



# Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit

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Transgenic tomato plants overexpressing a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter were able to grow, flower, and produce fruit in the presence of 200 mM sodium chloride. Although the leaves accumulated high sodium concentrations, the tomato fruit displayed very low sodium content. Contrary to the notion that multiple traits introduced by breeding into crop plants are needed to obtain salt-tolerant plants, the modification of a single trait significantly improved the salinity tolerance of this crop plant. These results demonstrate that with a combination of breeding and transgenic plants it could be possible to produce salt-tolerant crops with far fewer target traits than had been anticipated. The accumulation of sodium in the leaves and not in the fruit demonstrates the utility of such a modification in preserving the quality of the fruit.

Agricultural productivity is severely affected by soil salinity, and the damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. Much research is aimed toward the breeding of crop cultivars with improved salt tolerance. One school of thought has concluded that salt tolerance will be achieved only after pyramiding several characteristics in a single genotype, whereas each one alone could not confer a significant increase in salt tolerance<sup>1,2</sup>. Arguably, salt tolerance is a complex trait, and the long list of salt stress-responsive genes seems to support this<sup>3</sup>. However, the overexpression of a single gene recently was shown to improve salt tolerance in *Arabidopsis*<sup>4</sup>.

The detrimental effects of salt on plants are a consequence of both a water deficit resulting in osmotic stress and the effects of excess sodium ions on key biochemical processes. To tolerate high levels of salts, plants should be able to use ions for osmotic adjustment and to internally distribute these ions to keep sodium away from the cytosol. The presence of large, acidic-inside, membrane-bound vacuoles in plant cells allows the efficient compartmentation of sodium into the vacuole through the operation of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiports<sup>4,5</sup>. These antiports use the protonmotive force generated by the vacuolar H<sup>+</sup>-translocating enzymes, H<sup>+</sup>-adenosine triphosphatase (ATPase), and H<sup>+</sup>-inorganic pyrophosphatase (PP<sub>i</sub>ase) to couple the downhill movement of H<sup>+</sup> (down its electrochemical potential) with the uphill movement of Na<sup>+</sup> (against its electrochemical potential)<sup>6</sup>. The overexpression of *AtNHX1*, a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport from *Arabidopsis thaliana*, in *Arabidopsis* plants allowed the transgenic plants to grow in 200 mM NaCl (ref. 4), suggesting the possibility of engineering crop plants with improved salt tolerance.

Here, we show that transgenic tomato plants overexpressing a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport were able to grow, flower, and produce fruits in the presence of 200 mM sodium chloride. The high concentration of salt in the leaves and not in the fruit demonstrates the potential use of these transgenic plants for agricultural use in saline soils.

## Results and discussion

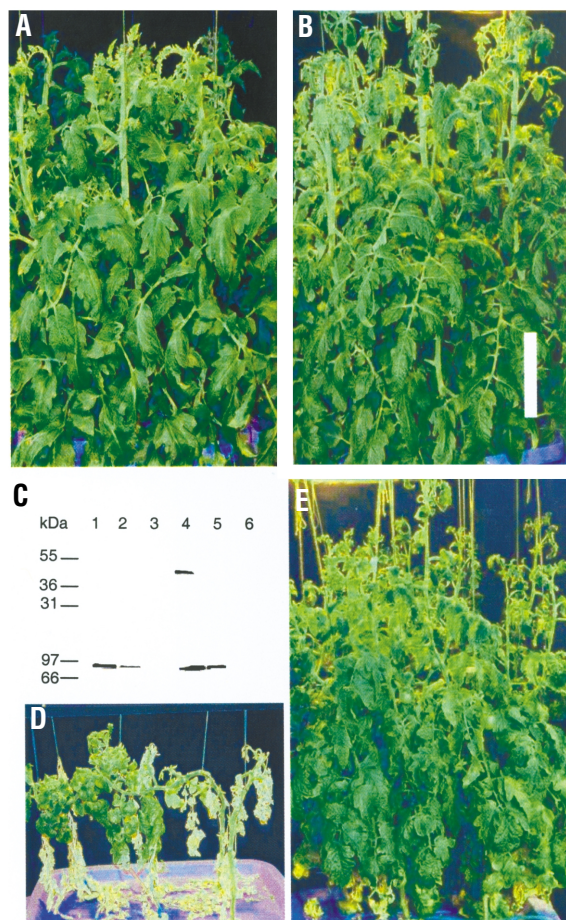
It has been suggested that the overexpression of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiports could serve for the engineering of salt-tolerant crops<sup>4</sup>. To

