1</i> <sup>a</sup>	<i>PpENA2</i>
Protonema	215 $\pm$ 59	312 $\pm$ 129	74 $\pm$ 11	30 $\pm$ 2	10 $\pm$ 2	1.5 $\pm$ 0.9
Gametophyte	309 $\pm$ 53	205 $\pm$ 49	127 $\pm$ 41	31 $\pm$ 2	49 $\pm$ 11	2.6 $\pm$ 1.4

<sup>a</sup>Genes are expressed significantly different in the protonema compared to the gametophyte ( $n = 4$ ;  $P < 0.01$ ).

B). In contrast, exposure to KCl had no effect on *PpENA1* expression (data not shown).

qRT-PCR was also performed for *PpENA2*, but the level of expression was too low in the protonemata to allow proper analysis (Table I).

To determine whether the induction of *PpENA1* was specific to Na<sup>+</sup> stress, protonemata were exposed to NaCl, osmotic stress, cold, oxidative stress, or ABA. Only NaCl treatment caused significant induction of *PpENA1* mRNA (Fig. 1B). To test whether the Na<sup>+</sup>-induced up-regulation of *PpENA1* was due to ionic or osmotic effects, protonemata were exposed to a similar decrease in water potential using either 60 mM NaCl or 30 mM polyethylene glycol (PEG) 1,500 for 4 and 8 h (Fig. 1C). After 8 h, osmotic treatment led to a 10-fold induction, compared to a 30-fold induction with NaCl, indicating that the induction of *PpENA1* expression under Na<sup>+</sup> stress is due to a combination of osmotic and ionic effects.

#### Tissue-Specific Expression of *PpENA1* and *PpENA2*

The expression of *PpENA1* and *PpENA2* in protonema and gametophyte were analyzed using RT-PCR and mRNA levels were compared to a selection of control genes (Table I). BLAST searches in the recently released draft of the genomic sequences (<http://moss.nibb.ac.jp>) confirmed that single copies of *PpENA1* and *PpENA2* are present in the *Physcomitrella* genome. The primers used for RT-PCR were designed to the 3' end of the genes based on publicly available EST sequences. Besides the nonspliced *PpENA2* mRNA, three putative splice variants have been identified (Benito and Rodríguez-Navarro, 2003). The primers used to detect *PpENA2* expression were therefore designed to anneal upstream of the alternative splice sites to ensure detection of all mRNA variants. Very low expression of *PpENA2* was detected in both protonema and gametophytes. In contrast, expression of *PpENA1* was 7- or 19-fold higher than *PpENA2* in protonema and gametophytes, respectively. A 5-fold higher level of expression of *PpENA1* in gametophytes compared to protonema was observed. Neither *PpENA2* nor the control genes showed this significant difference in expression levels between protonemal and gametophytic tissues (Table I).

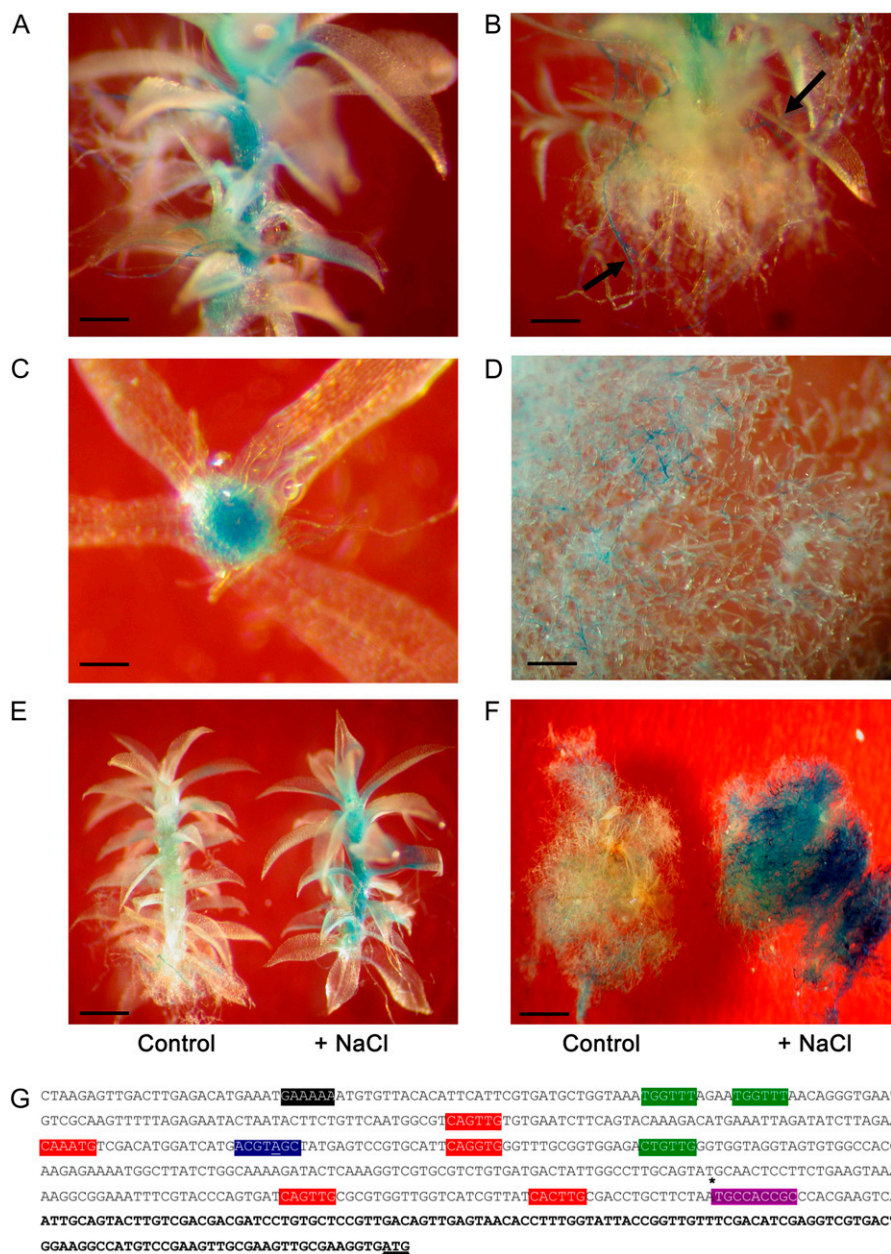
To determine whether *PpENA1* expression was confined to certain cell types, wild-type *Physcomitrella* was transformed with a construct containing a *PpENA1* promoter-GUS fusion. The promoter-GUS construct

