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Review

Why do plants lack sodium pumps and would they benefit from having one?

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Abstract. The purpose of this minireview is to discuss the feasibility of creating a new generation of salt-tolerant plants that express Na^+/K^+-ATPases from animals or green algae. Attempts to generate salt-tolerant plants have focussed on increase the expression of or introducing salt stress-related genes from plants, bryophytes and yeast. Even though these approaches have resulted in plants with increased salt tolerance, plant growth is decreased under salt stress and often also under normal growth conditions. New strategies to increase salt tolerance are therefore needed. Theoretically, plants transformed with an animal-type Na^+/K^+-ATPase should not only display a high degree of salt tolerance but should also reduce the stress response exhibited by the first generation of salt-tolerant plants under both normal and salt stress conditions. The biological feasibility of such a strategy of producing transgenic plants that display improved growth on saline soil but are indistinguishable from wild-type plants under normal growth conditions, is discussed.

Additional keywords: algal Na^+/K^+-ATPase, Na^+/K^+-ATPase, salt tolerance, vascular plants.

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Introduction

Plant growth is severely restricted by salts (primarily Na^+). Increased soil salinity represents a growing problem worldwide and, for example, can result from extensive irrigation with water containing salts, especially in arid regions (Food and Agriculture Organization 2000; Pitman and Läuchli 2002; Pimentel *et al*. 2004; Rengasamy 2006; Zhu 2007; Hasegawa 2013). Soils are considered saline when Na^+ concentrations are higher than 40 mM and, like in animal cells, normal cytoplasmic levels of Na^+ in plants are 10–30 mM, whereas toxic levels are less defined (Munns and Tester 2008). Salt induces osmotic stress by limiting water uptake from the soil, whereas the accumulation of toxic Na^+ ions in plant cells causes ionic stress, which impairs important biochemical processes (Serrano 1996; Hasegawa *et al*. 2000).

As salt tolerance in vascular plants is a polygenic trait (Flowers 2004), genetically engineering plants that are tolerant to this abiotic stress is not trivial. One of the strategies used to date is manipulating the expression of genes encoding transport proteins (Summarised in Fig. 1). Thus one strategy used to engineer salt-tolerant plants by keeping cytosolic Na^+ concentrations below toxic levels involves genetic manipulation of native or heterologous plasma membrane ion transporters to increase the plant cell's capacity for Na^+ extrusion and sequestration (Shi *et al*. 2002*a*; Nakayama *et al*. 2004), or exclusion from the shoots (Moller *et al*. 2009; Munns *et al*. 2012). These studies have resulted in transgenic plants with an improved ability to grow under saline conditions. However, attempts to translate this knowledge obtained in model plants to generate salt-resistant high-yielding crops have had limited success (Roy *et al*. 2014).

In contrast to animal growth, plant growth is slowed by the export of Na^+

To energise transport across the plasma membrane, plant cells rely on an electrochemical H^+ gradient that is generated by the plasma membrane H^+-ATPase (Fig. 2). Plant cells require a high intracellular K^+ concentration. To harvest this ion from the soil, root cells use several K^+ transporters that are driven by the plasma membrane's electrochemical gradient. Unfortunately, K^+ transporters and other transporters can give rise to Na^+ leaks, and therefore a constant influx of Na^+ is unavoidable (Rodríguez-Navarro and Rubio 2005; Maathuis *et al*. 2014; Volkov 2015; Nieves-Cordones *et al*. 2016). For plants to survive under saline conditions, they must maintain cytosolic Na^+ concentrations at levels that are nontoxic to the cells and that limit extensive water loss. Plant cells therefore transport Na^+ out of the cytoplasm to the external medium or sequester it in the vacuole.