assess this possibility, we overexpressed *AtNHX1* in tomato plants. Our assumption was that if the plants were genetically modified to have an enhanced ability to sequester sodium in their vacuoles, the transgenic tomato plants would be able to use salty water for cell expansion and growth. The enhanced capacity of the transgenic plants to accumulate Na<sup>+</sup> ions inside the vacuole would avert the toxic effects of Na<sup>+</sup> in the cell cytosol and also would maintain an osmotic balance by using Na<sup>+</sup> ions to drive water into the cells.

A construct containing the *A. thaliana AtNHX1*, coding for a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport, was introduced into the genome of *Lycopersicon esculentum* (cv. Moneymaker). Forty-seven transgenic plants and six homozygous lines from these transgenic plants were obtained in the T<sub>2</sub> generation (data not shown). Two of these homozygous lines were used in our experiments. These two lines were chosen because they grew more vigorously in high salinity. The overexpression of the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport did not affect the growth of the transgenic plants (only one line of transgenic plants is shown) because similar growth was observed when the wild-type and the transgenic plants were grown in the presence of 5 mM NaCl (Fig. 1A, B). Immunoblots of membrane fractions isolated from wild-type and transgenic plants only detected *AtNHX1* in the fractions enriched for tonoplast (vacuolar membrane) from transgenic plants (Fig. 1C), indicating the proper targeting of the Na<sup>+</sup>/H<sup>+</sup> antiport to the vacuoles. To assess whether the enhanced expression of the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport would allow plants to grow in high-salt conditions, we grew wild-type and transgenic plants in the presence of 200 mM NaCl, a concentration that inhibits the growth of almost all crop plants. The growth of the wild-type plants was severely affected by the presence of 200 mM NaCl in the growth solution, plant growth was inhibited, and most of the plants died or were severely stunted (Fig. 1D). On the other hand, the transgenic plants grew, flowered, and produced fruit (Fig. 1E).

To confirm that the presence of the Na<sup>+</sup>/H<sup>+</sup> antiport protein resulted in increased Na<sup>+</sup>/H<sup>+</sup> exchange, we monitored H<sup>+</sup>-dependent Na<sup>+</sup> movements in tonoplast vesicles isolated from leaves. The vesicular lumen was acidified by the activation of the vacuolar H<sup>+</sup>-PP<sub>i</sub>ase in the presence of K<sup>+</sup> ions, because the H<sup>+</sup>-PP<sub>i</sub>ase activity is K<sup>+</sup> dependent<sup>7</sup>. Once the pH gradient was established, the H<sup>+</sup>-pump activity was stopped by the addition of amino-methylene-

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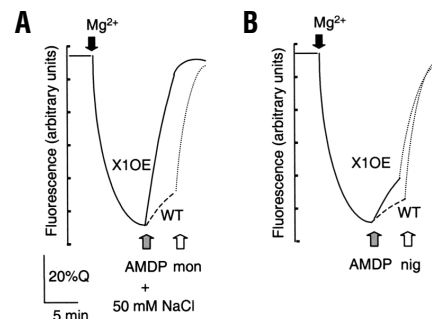