consisted of 1,311 bp upstream of the putative transcription start site and a 137-bp 5'-untranslated region (UTR). The publicly available EST sequences originate from a cDNA library with mainly full-length cDNAs and 5' UTR is defined as the sequence included in the ESTs (BJ188922 and BJ191872; Nishiyama et al., 2003). Twelve independent stable transformants were obtained, of which eight showed consistent, but variable, levels of GUS staining. Differences in GUS levels are a common phenomenon observed in transgenic plants, including *Physcomitrella* (Kamisugi and Cuming, 2005). In the nonstressed gametophyte, GUS staining was confined to the stem, the basal part of the leaves, and to a small number of rhizoids originating from the base of the gametophyte (Fig. 2, A–C). No staining was present in the apical part of the leaf and staining was less pronounced in the top leaves compared to the leaves near the base of the gametophyte. GUS staining was ubiquitous in the protonemata, although some apical and subapical protonematal cells showed more intense staining than others (Fig. 2D). The expression pattern in the gametophyte under NaCl stress was determined by dividing colonies of gametophytic tissue and exposing one-half to medium containing 100 mM NaCl for 24 h. Only gametophytes originating from the same transformation event were compared. In all eight transformants, GUS staining became much more pronounced after NaCl stress, but the staining was still confined to the same tissue types as in the untreated control (Fig. 2E). When protonemata were exposed to 100 mM NaCl for 24 h, staining became very intense throughout the whole colony (Fig. 2F).

In silico analysis of the promoter was performed to identify putative cis-regulatory elements, which could be responsible for the observed expression pattern (Fig. 2G). Several elements were identified that are known to be up-regulated under dehydration stress (Myb, Myc, and CE-1 coupling elements; Yamaguchi-Shinozaki and Shinozaki, 2005). The only putative ABA regulatory element contained a mismatch at a conserved position, which is likely to make it non-functional (Shen et al., 2004). In addition, the consensus sequence for a GT-1 element shown to be involved in salt-dependent up-regulation was identified in the promoter (Park et al., 2004).

#### K<sup>+</sup>/Na<sup>+</sup> Homeostasis in Wild-Type and *ena1* Knockouts

To determine the importance of *PpENA1* in *Physcomitrella*, mutants lacking Na<sup>+</sup>-ATPase were generated.



**Figure 2.** Tissue-specific expression of *PpENA1*. The *PpENA1* promoter (1,451 bp) was fused to a GUS-reporter gene and the construct transformed into *Physcomitrella*. **A**, GUS staining of a representative 5-week-old gametophyte grown on standard medium without NaCl, showing GUS staining in the stem and the basal part of the leaves. Bar = 0.4 mm. **B**, GUS staining of rhizoids from a nonstressed gametophyte. Arrows indicate the strongly stained filaments. Bar = 0.4 mm. **C**, Cross section of a gametophyte showing staining in the hydroids, cortex, and epidermis. Bar = 0.2 mm. **D**, Ubiquitous GUS staining in nonstressed protonemata. Bar = 0.4 mm. **E**, Comparison of GUS staining in two gametophytes originating from the same colony either left untreated or exposed to 100 mM NaCl for 24 h before GUS staining. The staining is significantly stronger, but still confined to the same tissues after salt stress. Bar = 0.6 mm. **F**, The effect of salt treatment on protonemata. A 10-d-old protonematal colony was divided in two and one-half of the colony was exposed to 100 mM NaCl for 24 h and the other half not treated. The strong GUS staining throughout the tissue is seen after NaCl stress. Bar = 1.5 mm. **G**, In silico *PpENA1*-promoter analysis. Putative cis-regulatory elements in the *PpENA1* promoter were identified searching PLACE or NSITE-PL. Because the importance of regulatory elements generally decreases with increasing distance from the transcription start site, only the first 500 bp upstream of the putative transcription start site were included in the analysis. Putative GT-1 box (GAAAAA, black), Myb (WAACCA, CTGTTG, green), Myc (CANNTG, red), ABRE (ACGTG<sup>\*</sup>GC, blue), and CE-1 coupling elements (TGCCACCGC<sup>\*</sup>, purple) are indicated. Mismatches in the consensus sequence are underlined and elements with an expected mean frequency <0.05 in 500 bp of random DNA sequence are marked with an asterisk. The putative 5' UTR based on available EST sequences is shown in bold.