The Na^+/H^+-antiporters located in the plasma membrane are the primary Na^+ efflux systems in plant cells (Zhu 2003; Deinlein *et al*. 2014). Plasma membrane-localised Na^+/H^+-antiporters are the only molecular system known to mediate the cellular export of Na^+ in vascular plants. Two genes encoding plasma membrane Na^+/H^+-antiporters have been identified in *Arabidopsis thaliana* (L.) Heynh., namely *AtSOS1* (Shi *et al*. 2000) and *AtNHX8*

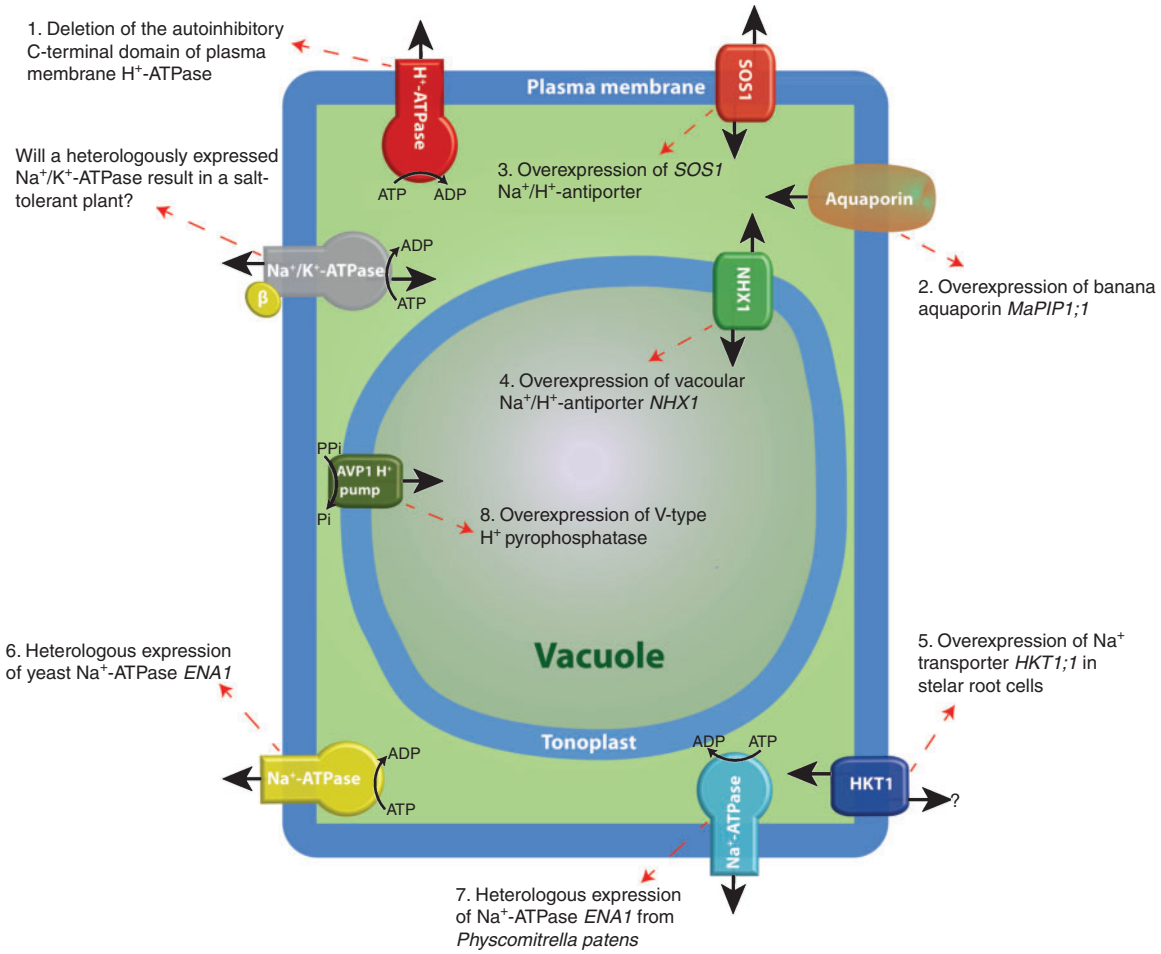

Fig. 1. Strategies to increase salt tolerance in plants. A selection of previous strategies that have increased salt tolerance in plants as well as a novel strategy. Overexpression of (1) the plasma membrane H^+-ATPases (Gevaudant *et al*. 2007) and (2) aquaporins (Xu *et al*. 2014) as well as (3–5) overexpression of endogenous (An *et al*. 2007; Moller *et al*. 2009; Shi *et al*. 2000) and (6, 7) heterologous (Jacobs *et al*. 2011; Nakayama *et al*. 2004) Na^+-transporters and (8) the V-type H^+-pyrophosphatase (Gaxiola *et al*. 2001) result in increased salt tolerance. The black arrows illustrate the direction of transport.