**Figure 1.** Salt tolerance of wild-type tomato plants and transgenic plants overexpressing AtNHX1 grown in the presence of 200 mM NaCl. (A) Wild-type plants grown in the presence of 5 mM NaCl. (B) Transgenic plants overexpressing AtNHX1, grown in the presence of 5 mM NaCl. (C) Western blots from leaf membrane proteins (5 mg) tested with antibodies raised against AtNHX1. (top) Lanes 1 and 4, tonoplast-enriched fraction; lanes 2 and 5, Golgi/endoplasmic reticulum-enriched fractions; lanes 3 and 6, plasma membrane fraction. Lanes 1–3 correspond to membranes from wild-type plants, whereas lanes 4–6 correspond to membranes from transgenic plants. Relative molecular masses are indicated on the left; (bottom) enrichment of the fractions with tonoplast membranes was assessed with antibodies raised against the vacuolar H<sup>+</sup>-PPase. Note there is some cross-reactivity of the antibodies with a PPase in Golgi. (D) Wild-type plants grown in the presence of 200 mM NaCl. (E) Transgenic plants overexpressing AtNHX1, grown in the presence of 200 mM NaCl. Plants shown after 11 weeks of growth. Bar in (B) = 25 cm.

**Table 1. Plant and fruit yield of wild-type (WT) tomato plants grown in the presence of 5 mM NaCl and T2 transgenic plants overexpressing AtNHX1 (OEX1) grown in the presence of 5 mM and 200 mM NaCl**

	WT (5 mM NaCl)	OEX1 (5 mM NaCl)	OEX1 (200 mM NaCl)
Height (cm)	124.0 ± 8.2	128.8 ± 9.5	107.6 ± 5.2
Fresh weight (g)(without fruit)	1,270 ± 103	1,329 ± 110	1,123 ± 134
Fruit per plant	17.2 ± 1.3	17.8 ± 0.6	18.4 ± 1.5
Fruit weight (g)	119.5 ± 13.4	116.7 ± 9.0	105.7 ± 6.7
Fruit water content (%)	90.8 ± 3.2	90.2 ± 2.2	90.7 ± 2.3
Solid solute content (°Brix)	4.2 ± 0.6	4.4 ± 0.7	4.2 ± 0.5

Plants were harvested 12 weeks after germination. Each value is the mean ± standard deviation ( $n = 10$  individual plants). Brix readings (°Brix) represent the concentrations of soluble solids as a percentage of total fresh weight.



