Heterologously expressed protein phosphatase calcineurin downregulates plant plasma membrane H+-ATPase activity at the post-translational level

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Abstract To investigate the effects of calcineurin expression on cellular ion homeostasis in plants, we have obtained a transgenic cell culture of tomato, expressing constitutively activated yeast calcineurin. Transgenic cells exhibited reduced growth rates and proton extrusion activity in vivo. We show that reduction of plasma membrane H+-ATPase activity by expression of calcineurin is the basis for the observed phenotypes. Transgenic calli and cell suspensions displayed also increased salt tolerance and contained slightly higher Ca^{2+} and K^+ levels. This demonstrates that calcineurin can modulate ion homeostasis in plants as it does in yeast by affecting the activity of primary ion transporters.
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Keywords: Calcineurin; H+-ATPase; Cell growth; Ion homeostasis; Tomato

1. Introduction

Calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase (PP2B) that consists of a catalytic subunit (CNA) and a tightly associated Ca^{2+} binding regulatory subunit (CNB), plays an important role in coupling Ca^{2+} signaling to cellular responses in both animals and fungi [1]. In mammalian cells, many of these effects are regulated at the transcriptional level through dephosphorylation dependent translocation to the nucleus of NFAT transcription factors. In yeast, calcineurin regulates gene expression through the CRZ1/TCN1/HAL8 transcription factor in a similar manner [2–4], and plays a central role in the maintenance of ion homeostasis in environmental stressful conditions by regulating expression of several P-type ATPases [5,6].

Apart from these transcriptional regulations, calcineurin regulates the activity of several ion transporters in yeast, like the potassium uptake systems TRK1 and TRK2, and the Ca^{2+}/H^+ antiporter VCX1 [6,7]. It was shown that the yeast plasma membrane P-type H+-ATPase PMA1 is an important target for post-translational regulation by calcineurin [8,9]. The *PMA1* gene is an essential gene whose product is required

for cytosolic pH homeostasis and maintenance of the electrochemical potential at the plasma membrane. The activity of this enzyme must be finely regulated to match the requirements for nutrient uptake, osmotic balance, ion homeostasis and stress tolerance [10].

Despite intensive efforts, no protein phosphatases similar to the catalytic subunit of calcineurin were found in the sequenced plant genomes. The functional homologs of calcineurin in plants have been suggested to be provided by the CBL/CIPK complexes, consisting of a family of calcineurin B-like proteins (CBLs or SCaBP) interacting reversibly with serine/threonine kinases (CIPKs or PKS) [11]. Nevertheless, patch clamp experiments have shown that calcineurin can regulate the activity of plant K^+ and Ca^{2+} channels [12,13]. This suggests that, although no calcineurin signaling pathway is present in plants, calcineurin can be used as a tool to regulate ion homeostasis in plant cells. In that sense, it was shown recently that the heterologous expression of yeast calcineurin improves salt tolerance in tobacco plants [14].

To study the possible effect of calcineurin on ion homeostasis in plants, we have created a transgenic cell culture expressing a constitutively activated form of yeast calcineurin. We show that yeast calcineurin affects growth rate, salt tolerance and ion homeostasis when expressed in tomato cells. These changes were related to a reduction in the activity of the plasma membrane proton ATPase and could be reversed by addition of fusicoccin to the growth medium. However, no significant changes in gene expression were found. These results demonstrate that calcineurin action in plants is produced at the post-translational level and indicate a negative role for phosphatase activity in regulation of the H+-ATPase.

2. Materials and methods

2.1. Plant transformation

For tomato plant transformation the PBTCan plasmid [14], harboring a gene encoding a truncated calcineurin catalytic subunit (CNAtr) and a gene encoding the regulatory subunit (CNB), was used (Gift from Drs. J.M. Pardo and T. Ruiz). In vitro plantlets of tomato were grown as described [15] and used for transformations, essentially as described [16]. Cotyledons of 7 days old plants were separated and incubated for 24 h on plates in shoot regeneration medium containing MS basal salts [17], Gamborg's B5 vitamins [18], 3% sucrose, 0.7% agar and 2 mg/L bencladenine. The cotyledons were incubated for 20

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min with transformed *Agrobacterium tumefaciens* strain LBA4404 (pAL4404), dried on filter paper and grown for 48 h on shoot regeneration medium. Thereafter, the cotyledons were transferred to selective shoot regeneration medium, containing 50 mg/L kanamycin and 500 mg/L carbenicilline. After 4–6 weeks at 27 °C, 16 h of light (20 mmol/m^2 s) and 8 h dark period, transgenic shoots developed on the selective culture medium.