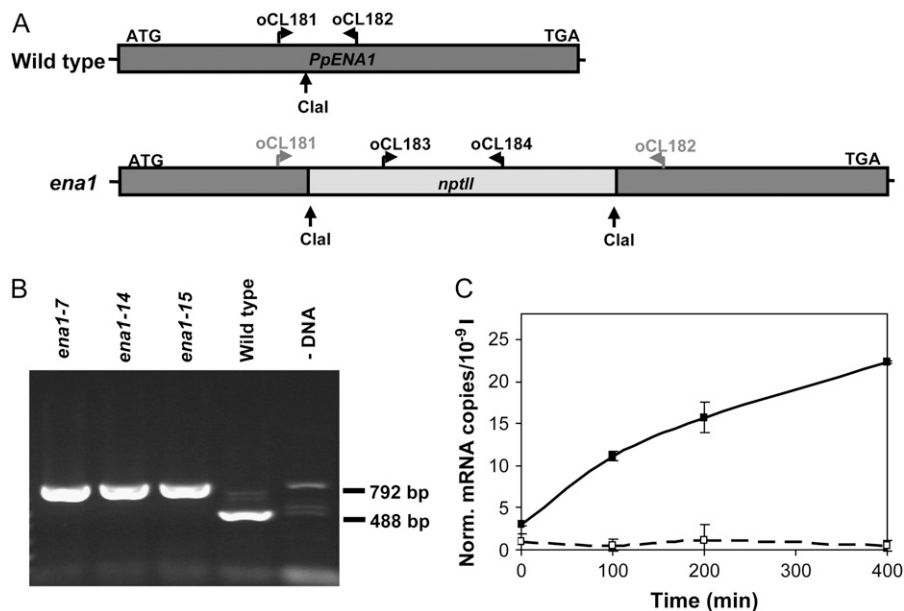
The *nptII* selective gene was inserted into a cDNA clone of *PpENA1* using a *Clal* site situated 1,189 bp downstream of the start ATG. This knockout cassette was used for transformation of wild-type *Physcomitrella*. After selection, 15 stable transformants were obtained. The stable transformants were PCR screened using genomic DNA as a template and primers specific to either side of the *Clal* restriction site (Fig. 3A). A short PCR fragment (488 bp) would be generated if the wild-type *PpENA1* gene was still present. A primer pair annealing to the *nptII* cassette was also included in the PCR reaction (792 bp) as a positive control. For three independent lines (*ena1-7*, *-14*, and *-15*), PCR reactions consistently gave the 792-bp *nptII* fragment and no 488-bp *PpENA1* fragment (Fig. 3B). To confirm that the knockout cassette had replaced the *PpENA1* gene, mRNA was purified from wild-type and *ena1* protonemata exposed to 100 mM NaCl. A clear increase in *PpENA1* mRNA was observed over time in the wild type, whereas the *PpENA1* transcript levels remained at the background level in the *ena1* mutants (Fig. 3C).

One of the reasons  $\text{Na}^+$  is toxic to plants is that  $\text{Na}^+$  can replace  $\text{K}^+$  in certain enzymes, rendering them nonfunctional. To test whether the lack of *PpENA1* affected the  $\text{K}^+$ -to- $\text{Na}^+$  ratio in *Physcomitrella* under NaCl stress, gametophytes were placed on medium containing 100, 200, or 400 mM NaCl for 1, 3, or 7 d. The  $\text{K}^+$ -to- $\text{Na}^+$  ratio in the untreated wild type was

$30 \pm 6$  ( $n = 6$ ), whereas the *ena1* lines had a slightly higher  $\text{K}^+$ -to- $\text{Na}^+$  ratio of  $39 \pm 1$  ( $n = 9$ ;  $P < 0.05$ ). Within 1 d of exposure to high levels of NaCl, the  $\text{K}^+$ -to- $\text{Na}^+$  ratio decreased dramatically (Fig. 4A). At 200 and 400 mM NaCl, the  $\text{K}^+$ -to- $\text{Na}^+$  ratio remained low and, even after 7 d, no significant recovery was observed. Upon exposure to 100 mM NaCl, however, the wild type was able to improve the  $\text{K}^+$ -to- $\text{Na}^+$  ratio by 50% after 3 d. In contrast, no significant recovery was observed in the *ena1* lines. To determine whether the ability of the wild-type plants to improve the  $\text{K}^+$ -to- $\text{Na}^+$  ratio at 100 mM was due to exclusion of  $\text{Na}^+$ , the intracellular  $\text{Na}^+$  and  $\text{K}^+$  concentrations were measured. Both wild type and *ena1* mutants experienced a dramatic increase in intracellular  $\text{Na}^+$  concentration in the first 24 h after transfer to 100 mM NaCl. In the wild-type gametophytes, however, no further increase was observed (Fig. 4B). In contrast, the intracellular  $\text{Na}^+$  concentration increased further by 60% to 100% in the *ena1* mutants (Fig. 4C;  $P < 0.001$ ) to be almost the same as the external  $\text{Na}^+$  concentration. The intracellular  $\text{K}^+$  concentration increased to the same extent in both wild type and *ena1* mutants.

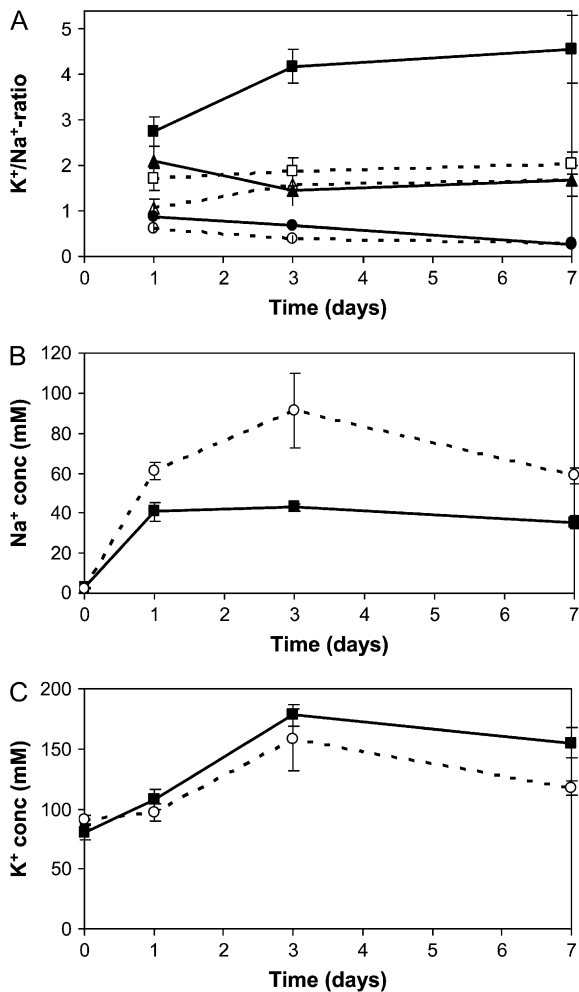
#### Growth of Wild-Type and *ena1* Plants under Salt Stress

To establish whether the inability to improve the  $\text{K}^+$ -to- $\text{Na}^+$  ratio had a negative effect on fitness,



**Figure 3.** Generating *PpENA1* knockouts by homologous recombination. **A**, cDNA clone of *PpENA1* was restriction enzyme digested at a unique *Clal* site and the *nptII* gene inserted to generate a knockout cassette. **B**, The transformants were screened by PCR on genomic DNA using a mix of primers specific to *PpENA1* (oCL181, oCL182) and *nptII* (oCL183, oCL184). A 488-bp band is expected in the wild type and a 792-bp band in the transformants where the *PpENA1* gene has been replaced by the knockout cassette (*ena1*). Although all four primers anneal to the *PpENA1* knockout cassette, the short fragment will be favored during the PCR. If the selective cassette is inserted in the genome, without replacing the *PpENA1* gene, both the 488- and 792-bp PCR fragments will be present. **C**, *PpENA1* mRNA levels. To ensure that *PpENA1* was not expressed in the *ena1* lines, mixed protonemata and gametophytes from wild type (black line) and *ena1* (stippled line) were exposed to 100 mM NaCl and tissue harvested at different time points used for qRT-PCR. *PpENA1* expression was normalized against four control genes (see "Materials and Methods").