(An *et al*. 2007), but only one, *OsSOS1*, has been identified in rice (*Oryza sativa* L.) (Martínez-Atienza *et al*. 2007). A mutation in *AtSOS1* renders *A. thaliana* hypersensitive to salt (Shi *et al*. 2000); likewise, a reduction of *SOS1* expression in the related extremophile *Thellungiella salsuginea* (Pall.) O.E.Schulz, which thrives in sea water, results in a plant that is as sensitive to salt as *A. thaliana* (Oh *et al*. 2009). Conversely, overexpression of *SOS1*-like genes in *A. thaliana* (Shi *et al*. 2002*a*; Yang *et al*. 2009; Feki *et al*. 2014) and other plant species (Yue *et al*. 2012; Yadav *et al*. 2012; An *et al*. 2014) confers increased tolerance to elevated levels of Na^+ in the medium. Taken together, the available evidence suggests that *SOS1* genes strongly influence a plant's ability to tolerate Na^+.

In an antiport system such as SOS1, Na^+ extrusion is hypothesised to be driven by H^+ influx through the same protein (Shi *et al*. 2000). In accordance with this notion, Na^+ fluxes are tightly correlated with H^+ fluxes across the plasma membrane in salt-tolerant plant species (Cuin *et al*. 2011; Bose *et al*. 2015). In salt-stressed plants, plasma membrane H^+-ATPase expression is increased (Braun *et al*. 1986; Niu *et al*. 1993; Perez-Prat *et al*. 1994) and overexpression of plasma membrane H^+-ATPase confers salt tolerance (Gevaudant *et al*. 2007). Thus the H^+ gradient across the plasma membrane appears to be essential for Na^+ export.

Although a H^+-driven antiporter may be an effective mechanism for extruding Na^+ in plants, it has, from an agricultural point of view, a major disadvantage: extrusion of Na^+ consumes the H^+ gradient used to drive the uptake of nutrients and, in turn, water, which is a prerequisite for growth. As a consequence, exposure to high concentrations of Na^+ would slow plant growth and decrease yields. In accordance with this hypothesis, plants with increased *SOS1* expression have increased tolerance to Na^+, but salt still slows growth (Shi *et al*. 2002*a*; Yang *et al*. 2009; Wang *et al*. 2011; Yue *et al*. 2012; Yadav *et al*. 2012; An *et al*. 2014; Feki *et al*. 2014). In other words, salt tolerance comes at a cost.

In animal cells, the handling of Na^+ is very different (Fig. 2). Here, the extrusion of Na^+ generates the electrochemical gradient that drives growth. Animals tolerate saline conditions because of their ability to actively extrude Na^+ via the Na^+/K^+-ATPase,