2.2. Induction of callus culture: determination of callus growth and ion content

Calli were initiated from sections of young shoots according to Rodríguez-Rosales et al. [15] in a medium supplemented with 25 mg/L kanamycin to select for transgenic cells. After 24 days, the calli were transferred to multiplication medium [15] and were subcultured in this medium every three weeks. For NaCl treatments, calli were transferred to fresh multiplication medium supplemented with 100 mm NaCl. Callus growth was quantified in terms of relative growth rate measured by the $(mf - mi)/mi$ ratio, where mf is the weight of the calli during growth and mi is the initial weight of the calli at the start of the growth curve. To determine the ion content, calli were dried by incubation at 80 °C for 72 h. Na^+, K^+ and Ca^{2+} content was determined by flame photometry [19]. Cl^- was quantified by potentiometric measurement with silver nitrate.

2.3. Preparation of cell suspension cultures

Cell suspension cultures were prepared from control and transgenic tomato calli as described previously [15,20]. The cultures were renewed every 11 days by transferring 10 g of cells into 100 ml fresh medium of the same composition as used for calli multiplication but without agar and kanamycin (standard culture medium). Cells were incubated in the dark at 27 °C and under continuous shaking at 120 rpm. After two months of culture, these cell lines were identified as control and transgenic cell suspensions and used for experiments. For some experiments, fusicoccin was added to the suspension cultures at a concentration of 0.5 μM after three days of culture (corresponding to the beginning of the exponential growth phase). Cell growth was expressed in terms of fresh weight in 11-day-old control and transgenic cell suspensions after filtering through a Whatman No. 4 filter paper. NaCl treatments consisted of the incubation of cell suspensions in the standard culture medium supplemented with 100 mM NaCl for one subculture.

2.4. Preparation of cDNA by RT-PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Residual DNA was eliminated by treating the samples with DNase (Qiagen) on the column prior to elution. Two μg of RNA was transcribed into first strand cDNA using the Enhanced Avian RT-PCR Kit (Sigma) with random nonamers as primers according to the manufacturer's instructions. In subsequent PCRs, control samples were included that were treated identically but without reverse transcriptase to exclude contamination with genomic DNA.

2.5. Detection of the calcineurin subunits in transformed calli by RT-PCR

To assay expression of CNA and CNB, cDNA was prepared from total RNA as described above and the genes were amplified by PCR using gene specific primers (CNAtr: 5′-AAT GGG TGC TGC TCC TTC CAA AAT-3′, 5′-TTA CAC ATC GTA TTG CAA TGT CAG-3′ and CNB 5′-AAT ACT GAG CAG ATA AAC GC-3′, 5′-ATT GCG ACA AGC ATC TCT GT-3′).

2.6. Real-time PCR

Real-time PCR was performed using platinum *Taq* DNA polymerase (Gibco) and SYBR-Green as fluorescent reporter in a Biorad iCycler. Gene specific primers were designed for the H^+-ATPase isoform LHA4 (forward 5′-AAAGCAGAGAGAGAGAGACG-3′, reverse 5′-AGCACCACCCATTGAAAGGG-3′). The identity of the amplified fragment was confirmed by gel electrophoresis and sequencing. Serial dilutions of cDNA were used to make a standard curve to optimize amplification efficiency. All reactions were performed in triplicate. Melt curves of the reaction products were generated and fluorescence data were collected at a temperature above the melting temperature of non-specific products. Relative expression data were calculated from the difference in threshold cycle (ΔC_t) between the studied gene (LHA4) and DNA amplified by primers specific for ribosomal DNA (5′-AAAAGGTCGACGCGGGGCT-3′ and 5′-CGACAGAAGGGACGAGAC-3′). The expression level was calculated from 2EXP[ΔC_t (control) − ΔC_t (sample)].