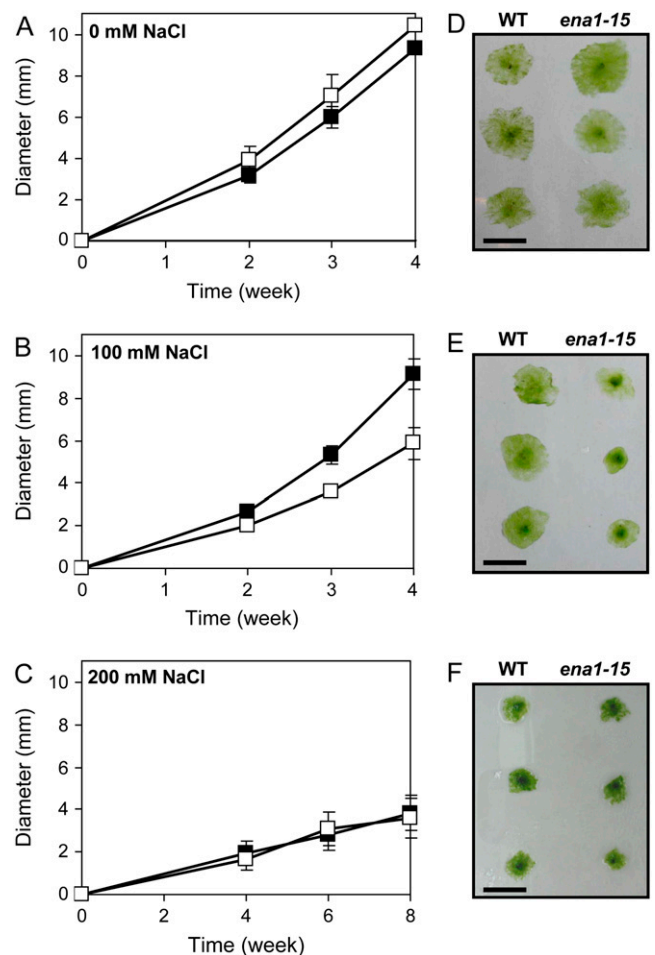


**Figure 4.** The intracellular K<sup>+</sup>-to-Na<sup>+</sup> ratio of wild-type and *ena1* plants exposed to varying NaCl concentrations. A, Five-week-old gametophytes of wild type (black;  $n = 6$ ) and *ena1* (stippled;  $n = 9$ ) were exposed to 100 (square), 200 (triangle), or 400 mM (circle) NaCl for up to 7 d. The data for *ena1* represent the average for three independent *ena1* lines. Intracellular Na<sup>+</sup> (B) and K<sup>+</sup> (C) concentrations in wild-type (black) and *ena1* gametophytes (stippled) were calculated after exposure to 100 mM NaCl. Water content of the tissue was determined by weighing the gametophytes before and after drying.

wild-type and *ena1* plants were grown on standard or salt-containing medium. The growth experiment was initiated using protoplasts to ensure that the starting material was homogeneous. Protoplasts were allowed to regenerate for 2 weeks before being transferred to medium containing 0, 100, or 200 mM NaCl. Growth was followed for 4 to 8 weeks by measuring the diameter of moss colonies.

On standard medium without added NaCl, no statistically significant difference in growth was observed between wild-type and *ena1* plants, although *ena1* appeared to grow marginally better (Fig. 5, A and D). When tissue was transferred to medium containing 100 mM NaCl, *ena1* grew significantly slower and, after 4 weeks, the diameter of the *ena1* colonies were 36%

smaller than wild type (Fig. 5, B and C;  $P < 0.005$ ). No growth impairment was observed for the wild type, which grew at a similar rate on standard or 100 mM NaCl medium. When tissue was transferred to medium containing 100 mM KCl and growth was followed for 4 weeks, no significant difference was observed between wild-type and *ena1* plants (colony diameter of wild type =  $6.8 \pm 0.9$  mm [ $n = 47$ ]; *ena1* =  $6.9 \pm 0.8$  mm [ $n = 44$ ]). When wild-type and *ena1* tissue was transferred to medium containing 200 mM of NaCl or KCl, both lines grew significantly slower than on standard medium, but no difference in growth was observed. Even after an extended growth period, none of the colonies exposed to 200 mM NaCl or KCl formed thallus, but remained in the protonemal state.



**Figure 5.** Effect of salt stress on growth of wild-type and *ena1* plants. Protoplasts from wild type and *ena1* were generated from 1-week-old protonemata to obtain identical starting material for the growth experiment. Protoplasts were allowed to recover for 2 weeks and then transferred to either standard medium (A) or medium with 100 (B) or 200 (C) mM NaCl. Growth of wild-type (black square) and *ena1* plants (white square) was followed by measuring the diameter of 40 to 60 colonies from each treatment. Representative wild-type and *ena1*-15 colonies after 4 weeks of growth on standard medium (D) or medium with 100 mM NaCl (E) or after 8 weeks of growth on 200 mM NaCl (F). Bar = 1 cm.