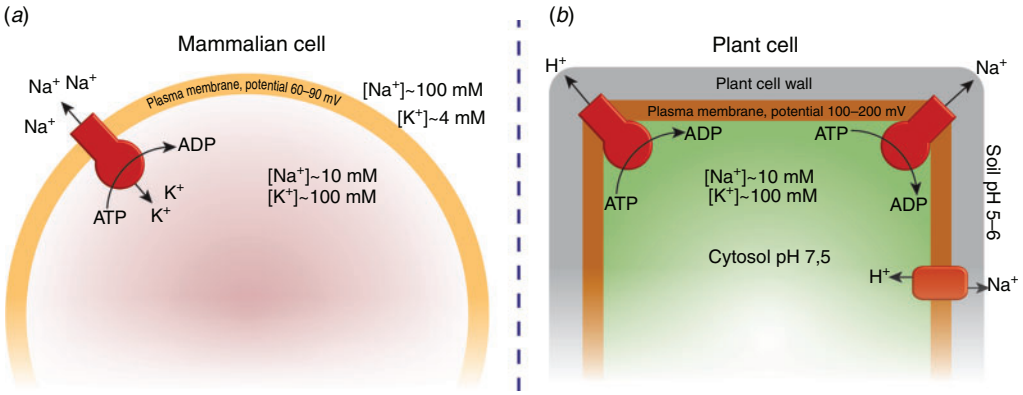

Fig. 2. The plasma membrane of all eukaryotic cells is energised by a P-type ATPase, which exports cations and separates charge across the membrane. (*a*) In animal cells, the plasma membrane is energised by the Na^+/K^+-ATPase, which exports three Na^+ ions and imports two K^+ ions for every ATP hydrolysed. (*b*) In fungi and plants, the plasma membrane is energised by a plasma membrane H^+-ATPase, which exports one H^+ for every ATP hydrolysed. The plasma membrane H^+-ATPase generates membrane potential of up to 200 mV in plant cells. To export Na^+ ions, vascular plant cells rely on a Na^+/H^+ antiporter that is energised by the electrochemical gradient, whereas the early land plants (bryophytes) have a Na^+-ATPase in addition to the Na^+/H^+ antiporter.

a P-type pump evolutionarily related to the plasma membrane H^+-ATPase. This system is extremely effective; at the expense of one ATP, this enzyme exports three Na^+ ions and imports two K^+ ions (Fig. 2). In comparison, the Na^+/H^+-antiporter indirectly consumes one ATP per Na^+ exported, making it export Na^+ at a higher cost. Furthermore, as a result of the activity of Na^+/K^+-ATPase, chemical Na^+ and K^+ gradients develop, as does an electrical charge difference across the plasma membrane. The combined electrochemical gradient represents stored potential energy that can be harvested by most other transport processes. Consequently, Na^+ extrusion is not only beneficial but is essential for animal growth.

Sodium pumps in primitive plants

In mosses, genes resembling the P-type *exitus natru* (ENA) Na^+-ATPases described in fungi have been identified (Pardo *et al*. 2006). Moss ENA Na^+-ATPases appear to act in concert with the SOS1-like Na^+/H^+ antiporters, whereas in fungi, ENA Na^+-ATPases are the major Na^+ exporters, even though Na^+/H^+ antiporters are present as well (Prista *et al*. 1997).

Some green algae harbour genes that encode proteins that appear to be related to animal Na^+/K^+-ATPases (Pedersen *et al*. 2012), such as *A8HX15* in the green alga *Chlamydomonas reinhardtii*. However, similar genes have not been identified in fungi and mosses. Even though we still do not know whether Na^+/K^+-ATPase-like pumps in green algae actually transport Na^+, it appears that both Na^+- and Na^+/K^+- transporting ATPases were present in the ancestors of vascular plants.

The positive association between *ENA1* expression and salt tolerance in the moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. raises the possibility that the heterologous expression of *ENA1* would improve the salt tolerance of crop plants. Accordingly, *ENA1* from *P. patens* has been heterologously expressed in rice using the strong constitutive 35S-promoter (Jacobs *et al*. 2011). Although the expression of ENA imparted tolerance to salinity stress, the transgenic plants were, in some cases, less fit than control plants under normal conditions (Jacobs *et al*. 2011). Furthermore, ENA expression could not be clearly linked with the cellular content of Na^+ and K^+ ions, possibly because ENA pumps have a limited ability to discriminate between these two ions (Nakayama *et al*. 2004; Kong *et al*. 2008; Jacobs *et al*. 2011). In concordance, heterologous expression of the yeast homologue of *ENA1* in tobacco (*Nicotiana tabacum* L.) cells showed export of K^+ ions as well, which is clearly undesirable if the purpose is to improve growth (Nakayama *et al*. 2004).

Under nonstressful conditions, wild-type *P. patens* appears to grow slightly more slowly than an *ena1* mutant devoid of Na^+ ATPases (Lunde *et al*. 2007). Hence, in the absence of salt stress, there might be a small growth penalty associated with the presence of ENA1. This would suggest that ENA1 is not a housekeeping enzyme but rather has a specialised function during conditions of salt stress and that loss of the gene seems favourable when salt is absent.