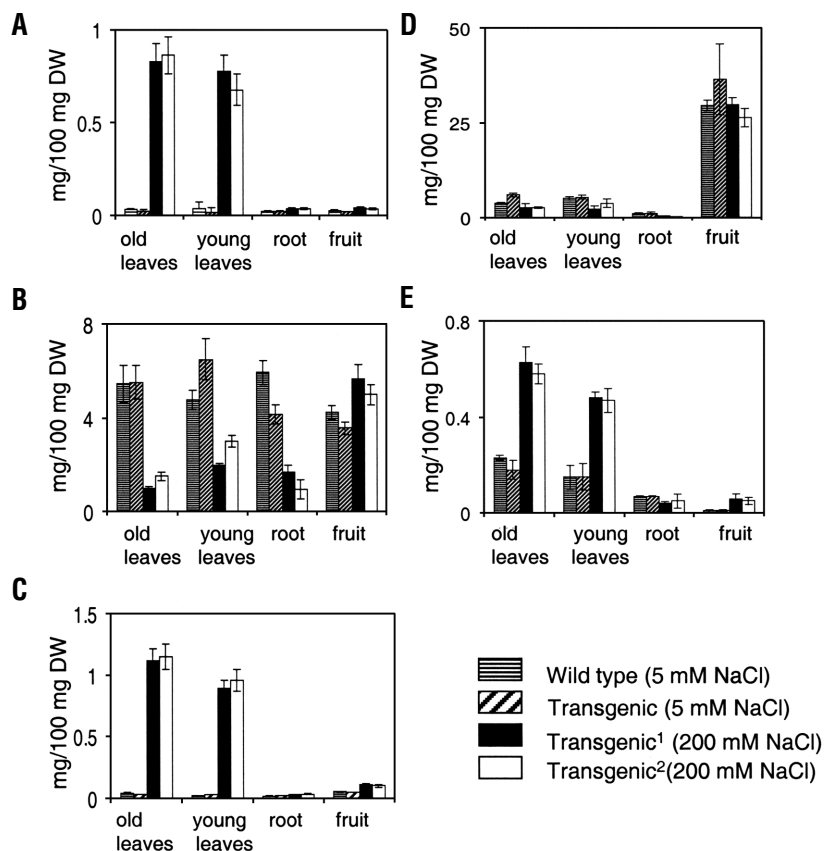
**Figure 2.** Na<sup>+</sup>/H<sup>+</sup> exchange activity in leaf tonoplast vesicles. Membrane fractions were purified from leaves using the method described<sup>5</sup> with the modifications described<sup>4</sup>. At the indicated times, the vacuolar H<sup>+</sup>-PPase was activated by the addition of Mg<sup>2+</sup>. When a steady state pH gradient (acidic inside) was formed, the PP<sub>i</sub>-dependent H<sup>+</sup> transport activity was stopped by the addition of AMDP, and the rates of cation/H<sup>+</sup> exchange were determined in vesicles isolated from wild-type plants (WT) and transgenic plants overexpressing AtNHX1 (X1OE). (A) Na<sup>+</sup>-dependent H<sup>+</sup> exchange; (B) K<sup>+</sup>-dependent H<sup>+</sup> exchange. The addition of monensin (mon), an artificial Na<sup>+</sup>/H<sup>+</sup> antiporter, or nigericin (nig), an artificial K<sup>+</sup>/H<sup>+</sup> antiporter, abolished the pH gradient, and the fluorescence was fully recovered (dotted lines). The solid lines indicate rates of cation/H<sup>+</sup> exchange in transgenic vesicles after AMDP addition, whereas the dashed lines indicate the rate in wild-type vesicles. The figure shows a typical recording.

diphosphonate (AMDP) (ref. 8), NaCl was added, and the rates of Na<sup>+</sup>/H<sup>+</sup> exchange were measured (Fig. 2A). Tonoplast vesicles isolated from transgenic plants displayed Na<sup>+</sup>/H<sup>+</sup> exchange rates sevenfold higher than those from vesicles isolated from wild-type plants.

Interestingly, K<sup>+</sup>/H<sup>+</sup> exchange also was observed in the tonoplast vesicles after the addition of AMDP, in the absence of external Na<sup>+</sup> (Fig. 2B), and the rates of K<sup>+</sup>/H<sup>+</sup> exchange were significantly higher in vesicles isolated from the transgenic plants. These results indicate that the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter also was able to mediate K<sup>+</sup>/H<sup>+</sup> exchange, albeit with a lower specificity for K<sup>+</sup> than for Na<sup>+</sup>. K<sup>+</sup> ions are involved in a variety of physiological processes, and vacuolar pools generate the turgor needed to drive cell expansion<sup>9</sup>. Under K<sup>+</sup> deficient growth conditions, vacuolar K<sup>+</sup> concentrations decline, whereas the cytosolic K<sup>+</sup> concentrations remain relatively constant<sup>10</sup>. Cytosolic K<sup>+</sup> concentrations decline only when the vacuolar K<sup>+</sup> concentrations decrease to values of roughly 20 mM (ref. 11). The decrease in cytosolic K<sup>+</sup> concentrations with the concomitant increase in cytosolic Na<sup>+</sup>/K<sup>+</sup> ratio is the basis of cytosolic Na<sup>+</sup> toxicity<sup>12</sup>. Given the cytosol-negative electrical potential difference at the tonoplast, an active K<sup>+</sup> translocation mechanism into the vacuole has to be considered. Evidence of a K<sup>+</sup>/H<sup>+</sup> antiporter was found in tonoplast-enriched fractions from different plants<sup>6</sup>. Although the *Arabidopsis* sequencing project is completed, only putative K<sup>+</sup>/H<sup>+</sup> antiports with similarity to the