Exposure to stressful levels of NaCl often leads to loss of chlorophyll and development of chlorotic areas. Although *ena1* growth was impaired at 100 mM NaCl, the colonies did not appear paler or more chlorotic than wild type under these conditions. Total chlorophyll was extracted from wild-type and *ena1* plants grown on standard or 100 mM NaCl medium to determine more precisely whether the total level or composition of chlorophyll was changed. The chlorophyll in nonstressed tissue was  $21 \pm 3$  and  $19 \pm 1$  mg chl/g dry weight for *ena1* and wild type, respectively. No significant difference was observed between *ena1* and wild-type plants grown at 100 mM NaCl having  $18 \pm 2$  and  $19 \pm 1$  mg chl/g dry weight, respectively. However, a small decrease in the chl *a/b* ratio was observed in the *ena1* tissue compared to wild type grown at 100 mM being  $2.01 \pm 0.03$  and  $2.14 \pm 0.09$ , respectively ( $P < 0.05$ ).

## DISCUSSION

*Physcomitrella* is tolerant of high levels of NaCl in the surrounding medium and can maintain growth at  $\text{Na}^+$  concentrations that would be detrimental to most vascular plants (Benito and Rodríguez-Navarro, 2003; Frank et al., 2005). A gene encoding an AtSOS1 homolog and two ENA-type ATPases has previously been identified in *Physcomitrella* (Benito and Rodríguez-Navarro, 2003). The physiological importance of having two  $\text{Na}^+$  efflux systems in plants is not known, but it is possible that PpENA1, like  $\text{Na}^+$ -pumping ATPases in fungi, is responsible for the extrusion of  $\text{Na}^+$  into the surrounding medium and hence contributes to the high NaCl tolerance of *Physcomitrella*. In yeast, expression of the plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter *sod2* from *Schizosaccharomyces pombe* can complement the salt-sensitive *S. cerevisiae ena* knockout line and the expression of *ScENA* can complement the *sod2::ura4* mutation in *S. pombe* (Bañuelos et al., 1995; Hahnenberger et al., 1996). Hence, under certain stress conditions, the two systems appear to be functionally redundant.

### How Is PpENA1 Regulated?

The large (25- to 30-fold) transcriptional up-regulation, specifically in response to NaCl, implies an important functional role of PpENA1 in salinity tolerance (Fig. 1). This up-regulation is much more substantial than the 2- to 5-fold up-regulation of the salt-responsive  $\text{Na}^+/\text{H}^+$  antiporters observed in vascular plants (Shi et al., 2002; Shi and Zhu, 2002; Yokoi et al., 2002; Kant et al., 2006). Except for osmotic stress, none of the other abiotic stresses tested led to increased PpENA1 transcript levels, confirming the specificity of the response. It is intriguing that exogenously added ABA had no effect on PpENA1 expression. One explanation could be that the regulation of PpENA1

has to be highly controlled and specific to salt stress. In silico analysis of the PpENA1 promoter revealed the presence of several putative regulatory elements. Although the elements used for the search mainly originated from vascular plants, it has previously been shown that ABA regulatory elements are conserved through plant evolution (Knight et al., 1995).

A 6-nucleotide element (GT-1) was recently identified in the promoter of a calmodulin from soybean (*Glycine max*; Park et al., 2004). The promoter was not responding to ABA, but the presence of this GT-1 element led to strong up-regulation upon salt treatment. An identical element was identified within the first 500 bp of the PpENA1 promoter, suggesting that this element could be the core-regulating cis-element.

In vascular plants, regulation of the two  $\text{Na}^+/\text{H}^+$  antiporters (NHX1 and SOS1) appears to be very similar to PpENA1, although differences are also apparent. In Arabidopsis, AtNHX1 is up-regulated after exposure to high levels of NaCl or KCl (Gaxiola et al., 1999; Shi and Zhu, 2002; Yokoi et al., 2002). A similar induction of OsNHX1 is observed in rice (*Oryza sativa*) after treating plants with NaCl or with isoosmotic levels of mannitol. In accordance with PpENA1 results, osmotic stress only caused partial induction of OsNHX1 compared to ionic stress (Fukuda et al., 2004). Although this could suggest a similar type of regulation, a significant difference is that NHX1 up-regulation is induced by ABA (Shi and Zhu, 2002; Yokoi et al., 2002). ABA dependence could, however, be species specific because no increase in  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux was observed in the tonoplasts from *Mesembryanthemum crystallinum* after ABA treatment (Barkla et al., 1999). Like PpENA1, AtSOS1 expression is up-regulated by NaCl, but no up-regulation is observed after ABA treatment (Shi et al., 2000).

In vascular plants, the salt-specific response is also posttranslationally coordinated via the SOS pathway consisting of a Ser-Thr kinase (SOS2) and a calcium-binding protein (SOS3; Shi et al., 2000; Gong et al., 2004). It was recently shown that the SOS pathway not only regulates the activity of SOS1, but is also involved in regulating the vacuolar ion-exchange activity of AtNHX (Qiu et al., 2004). The finding of *Physcomitrella* ESTs showing 69% and 67% identity at the amino acid level to AtSOS2 (BJ187011, BJ589574) and AtSOS3 (BJ201990, BJ609228) opens the possibility that a SOS pathway also coordinates the salt response in *Physcomitrella*. If similar coordination occurs in *Physcomitrella*, it will be interesting in future studies to see whether PpENA1 activity is regulated by the SOS pathway.

### What Is the Physiological Role of PpENA1?

The capacity of plants to maintain a high  $\text{K}^+$ -to- $\text{Na}^+$  ratio is likely to be a key determinant in salt tolerance. The steady-state level of both ions will ultimately reflect the sum of the many transporters involved in uptake, efflux, and compartmentation. To determine

the physiological advantage of having Na<sup>+</sup>-ATPase in plants, the K<sup>+</sup>-to-Na<sup>+</sup> ratio was determined at different NaCl concentrations (Fig. 4A). The salt stress experiment clearly showed that wild-type *Physcomitrella* was able to maintain a higher K<sup>+</sup>-to-Na<sup>+</sup> ratio at 100 mM NaCl than the *ena1* mutant. However, at higher NaCl concentrations, the advantage of having PpENA1 disappeared and the K<sup>+</sup>-to-Na<sup>+</sup> ratio in wild type and *ena1* was similar. Under severe stress (200–400 mM NaCl), it appears that PpENA1 is overwhelmed and no difference in the growth or K<sup>+</sup>-to-Na<sup>+</sup> ratio of *ena1* and wild-type plants could be observed (Figs. 4A and 5C).