Why do higher plants lack Na^+ pumps?

A notable difference between primitive plants (i.e. green algae and mosses) and flowering plants is the complete absence in the latter of genes that encode Na^+/K^+-ATPases, Na^+-ATPases or both. Considering that Na^+ pumps give green algae and mosses a selective advantage under salt stress, it is intriguing that these ATPases became lost during the course of evolution of vascular plants.

A possible explanation for why higher plants lack Na^+ pumps could relate to the environment when the plants colonised the land. A freshwater origin is likely for the first land plants that colonised early land around 500 million years ago (Kenrick and Crane 1997). The absence of Na^+ made it became impossible to generate a steep electrochemical gradient using Na^+, and another ion had to be used to energise the plasma membrane. Instead, primitive plants selected the omnipresent proton as the energising cation and Na^+ pumps were subsequently lost. A plasma membrane H^+-ATPase was already present in the

ancestors of land plants (Falhof *et al*. 2016), possibly serving a role in regulating cytoplasmic pH.

A reason for the loss of ENA-type Na^+-ATPases could be that their expression is a burden under nonsaline conditions, as described above. Structural studies of the mammalian Na^+/K^+-ATPase suggest that the high selectivity for Na^+ over K^+ at the transport sites exposed to the cytoplasm is obtained by the presence of three Na^+-binding sites in a row (Kanai *et al*. 2013). Binding of the first Na^+ ion facilitates binding of the second and subsequently the third Na^+ ion, and specificity is increased in each step. In contrast, homology modelling of ENA pumps suggests that these Na^+-ATPases bind Na^+ in a single ion-binding pocket situated between transmembrane Helices 4 and 6 (Drew *et al*. 2011). The ability to bind a single cation only may explain why ENA pumps are less specific and ineffective at discriminating efficiently between Na^+ and K^+. This intrinsic problem makes it difficult to envision a strategy to increase the Na^+ specificity of ENA pumps.

The Na^+/K^+-ATPases do not have the specificity problem of Na^+-ATPases, so why were they lost? A possible option relates to the biophysical properties of H^+- and Na^+/K^+-ATPases. In flowering plants, H^+ pumps create steep electrochemical gradients, composed of two parts: an H^+ gradient of more than 100-fold (two pH units) between the cytoplasm and the exterior, and a membrane potential that can exceed 200 mV (negative on the inside; Hirsch *et al*. 1998). The resulting proton motive force allows plant roots to efficiently mine the soil for mineral nutrients (Palmgren 1998). By comparison, in animal cells, Na^+/K^+ pumps typically produce membrane potentials of only around 60 mV, and work against 10-fold Na^+ concentration gradients. Thus H^+ pumps appear to be more efficient than Na^+ pumps at creating very large electrochemical gradients to support nutrient uptake.

The activity of the Na^+/K^+-ATPase is voltage-dependent (De Weer *et al*. 1988). For example, the squid (*Loligo pealeii*) Na^+/K^+-ATPase which operates in a marine environment, has optimal active activity at zero voltage and negligible activity as the membrane potential approaches –200 mV (Colina *et al*. 2007). Theoretically, under the ionic conditions of animal cells, membrane potentials exceeding around –230 mV would cause the Na^+/K^+-ATPase pump to operate in the reverse direction, turning it into a Na^+ uptake system (Glitsch 2001). Reversal of the Na^+/K^+-ATPase can also be induced by varying the concentrations of Na^+ and K^+ (Garrahan and Glynn 1967; Glitsch 2001). Furthermore, in the presence of a steep H^+ gradient across the plasma membrane, inwardly directed H^+ fluxes can occur through animal Na^+/K^+-ATPases even under native conditions (Mitchell *et al*. 2014; Vedovato and Gadsby 2014). It is thus possible that large H^+-based electrochemical gradients may have caused the ancestral Na^+ pump to malfunction and result in inwardly directed leaks of H^+, Na^+ or both. As a result, during evolution, the survival of vascular plants might have depended on the loss of Na^+ pumps to support the functioning of the more effective H^+ pumps.

Are H^+ and Na^+ pumps able to operate together in the same membrane?