2.7. Determination of in vivo proton extrusion

For the measurement of H^+ extrusion, a Methromh pH stat system (Methromh AG CH-9100, Herisau, Switzerland) was used. Tomato cells obtained after calli disaggregation were collected by filtration and washed three times with 1.6% sorbitol [21]. Cells (6 g) were incubated in a thermoregulated (27 °C) Buchner funnel through which CO_2-free air was pumped. The reaction medium (20 ml) consisted of 1.6% sorbitol, 2 mM K_2SO_4 and 0.1 mM $CaSO_4$. The initial pH of the medium was adjusted to 6.8 and the apparent H^+ extrusion was estimated over a 6-h period by the amount of 5 mM KOH needed to maintain the pH value at 6.8. When indicated, 100 mM NaCl, 2 μM fusicoccin or 100 μM vanadate was added to the reaction medium.

2.8. Plasma membrane H^+-ATPase activity and ATP-dependent H^+-transport

Plasma membrane was purified from microsomes by two-phase partitioning according to [21]. ATP hydrolytic activity and ATP dependent H^+-pumping of the H^+-ATPase in purified plasma membranes were measured according to [15,21]. ATP dependent H^+-pumping assays were made using the pH gradient probe ACMA to monitor the formation of an acid inside pH gradient [21] and 0.075% (w/v) polyoxyethylene 20 acetyl ether (Brij 58) to create inside–out plasma membrane vesicles [22].

2.9. Immunodetection of plasma membrane H^+-ATPase

Membrane fractions (10 μg of protein) were separated by SDS–PAGE on 10% acrylamide. Western blotting was performed as described in [21]. After protein electrotransfer to a PVDF membrane (Pall Gelman), the blot was incubated with a polyclonal antibody against the plasma membrane proton ATPase.

2.10. Protein determination

Protein was determined by the method of Schaffner and Weissmann [23] with BSA as standard.

3. Results

3.1. Establishment of the transgenic cell cultures

Transgenic and control cell lines were generated as described in Section 2. From a total of 200 transformed cotyledon sections, 40 gave rise to shoots growing on medium containing up to 400 mg/L kanamycin, but only from three regenerated shoots viable calli were obtained on the selection medium containing 25 mg/L kanamycin. A total of 12 transgenic lines were obtained from different fragments of these three shoots. From the first transgenic regenerated shoot, only callus line L1 was obtained. From the second regenerated shoot, callus lines L2, L3, L4, L5 and L6 were obtained. From the third regenerated shoot, callus lines L7, L8, L9, L10, L11 and L12 were obtained. In all subsequent studies, callus lines originating from each of the three different regenerated shoots were compared. The transgenic lines showed similar levels of expression of both the truncated catalytic subunit (CNAtr) and the regulatory subunit (CNB) as assayed by RT-PCR (Fig. 1).

3.2. Callus growth and ion content

In normal growth conditions, growth of the transgenic calli was lower as compared to the control calli. When calli were grown in the presence of 100 mM NaCl, the transgenic calli
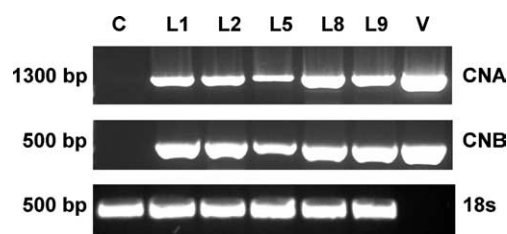
Fig. 1. RT-PCR. PCR was performed on cDNA prepared by RT-PCR as described in Section 2. PCR products were visualized in agarose gels stained with ethidium bromide. C, control calli; L1 to L9, transgenic calli; V, vector DNA containing the CNAtr and CNB genes.
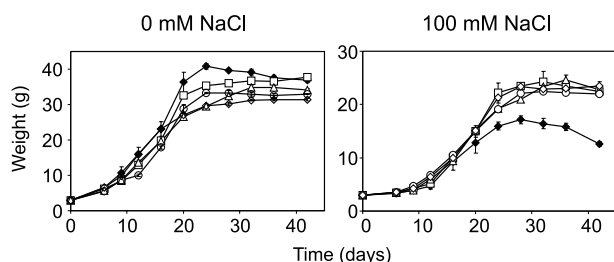