Growth impairment correlated well with the intracellular K<sup>+</sup>-to-Na<sup>+</sup> ratio; when the ratio was 3 or above, growth was similar to the nonstressed control, but at 2 or below, growth was impaired. A similar observation has been made in yeast, where normal growth was observed only when the K<sup>+</sup>-to-Na<sup>+</sup> ratio was above 2 (Bañuelos et al., 1998). The K<sup>+</sup> concentration increased in both wild-type and *ena1* plants upon exposure to NaCl. This effect is not due to shrinking of the tissue because no changes in water content per unit dry weight were observed (data not shown) and the increased uptake or decreased efflux of K<sup>+</sup> could be a compensating response to the increased intracellular levels of Na<sup>+</sup>. The growth difference was observed at pH 6.5, where PpSOS1 would be expected to be active and yet the presence of PpENA1 gave the wild type a growth advantage. A similar complementary advantage of having both the ENA-type ATPases (*ENA1-4*) and Na<sup>+</sup>/H<sup>+</sup> antiporter (*NHA1*) under salt stress has been observed in yeast (Bañuelos et al., 1998). At alkaline pH, *ENA1-4* became more important for salt tolerance. A growth experiment at pH 8 was also attempted with wild-type and *ena1* plants in the presence of 100 or 200 mM NaCl. However, both lines were stressed and grew poorly even in the absence of NaCl (data not shown). The difficulty of cultivating and performing stress experiments with *Physcomitrella* at alkaline pH has also been noted by Benito and Rodríguez-Navarro (2003).

One of the reasons for Na<sup>+</sup> toxicity is that Na<sup>+</sup> can replace K<sup>+</sup> in essential enzymes and thereby inactivate them. Under severe Na<sup>+</sup> toxicity, metabolism and photosynthesis will be affected, leading to decreased levels of chlorophyll. No difference in chlorophyll content per unit dry weight was observed between stressed and nonstressed tissue or between *ena1* and wild-type plants. Hence, it appears that, although *ena1* plants contain more intracellular Na<sup>+</sup>, it is able to protect the sensitive components either by sequestering the Na<sup>+</sup> in the vacuole, synthesizing compatible osmolytes, or up-regulating levels of protective proteins. Up-regulation of a dehydrin-like protein essential for the recovery of *Physcomitrella* after salt stress and of an enzyme involved in protection against reactive oxygen species has recently been shown (Lunde et al., 2006; Saavedra et al. 2006). The lack of visual signs of stress implies that, even in *ena1* plants, Na<sup>+</sup> does not build up to toxic concentrations in the

cytosol and that both wild-type and *ena1* plants are able to manage the intracellular Na<sup>+</sup>. However, the impaired growth of *ena1* plants on salt suggests that the metabolic price for doing so is higher in *ena1* than wild type.

In Arabidopsis, *AtSOS1* is mainly expressed in the epidermis cells of the root tip, the pericycle, and the parenchyma cells bordering the xylem, suggesting that *AtSOS1* is involved in xylem loading-unloading of Na<sup>+</sup> and some exclusion into the soil (Shi et al., 2002). The tissue in *Physcomitrella* is generally only one cell layer thick and mineral transport does not occur in a vascular system, but either from cell to cell or in specialized cells, called hydroids, found in the stem of the gametophyte (Reski, 1998). GUS staining suggested that Na<sup>+</sup> exclusion is particularly important in the rhizoids, which are in direct contact with the medium. Strong expression was also observed in the mature stem, in the central hydroids, the surrounding cortex, and the epidermal cell layer. In addition, the basal part of the one cell-layer-thick leaves was GUS stained. Although vascular plants and mosses are very different in their morphology, *PpENA1* is expressed like *AtSOS1* in tissue involved in nutrient transport, thereby minimizing the amount of Na<sup>+</sup> reaching the photosynthetically active cells. The strong and ubiquitous expression of *PpENA1* in protonemata, suggesting a more efficient Na<sup>+</sup> extrusion from this tissue, could explain why *Physcomitrella* does not differentiate into gametophytes at high salt concentrations.

#### What Is the Functional Role of PpENA2?

The functional role, if any, of the PpENA1 homolog, PpENA2, is still unknown. However, it is clear that *PpENA2* expression is significantly lower than *PpENA1* and this low expression level is not able to complement the *PpENA1* knockout at 100 mM NaCl (Table I; Fig. 5, B and E). This is in line with previous experiments showing that *PpENA2*, in contrast to *PpENA1*, could not complement a salt-sensitive yeast strain (*ena1-4*, *nha1*) on high-salt medium (Benito and Rodríguez-Navarro, 2003). BLAST searches among more than 100,000 *Physcomitrella* EST sequences reveal four EST sequences (BJ580267, BJ581369, BJ583232, BJ594559) representing the 3' end of *PpENA2*. These sequences align with the genomic sequences and do not suggest that the *PpENA2* gene contains any introns. However, three splice variants were identified in the 3' end by Benito and Rodríguez-Navarro (2003) and, whereas the nonspliced *PpENA2* gives rise to a protein of similar length to *PpENA1* (972 and 967 amino acids, respectively), the three splice variants cause PpENA2 to be either truncated or extended. Although this needs to be investigated further, it is possible that the low level of *PpENA2* expression combined with the alternative splicing of the 3' end gives rise to very little functional product. Redundancy of the Na<sup>+</sup>-ATPases has also been observed in yeast (Garcia-deblas et al., 1993).