A major question therefore remains: is it possible at all for Na^+/K^+-ATPases and advanced plasma membrane H^+-ATPases to coexist in the same cell type?

Putative Na^+ and H^+ ATPases are present in green algae, most of which are marine organisms, but we do not know whether both types of pumps contribute to energising the plasma membrane. Neither do we know whether secondary active transport systems in marine green algae accept both Na^+ and H^+ as a coupling ion. Based on genomic and physiological evidence, respectively, it has been suggested that the chlorophyte alga *C. reinhardtii* and the streptophyte alga *Cara corallina*, both living in freshwater, have distinct H^+- and Na^+-coupled secondary transport systems (Taylor *et al*. 2012). Unfortunately, no molecular evidence is available to support the notion that Na^+ and H^+ could both function as the coupling ion in the same cell system.

As hypothesised above, classical Na^+/K^+ pumps may be limited in their function by the electrochemical H^+ gradient generated by plant plasma membrane H^+-ATPase. However, if the Na^+/K^+-ATPase is not operational under normal conditions in a plant cell, it would at least not pose a threat. When Na^+ flows into the plant cell under conditions of elevated salt, the membrane becomes depolarised (Bose *et al*. 2015) and, as the combined result of reduced membrane potential and elevated Na^+, the Na^+/K^+-ATPase would be activated. Furthermore, under conditions of salinity, the potential threat of H^+ leakage through the Na^+/K^+-ATPase might be absent, as inflow of H^+ through this pump is inhibited at extracellular Na^+ concentrations higher than 10 mM (Mitchell *et al*. 2014; Vedovato and Gadsby 2014). The combined effect of Na^+ depolarisation of the membrane and high-salt inhibition of inwardly directed H^+ leaks may provide a mechanism for the co-occurrence of plasma membrane H^+-ATPases and Na^+/K^+-ATPases.

A way to experimentally test whether land plants would benefit from a Na^+/K^+-ATPase could be to introduce one such enzyme from a heterologous source and test its impact on salt tolerance. The expression of an animal Na^+/K^+-ATPase in plants is challenged by the presence of a highly glycosylated β-subunit in these pumps (Vagin *et al*. 2006). The β-subunit has to be coexpressed with the catalytic subunit but may not be glycosylated correctly in a heterologous expression system such as plants. This problem may be avoided by expressing putative Na^+/K^+-ATPases from green algae because of their closer relationship with plants. Although a β-subunit resembling that of the animal pump has not been identified in any green algal Na^+/K^+-ATPases, a more distantly related subunit may be present and required for pump function (Fig. 3). All predicted algal counterparts of Na^+/K^+-ATPase contain a conserved insert between transmembrane Segments 7 and 8 that is lacking in the animal pumps and is predicted to be exposed to the extracellular side of the membrane. With a length of ≈50 amino acid residues, this insert roughly corresponds in size to the extracellular part of the β-subunit of the animal pumps (Fig. 3). It is tempting to speculate that this structure in algal pumps has an equivalent role as the β-subunit of the animal Na^+/K^+-ATPases but so far, this hypothesis lacks experimental support.

To test if animal or algal Na^+/K^+-ATPases and plant plasma membrane H^+-ATPases can in fact coexist, they would need to be coexpressed in the same cell type. After developing this type of coexpression system, it would be possible to monitor the transport activities of both enzymes simultaneously and to study their combined impact on ion fluxes and cell viability. Heterologously expressing an algal Na^+/K^+-ATPase in plants