glutathione-regulated potassium efflux system of *Escherichia coli*<sup>13</sup> have been sequenced (GenBank accession numbers AAF78418, AAD10158, CCAB80872). Although their putative function has not yet been characterized in plants, in bacteria and yeast these transporters function as plasma membrane-bound potassium exchangers<sup>13,14</sup>. Although the role of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiports in glycophytes has yet to be established, its ubiquity in plants (E. Blumwald, unpublished data) and its ability to mediate K<sup>+</sup> transport suggest that the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter also could play a role in cellular K<sup>+</sup> homeostasis.

We determined the ion, sugar, and proline contents of wild-type and transgenic plants



**Figure 3.** Ion, sugar, and proline contents of wild-type and transgenic plants grown at various salt concentrations. Wild-type (hatched line bars) and transgenic plants (cross-hatched line bars) were grown in the presence of 5 mM NaCl. Two independent transgenic lines (black and white bars) were grown in the presence of 200 mM NaCl. (A)  $\text{Na}^+$  contents; (B)  $\text{K}^+$  contents; (C)  $\text{Cl}^-$  contents; (D) soluble sugar contents; (E) proline contents. For each determination, leaves, roots, and fruits from 10 plants were collected from each hydroponic tank and pooled. Values are the mean  $\pm$  standard deviation from material collected from three hydroponic tanks ( $n = 3$ ). DW, dry weight.

otic and eukaryotic organisms accumulate proline during osmotic and salt stress<sup>15,16</sup>. Proline contributes to osmotic adjustment<sup>17</sup> and the protection of macromolecules during dehydration<sup>18</sup> and as a hydroxyl radical scavenger<sup>19</sup>. Evidence supporting the role of proline during salt stress was obtained based on salt tolerance in transgenic tobacco plants with enhanced levels of proline biosynthesis<sup>20</sup> and salt tolerance of *Arabidopsis* with suppressed levels of proline degradation<sup>21</sup>.

Taken together, our results demonstrate the ability of the transgenic plants to use salty water for growth. Despite the high  $\text{Na}^+$  and  $\text{Cl}^-$  content in the leaves of the transgenic plants grown at 200 mM NaCl, only a marginal increase in the  $\text{Na}^+$  and  $\text{Cl}^-$  content of the fruit was observed. The  $\text{K}^+$  content of the leaves from transgenic plants grown in salt decreased whereas the  $\text{K}^+$  content of the transgenic fruits was higher than the  $\text{K}^+$  content of the fruit from plants grown at low salinity.

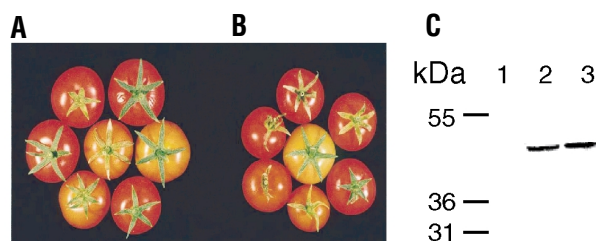
These results clearly demonstrate that the enhanced accumulation of  $\text{Na}^+$ , mediated by the vacuolar  $\text{Na}^+/\text{H}^+$  antiport, allowed the transgenic plants to ameliorate the toxic effects of  $\text{Na}^+$  and that the transgenic plants overcame salt-induced impaired nutrient acquisition<sup>7</sup>. Notably, transgenic plants grown in the presence of 200 mM NaCl produced fruit (Fig. 4A, B and Table 1). Although the transgenic leaves accumulated  $\text{Na}^+$  to almost 1% of their dry weight, the fruit displayed only a marginal increase in  $\text{Na}^+$  content and a 25% increase in  $\text{K}^+$  content. The number of fruit per plant was similar, and although the fruit from the transgenic plants grown in 200 mM NaCl were somewhat smaller, no significant difference was observed in their water content or total soluble solids content (Table 1). The low  $\text{Na}^+$  content of the transgenic fruit cannot be attributed to the lack of vacuolar  $\text{Na}^+/\text{H}^+$  antiport because the protein was present in the fruit tissue (Fig. 4C). It has been demonstrated that in expanding fruit of many plant species, including tomato, greater than 90% of the water transported into the fruit occurs through the phloem<sup>22–24</sup>. Thus, the ability to maintain a high cytosolic  $\text{K}^+/\text{Na}^+$  concentration ratio along the symplastic pathway was most probably responsible for the low  $\text{Na}^+$  content of the fruit.