## Why Has PpENA1 Been Maintained in *Physcomitrella*?

When *PpENA1* apparently gives *Physcomitrella* a clear selective advantage under moderate salt stress, it is intriguing that Na<sup>+</sup>-ATPases are absent in vascular plants. The presence of both a Na<sup>+</sup>,K<sup>+</sup>-ATPase and a SOS1 homolog in the red alga, *Porphyra*, living in the sea at high salinity and alkaline pH also suggests that having both types of pumps is important. The early land on which plants evolved was likely to be oligotrophic and low in Na<sup>+</sup> (Rodríguez-Navarro and Rubio, 2006), so it is possible that Na<sup>+</sup>-ATPases were lost due to the low selective pressure. Although not statistically significant, wild-type plants appear to grow slightly more slowly than *ena1* plants under nonstressful conditions. Hence, under these conditions, there might be a small growth penalty of having PpENA1, which could explain the evolutionary loss. Under nonstressful conditions, the wild type had a slightly lower K<sup>+</sup>-to-Na<sup>+</sup> ratio than *ena1* plants and it is possible that PpENA1 at low Na<sup>+</sup> concentrations excludes essential K<sup>+</sup> instead. From complementation studies in yeast, it appears that PpENA1, like ScENA, has a certain affinity for K<sup>+</sup> (Benito and Rodríguez-Navarro, 2003). In yeast, the dual affinity for both Na<sup>+</sup> and K<sup>+</sup> was shown to lead to concomitant efflux of K<sup>+</sup> with Na<sup>+</sup> under low Na<sup>+</sup> conditions (Benito et al., 2002). In contrast, no K<sup>+</sup> efflux was observed at high Na<sup>+</sup> concentrations. A similar scenario could be envisioned for PpENA1 in *Physcomitrella*.

One explanation for why *PpENA1* apparently has been conserved in *Physcomitrella* could be that moss and fungi, unlike vascular plants, are able to undergo significant water loss and still be viable (Frank et al., 2005). Na<sup>+</sup>-ATPases could ensure that Na<sup>+</sup> does not build up under these conditions. However, no up-regulation of *PpENA1* was observed in *Physcomitrella* under severe drought imposed by placing protonemata on Whatman paper (data not shown).

The correlation between *PpENA1* expression and salt tolerance raises the possibility that heterologous expression of *PpENA1* may improve the salt tolerance of crop plants. Heterologous expression of *AtNHX1* in diverse plants like *Brassica*, tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*) allowed all three crops to grow under highly saline conditions and even produced a higher yield with similar properties to wild-type plants growing under nonstressful conditions (Zhang et al., 2001; Zhang and Blumwald, 2001; Xue et al., 2004). These results confirm that introducing a more efficient system for dealing with Na<sup>+</sup> by changing a single trait is possible and can confer salt tolerance to crops not only in the greenhouse, but also in field trials (Xue et al., 2004). It will be of great interest to see whether the expression of *PpENA1* in, for example, roots, where Na<sup>+</sup> can be extruded to the large volume of growth medium, will be able to improve salt tolerance of vascular plants.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Physcomitrella patens* (Hedw.) derived from a wild-type specimen collected in Gransden Wood in Huntingdonshire, UK (Ashton and Cove, 1977) was grown at 22°C on cellophane discs placed on solid minimal medium (Ashton et al., 1979), supplemented with ammonium tartrate when protonemata were required (0.5 g/L). Standard irradiance conditions were 16-h white light (fluorescent tubes; GRO-LUX; 100 μmol m<sup>-2</sup> s<sup>-1</sup>) and 8-h darkness. Stress experiments were carried out on protonemata homogenized 10 d prior to the stress exposure or on 5-week-old gametophytes. Abiotic stress was induced by transferring the cellophane with *Physcomitrella* protonemata to medium or filter discs containing 100 μM methyl viologen (Sigma), 50 μM ABA (Sigma), 60 or 100 mM NaCl, 60 or 100 mM KCl, or 30 mM PEG 1,500 (Sigma). Supplemental CaCl<sub>2</sub> was added to obtain the same level of free Ca<sup>2+</sup> under all treatments. The amount of added CaCl<sub>2</sub> required was calculated using MinTeq, version 2.30 (<http://www.lwr.kth.se/English/OurSoftware/vminteq>). The osmolarity of PEG 1,500 and NaCl was calculated according to the Merck Index (11th ed., 1989). Cold stress was imposed by placing the plates on ice. UV-B stress was imposed by placing *Physcomitrella* under a UVLMS-38 lamp fitted with an 8-W 302-nm tube (Ultra-Violet Products). The tissue was exposed to 0.23 mW cm<sup>-2</sup> measured using an optical radiometer (MS-100; Ultra-Violet Products) fitted with a UV-B sensor (MP-131). Samples were frozen in liquid nitrogen upon harvesting and stored at -80°C until RNA was extracted.

The growth rate of wild-type and *ena1* knockout lines under salt stress was determined by generating protoplasts (Schaefer and Zryd, 1997). After 2 weeks, the recovered protoplasts were transferred to standard medium, containing 100 or 200 mM of NaCl or KCl. Growth was followed for 4 to 8 weeks by measuring the diameter of 40 to 60 colonies per treatment.

### RNA Extraction, cDNA Synthesis, and Real-Time qRT-PCR

Total RNA was extracted from protonemata or gametophytes using TRIzol (Invitrogen) according to the manufacturer's instructions. Purified RNA was treated with DNaseI using the DNA-free kit (Ambion). RNA integrity was checked on a 1% (w/v) agarose gel containing ethidium bromide. cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen) and 2.5 μM oligo(dT) (18–20) primer according to the manufacturer's protocol. Four control genes—*histone3* (BT003326), *elongation factor 1α* (P13905), *actin2* (NP\_188508), and *tubulin β1* (NP\_177706)—were used in each experiment, as described in Lunde et al. (2006). Primers specific to *PpENA1* were designed, a forward primer (5'-AAGGCATTACCTGGGAGTGGAT) annealing to the coding region and a reverse primer (5'-ACAGCATGGGTGCGGATTCT) annealing to the 3' UTR. Three splice forms of *PpENA2* have been identified (Benito and Rodríguez-Navarro, 2003). PCR primers annealing to *PpENA2* (5'-ATGGC-GACCTTGGCAGTCAG, 5'-CAGGGTGGGAAGTGGCACC) were designed to the coding region upstream of the splice sites to include all splice variants. Primers used for *PpMdh2* (monodehydroascorbate reductase) are described in Lunde et al. (2006). qRT-PCR analysis was performed essentially as described in Burton et al. (2004). For each primer pair, the specific PCR fragment was generated, purified, and quantified using a known DNA standard (pUC19/*HpaII* digested; Geneworks). The size in base pairs and identity were confirmed by sequencing. A dilution series of each PCR fragment was prepared covering seven orders of magnitude (10<sup>7</sup> – 10<sup>1</sup> copies of PCR fragment per microliter). Three replicates of each of the seven standard dilutions were included in each RT-PCR experiment to allow quantification of the corresponding cDNA using Rotor-Gene version 4.6 software (Corbett Research). qRT-PCR results were normalized to the four control genes described above to obtain the normalized mRNA copies per nanoliter cDNA. In the abiotic stress experiment, the relative changes in mRNA were obtained by dividing the qRT-PCR results (normalized mRNA copies per nanoliter cDNA) for the treated tissue with the qRT-PCR results for the corresponding untreated control tissue.