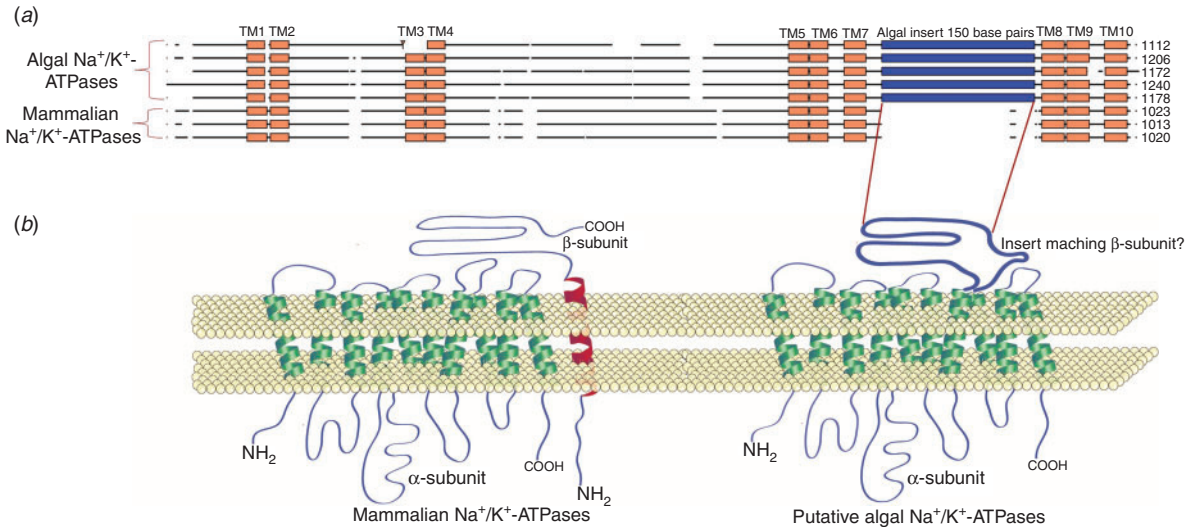

Fig. 3. Comparison of algal and mammalian Na^+/K^+-ATPases. (*a*) An alignment of five putative Na^+/K^+-ATPases from algae and three mammalian α-subunits from Na^+/K^+-ATPases. The transmembrane segments are marked. In all five algal genes, an insert (blue bar) of ~150 amino acids was observed between transmembrane (TM) Helix 7 and 8, which might constitute an inbuilt β-subunit. From top, *Chlamydomonas reinhardtii* (*XP_001696293.1*) with a 'β-subunit'-like insert comprising Residues 820–990; *Volvox carteri* f. *nagariensis* (*XP_001415408.1*), insert at Residues 914–1085; *Osterococcus tauri* (*XP_003074149.1*), insert at Residues 892–1048; *Chlorella variabilis* (*XP_005847906.1*), insert at Residues 934–1120; *Flabellia petiolata* (CAI99406.1), insert at Residues 899–1058 and *Homo sapiens* (*ATA1*, *ATA2*, *ATA3*). The predicted coding sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program. (*b*) A schematic representation of a mammalian Na^+/K^+-ATPase with the β-subunit and an algal Na^+/K^+-ATPase.

would provide insight into how to secure the co-existence of Na^+/K^+-ATPases and H^+-ATPases in plant cells and reveal whether the β-subunit is needed to generate functional algal Na^+/K^+-ATPases. As a starting point for such studies, one might express a Na^+/K^+-ATPase in specific cell types, exposed to salt and already harbouring a plasma membrane-localised H^+-ATPase. The *SOS1* promotor is active in roots and induced by salt (Shi *et al*. 2002*b*; Goyal *et al*. 2013). Usage of this or related promoters may ensure that the Na^+/K^+-ATPase protein is expressed only where its action is needed and thereby minimise the metabolic stress endured by plants when such pumps are present under normal conditions.

An alternative strategy for engineering salt-tolerant plants based on improving the K^+ : Na^+ ratio

Endeavours to improve salt tolerance in plants have hitherto focussed on either removing Na^+ from the plant cytosol or on increasing the intracellular K^+ content through overexpressing either Na^+ or K^+ transporters. No studies have, to our knowledge, attempted to improve the K^+ : Na^+ ratio in plant cells by simultaneously removing Na^+ ions and increasing the level of K^+. However, manipulating the levels of both alkali cations in plant cells simultaneously would improve the salt tolerance profile. Potassium is an essential macronutrient required for numerous cellular processes and it is the most abundant inorganic cation in plant cells. Various reports indicate that increased cytosolic K^+ levels relative to Na^+ (i.e. an increased intracellular K^+ : Na^+ ratio) are correlated with salt tolerance in plants (reviewed in Hasegawa *et al*. (2000)).

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