Worldwide, more than 60 million hectares of irrigated land (representing 25% of the total irrigated acreage in the world) have been damaged by salt<sup>25</sup>. Our findings suggests the feasibility of producing salt-tolerant transgenic plants that will produce edible crops.

### Experimental protocol

**Plant material and transgenic plants.** *Lycopersicon esculentum* (cv. Moneymaker) seeds were germinated on Murashige and Skoog medium (MS). Cotyledon explants were excised from seven-day-old seedlings, cut in half, and cultured overnight on a one-day-old feeder layer consisting of 3 ml of a seven-day-old sugar beet suspension culture plated and overlaid with a sterile Whatman filter paper. The binary Ti vector pBI121 was used for transformation. The *GUS* gene<sup>26</sup> of the binary vector was replaced with the *AtNHX1* gene at the *SalI* and *SstI* restriction sites to gain the new expression construct pHZX1. pHZX1 was electroporated into *Agrobacterium tumefaciens*.

grown at low (5 mM) NaCl and two independent transgenic lines grown at high (200 mM) NaCl (Fig. 3). Note that a comparison with wild-type plants grown at high salinity was not possible because all of the wild-type plants grown in these conditions were dead. At low salinity, no significant differences were seen in the content of  $\text{Na}^+$  (Fig. 3A),  $\text{K}^+$  (Fig. 3B),  $\text{Cl}^-$  (Fig. 3C), soluble sugars (Fig. 3D), or proline (Fig. 3E) in any of the tissues tested. Marked changes were seen in transgenic plants grown at high salinity. A 28- and 20-fold increase in  $\text{Na}^+$  content was seen in fully developed mature (old) and developing (young) leaves, respectively (Fig. 3A), and a similar increase in  $\text{Cl}^-$  content also was observed (Fig. 3C). The  $\text{K}^+$  content of old leaves, young leaves, and roots decreased five-, two-, and fourfold, respectively (Fig. 3B). Although no significant difference in soluble sugars was observed during growth in high salinity (Fig. 3D), a three- and fivefold increase in proline content was seen in leaves and fruits, respectively (Fig. 3E). The accumulation of proline in response to high salinity is well documented. Many prokary-



**Figure 4.** Fruits from wild-type and transgenic plants. (A) Tomato fruits from wild-type plants; (B) tomato fruits from transgenic plants. (C) Western blots from fruit tonoplast proteins (5 mg) tested with antibodies raised against AtNHX1. Wild-type plants grown in the presence of 5 mM NaCl (lane 1). Two independent transgenic lines grown in the presence of 200 mM NaCl (lanes 2 and 3).





*ciens* strain LBA4404. For cocultivation, 1 ml of pHZX1 containing *Agrobacterium* was inoculated into 15 ml Luria–Bertani medium containing 50 mg/l kanamycin, 50 mg/l rifampicin, and 200 mM acetone-syringone. After two days of cocultivation with *Agrobacterium*, the explants were transferred to selective regeneration medium<sup>27</sup>. Regenerated shoots were transferred to fresh medium biweekly. When the green shoots were 1–2 cm tall, they were separated from the calli and transferred onto rooting medium containing modified MS salts<sup>27</sup>. Approximately 98% shoots can form roots in two weeks. Rooted shoots were transplanted to soil and plants regenerated. T1 seeds were grown on plates containing MS medium and 100 mg/l kanamycin, and homozygous seeds were selected.