### Genomic DNA Purification, Genomic Walking, Promoter Analyses, and BLAST Searches

Genomic DNA was purified as in Schlink and Reski (2002). The *PpENA1* promoter sequence was obtained by genomic walking as described in Siebert et al. (1995). Genomic DNA (15 μg) was digested overnight with 100 units of *DraI*, *NruI*, *HpaI*, *StuI*, *SmaI*, *EcoRI*, *HincII*, or *PvuII*, phenol-chloroform

purified and ligated to a 5' adaptor to generate eight different templates. The 5' sequences were obtained by nested PCR using gene-specific and adaptor-specific primers. The overlapping sequences obtained from the eight templates were aligned using Contig Express (Vector NTI 8). The promoter sequence was analyzed using NSITE-PL ([www.softberry.com](http://www.softberry.com)) and PLACE (<http://www.dna.affrc.go.jp/PLACE>). ESTs encoding PpENA1 and PpENA2 were obtained by BLAST searches in the two *Physcomitrella* databases ([www.cosmos.org](http://www.cosmos.org); <http://moss.nibb.ac.jp>). Corresponding genomic sequences were obtained from BLAST searches of the draft release of the whole-genome shotgun sequences (<http://moss.nibb.ac.jp>).

### Generating PpENA1 Knockouts and GUS-Promoter Fusions

To obtain stable knockouts of *PpENA1*, a cassette containing p35S-nptII-CamVter was excised from pMBL6 (<http://www.biology.wustl.edu/moss/pmb6.jpg>) using *Clal* and inserted into a unique *Clal* site in the *PpENA1* gene (Benito and Rodríguez-Navarro, 2003), generating pCL247. To increase the efficiency of homologous recombination, pCL247 was digested with *EcoRI*, which cuts twice in the vector backbone. *Physcomitrella* was transformed with 10 to 30 µg DNA and selected on geneticin (Gibco) according to Schaefer and Zryd (1997). To confirm integration, several independent PCR reactions were performed on genomic DNA purified from resistant colonies using primers oCL183 (5'-ATTGAACAAGATGGATTGCACGC) and oCL184 (5'-TCAGAA-GAAGCTCGTCAAGAAGGCG) annealing to the selective cassette (p35S-nptII-CamVter) and primers oCL181 (5'-AACGGGTACGCTGACGGAGG) and oCL182 (5'-CGTCCCAGAGATGCTATGT) annealing 5' or 3' of the *Clal* site, respectively (Fig. 3). Genomic DNA was checked by PCR using primers annealing to the 5' and 3' end of *PpENA2*, respectively (5'-ATGGTCCG-CATCCGAGAGTTGA, 5'-CTACATAGTGTAAGGGTGGTG), to ensure that *PpENA2* was intact in the transformants.

The 1,448-bp DNA fragment directly upstream from the *PpENA1* coding region was obtained by nested PCR using genomic DNA as template. The first PCR was done using oCL136 (CTGTGCCAGAGCCCTCC) and oCL138 (GCAGGTAGCTTCCAATTTCTCT), and the second PCR using oCL137 (CACCTTCGCAACTTCGCAAC) and oCL139 (CGTACACCAGCAGACAT-TAGTG). The fragment was cloned into PCR8/GW/TOPO (Invitrogen) and transferred to a destination vector containing the GUS reporter gene (pMDC162; Curtis and Grossniklaus, 2003) using Gateway technology according to the manufacturer's protocol (Invitrogen). The *PpENA1* promoter-GUS construct, due to sequence-specific homologous recombination, is precisely targeted to the native *PpENA1* gene. GUS staining was performed according to Jefferson (1989) and visualized using a stereomicroscope.

### Intracellular Na<sup>+</sup> and K<sup>+</sup> Levels and Chlorophyll Content

Five-week-old gametophytes were transferred from standard medium to medium containing 100, 200, or 400 mM NaCl. In the standard medium, 1 mM CaCl<sub>2</sub> was added and 1.3, 1.7, 2.0 mM CaCl<sub>2</sub>, respectively, were added to the NaCl-containing medium to keep the level of available Ca<sup>2+</sup> constant (see above). After 1, 3, or 7 d of exposure, gametophytes were washed for 5 s in 10 mM CaCl<sub>2</sub> and allowed to drain briefly on Whatman filter paper. The single short wash was selected to minimize leakage and loss of intracellular K<sup>+</sup> and Na<sup>+</sup> during washing. Preliminary experiments had shown that, for tissue exposed to 100 or 200 mM NaCl for 4 h, a 5-s wash led to a 75% and 87% reduction in NaCl compared to an unwashed control. A subsequent second washing step led to only a further 2% to 4% decrease in tissue Na<sup>+</sup>.

The water content of the gametophytes was determined by weighing the samples before and after drying overnight at 80°C. Na<sup>+</sup> and K<sup>+</sup> content was determined after digestion in 10 mM nitric acid overnight at 80°C and analyses using a flame photometer (Sherwood 420).

The total chlorophyll content of protonemata was determined by measuring the absorbance of acetone extracts at 652, 647, and 663 nm (Arnon, 1949; Frank et al., 2005). All *P* values for statistical significance were determined using a two-tailed, Student's *t* test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF683141.

### ACKNOWLEDGMENTS

We acknowledge Dr. Jesse Machuka as part of the *Physcomitrella* EST Program at the University of Leeds and Washington University for supplying

pMBL6. We also thank Neil J. Shirley for excellent technical assistance and Alonso Rodríguez-Navarro for kindly supplying the vector containing *PpENA1*.

Received December 20, 2006; accepted June 4, 2007; published June 7, 2007.

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