Fig. 2. Growth curves. Callus growth was determined as indicated in Section 2. ◆, control calli; open symbols, transgenic calli; □, L1; ◇, L2; ○, L5; △, L8. Data are means ± S.D. of four separate growth curves with duplicate determinations of fresh weight.

showed a higher growth rate than the control calli (Fig. 2). A similar behavior was observed for the control and transgenic cell suspensions grown in the presence and absence of NaCl (data not shown). Transgenic calli contained slightly higher levels of K^+ and Ca^{2+} as compared to control calli both in the absence or presence of NaCl (Fig. 3). In conditions of NaCl stress, transgenic calli accumulated more Na^+, but less Cl^- as compared to control calli (Fig. 3). The differences in ion content were similar when data were expressed on a dry weight basis and thus were not a result of water content (data not
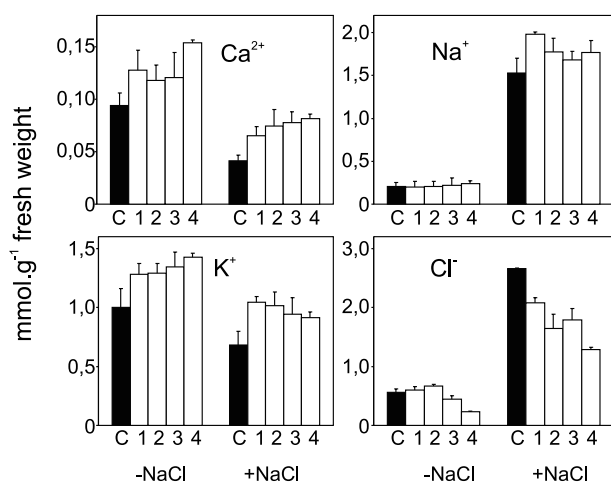

Fig. 3. Ionic content. The content in Ca^{2+}, Na^+, K^+ and Cl^- in control (C) and L1 (1), L2 (2), L5 (3) and L8 (4) transgenic calli was determined after 5 weeks of incubation on multiplication medium supplemented or not with 100 mM NaCl. Data are means ± S.D. of six independent experiments with determinations of ionic content in triplicate for each experiment.

Table 1
Na^+/K^+ ratio in tomato control and transgenic calli grown in multiplication medium supplemented with 100 mM NaCl

	K^+/Na^+ ratio
C	0.75
L1	0.90
L2	0.98
L8	0.96
L9	0.88

Data are calculated from values in Fig. 3.

shown). The variation in Na^+ and K^+ content resulted in a higher K^+/Na^+ ratio in transgenic cell lines when grown in the presence of NaCl (Table 1).

3.3. In vivo proton extrusion

Transgenic and control calli were disaggregated in unbuffered medium. Here, it was observed that the transgenic cells equilibrated at higher pH as compared to control cells, indicating a lower H^+ extrusion activity. This was confirmed by the H^+ extrusion measurements, which showed a substantial lower activity in the transgenic cells than in the controls (Fig. 4). H^+ pumping doubled by the addition of fusicoccin, indicating that the plasma membrane H^+-ATPase contributed significantly to the observed H^+ pumping (data not shown).

3.4. In vitro ATPase activity and H^+ pumping by the plasma membrane H^+-ATPase

Reduced H^+ extrusion in vivo could be caused either by a reduced H^+-ATPase activity, or an increased activity of secondary ion transporters causing membrane potential dissipation or backtransport of protons. We could show that in the transgenic lines, the ATP hydrolysis and proton transport by the plasma membrane H^+-ATPase were lower and that the H^+-transport/ATP hydrolysis ratio was reduced as compared to the control lines (Table 2).