For salt tolerance experiments, wild-type and two independent lines (T2) of transgenic plants were grown hydroponically. Seeds were germinated in agar plates containing MS medium under continuous light at 25°C. Two weeks after germination, 60 of each wild-type and transgenic seedlings were transferred to six hydroponic tanks, containing 20 seedlings in each tank and grown in the greenhouse. Day temperature was maintained at 26°C ± 2°C and night temperature was 22°C ± 2°C. Relative humidity was maintained at 50% ± 10%. Plants were grown under a 14 h light/10 h dark photoperiod. Supplemental lighting consisted of eight high-pressure sodium lamps and resulted in a total (sunlight and supplemental light) of ~1,250 mmol/m<sup>2</sup>s. The nutrient solution was obtained by mixing 1.2 g per liter of stock fertilizer (tomato fertilizer; Plant-Prod, Brampton, Ontario, Canada) and 1 g per liter of CaNO<sub>3</sub>. The final nutrient solution contained (in milligrams per liter) 200 N, 54 P, 256 K, 147 Ca, 42 Mg micronutrients and was supplemented with 5 mM or 200 mM NaCl. The nutrient solution was replaced every six days, and the roots were kept under constant aeration.

**Membrane isolation and western blots.** Membrane fractions were isolated from shoots of four-week-old plants or tomato fruits from mature plants as described<sup>5</sup>. Western blots of the different membrane fractions were performed as described<sup>4</sup>.

**Transport assays.** The cation/H<sup>+</sup> exchange activity was measured by following the pH-dependent fluorescence quenching of acridine orange<sup>5</sup>. An acidic-inside pH gradient across the tonoplast vesicles was obtained by activation of

the vacuolar H<sup>+</sup>-PPase. Twenty micrograms of tonoplast vesicles was added to 0.8 ml buffer containing 0.25 M mannitol, 5 mM Tris/MES (pH 8.0), 2 mM dithiothreitol, 25 mM KCl, 0.8 mM Tris-PPi, and 5 mM acridine orange. Proton translocation was initiated by the addition of 1 mM Mg<sup>2+</sup>, and the change in fluorescence was monitored as described<sup>5</sup>. When a steady state pH gradient (acidic inside) was formed, PPi-dependent H<sup>+</sup>-transport activity was stopped by the addition of AMDP, and the changes in rate of fluorescence recovery were determined in the presence and absence of 50 mM NaCl.

**Leaf and fruit chemical analysis.** We performed chemical analysis from three-month-old plants. Fully expanded mature leaves from the six most lower basal nodes (old leaves), developing leaves from the six most upper apical nodes (young leaves), roots, and fruits were collected and dried at 70°C for 24 h, and the material was ground to a fine powder. Tomatoes were collected at the mature green/red ripe stage and were allowed one week of further maturation at the bench at room temperature (22°C) before analysis. For the determination of soluble sugars, each sample (100 mg) was resuspended in 2 ml of water, sonicated, and centrifuged for 10 min at 2,500 g. Soluble sugar and proline contents were determined in the supernatant as described<sup>28,29</sup>. Ion contents were determined by atomic absorption spectrophotometry and chloride content by titration. Water content was calculated as (FW – DW)/FW, where FW and DW are the fresh and dry weight, respectively. Dry weight was obtained by drying the material at 70°C until a constant weight was obtained. For the determination of soluble solid contents, the tomatoes were strained through a 20 mm mesh, and Brix readings of the juice were obtained by refractometry. Brix readings (°Brix) represent the concentrations of soluble solids as a percentage of total fresh weight.

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