3.5. Analysis of plasma membrane H^+-ATPase content and expression by immunoblot analysis and real-time RT-PCR

The reduced activity of the plasma membrane H^+-ATPase could be caused by reduced amounts of enzyme in the transgenic calli. Immunoblot analysis showed, however, that the transgenic cell lines contained similar amounts of plasma
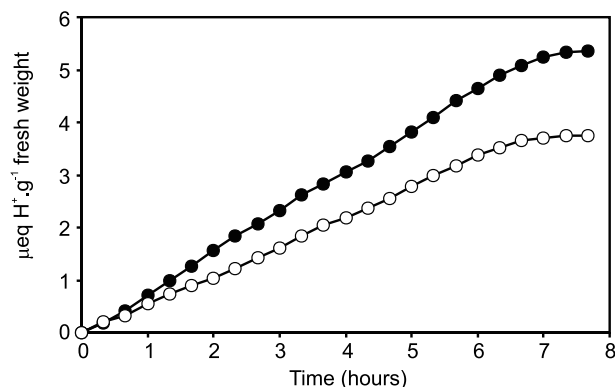

Fig. 4. Proton extrusion. Control and transgenic calli were disaggregated and resuspended in the proton extrusion medium at pH 7.3 as indicated. Data are means of four independent experiments with values not differing by more than 5% from the mean.

Table 2
ATP hydrolysis and ATP-dependent proton transport activities in plasma membrane vesicles purified from control and transgenic tomato calli

	ATP hydrolysis (μmol Pi mg^{-1} h^{-1})	H$^+$ transport (% quenching mg^{-1} h^{-1})	H$^+$/ATP
Control	18.16 ± 2.80	614 ± 75	33.8
L1	13.83 ± 2.40	217 ± 90	15.7
L2	11.05 ± 2.80	157 ± 39	14.2
L8	10.17 ± 4.91	185 ± 19	18.2

Data are means $\pm$ S.D. of four experiments using different membrane preparations.
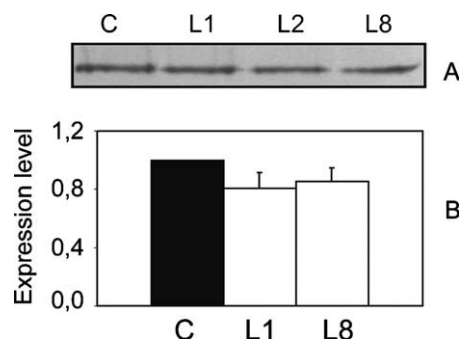

Fig. 5. Immunodetection of plasma membrane H$^+$-ATPase (A) and quantification by real-time PCR of the transcript levels of H$^+$-ATPase isoform LHA4 (B). Plasma membrane fractions (5 μg) isolated from control and transgenic cell lines were separated by electrophoresis, blotted onto nitrocellulose membranes and probed with a polyclonal antibody against the plasma membrane H$^+$-ATPase. Real-time PCR was performed as described in Section 2. Data are means $\pm$ S.D. of results obtained using three different RNA extractions. C, control cell lines; L1, L2, L8, transgenic cell lines.
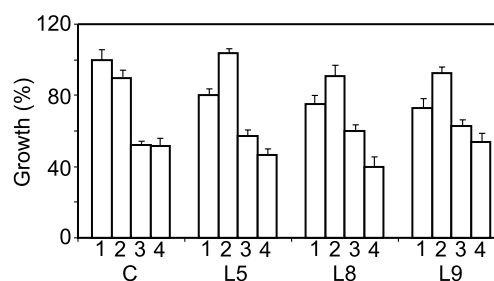

Fig. 6. Effect of fusicoccin on growth of control and transgenic cell cultures. Growth was measured as indicated in Section 2 in the presence or the absence of 0.5 μM fusicoccin. 100% growth corresponds to growth of control cells in the absence of fusicoccin or NaCl. C, control cell line; L5, L8, L9, transgenic cell lines. 1, No additions; 2, 0.5 μM fusicoccin; 3, 100 mM NaCl; 4, 0.5 μM fusicoccin and 100 mM NaCl.

membrane H$^+$-ATPase as compared to control lines (Fig. 5A). These data were confirmed by quantitative real-time PCR using primers specific for the main isoform LHA4 (Fig. 5B), indicating that in transgenic calli the activity of the H$^+$-ATPase is regulated at a post-translational level.

3.6. Effect of fusicoccin on growth of transgenic cell cultures

Fusicoccin is a specific activator of the plant plasma membrane H$^+$-ATPase and could therefore be used to test whether the lower H$^+$-ATPase activity in transgenic calli as compared to the control calli could be responsible for the observed growth effects. Fusicoccin (0.5 μM), added to 3 day old cell suspensions, stimulated growth to about 30% in transgenic cell lines, whilst it was slightly inhibitory in control cell lines (Fig. 6). As observed for the transgenic calli, the growth of transgenic cells was less inhibited as compared to control cells in the presence of NaCl (Fig. 6). In these conditions, the addition of fusicoccin had little effect in control cell lines grown in the presence of NaCl, but inhibited growth of transgenic cells cultivated in the presence of NaCl.

4. Discussion

In this paper, we have shown that the expression of a constitutively activated yeast calcineurin in higher plants has profound effects on cell growth and ion homeostasis. At the molecular level, the most clear-cut effect that we could demonstrate is a reduction in the activity but not content of the plasma membrane H$^+$-ATPase (Fig. 5, Table 2). Reduction of the activity of the closely related H$^+$-ATPase PMA1 has also been reported in yeast cells overexpressing a constitutively activated allele of calcineurin [9]. Furthermore, mutations in PMA1 that reduce its activity suppress multiple phenotypes of calcineurin deficient yeast, and confer slow growth, reduced nutrient uptake, sensitivity to low pH and resistance to Na$^+$ and the aminoglycoside antibiotic Hygromycin B [24–26].

Like in yeast, the plant plasma membrane H$^+$-ATPase is the primary energy source for uptake of many ions through secondary transport systems, driving cell expansion and growth [27]. We show that expression of calcineurin in plants also causes a reduction in activity of the plasma membrane H$^+$-ATPase, and that this reduction provokes similar phenotypes as compared to yeast, confirming the fundamental role of this enzyme in plants: Tomato calli expressing calcineurin grow at reduced rates at higher external pH and display increased salt tolerance. The fact that we could reverse these phenotypes by the addition of fusicoccin shows that the effect is specific for the proton ATPase.

In yeast, it is generally suggested that the decrease in plasma membrane H$^+$-ATPase activity will provoke a diminution of membrane potential driven uptake of toxic cations, thereby explaining the increased salt and hygromycin resistance of pma1 mutants [26]. The transgenic calli were also more resistant to Na$^+$, but accumulated more cations, especially K$^+$ and Ca^{2+}, and less anions (Cl$^-$) as compared to the control calli (Fig. 3). Possibly, calcineurin additionally regulates other secondary ion transport systems that can modify membrane potential and energize cation or anion transport. Indeed, it was shown in patch clamp experiments that calcineurin inhibits inward rectifying K$^+$ channels and a Ca^{2+} permeable slow vacuolar channel in plant cells [12,13]. On the other hand, it is possible that the reduction of cytoplasmic pH caused by plasma membrane H$^+$-ATPase inhibition induces secondary transporters involved in the uptake of cations. Notably, it has been reported in yeast that pma1 mutations enhance sequestration of Na$^+$ in vacuoles through induction of the vacuolar Na$^+$/H$^+$ antiporter NHX1 [25].

The fact that calcineurin regulates H$^+$-ATPase activity both in yeast and plants, despite the absence of a homologous phosphatase in plants, suggests a relatively direct interaction. The mechanism of post-translational regulation is similar in

plants and yeast, and involves activation by phosphorylation of specific residues in the c-terminal autoinhibitory domain. The yeast H^+-ATPase is activated by phosphorylation in response to glucose, causing both an increase in ATP hydrolytic activity and H^+/ATP coupling ratio [28–30]. In response to specific stimuli, the plant H^+-ATPase is phosphorylated at a threonine residue at the penultimate position of the c-terminal domain leading to 14-3-3 binding and activation of the ATPase [27]. An increase in H^+/ATP coupling ratio during this activation has also been described [31,32]. In this study, we have observed a decrease in H^+/ATP coupling ratio in the plasma membrane H^+-ATPase activity obtained from transgenic calli as compared to the control calli (Table 2), which suggests that calcineurin could be directly involved in dephosphorylation and deactivation of the H^+-ATPase. A role in the downregulation of H^+-ATPase activity has been suggested for endogenous phosphatases [33] and calcineurin could thus act accordingly. However, our attempts to inhibit plasma membrane ATPase activity in vitro using bovine calcineurin were not successful (data not shown). This was previously also reported for the interaction of calcineurin with the yeast PMA1 ATPase [8], indicating that calcineurin action is more indirect, possibly by inhibiting the activating kinase.

In conclusion, our study shows that the expression of a regulatory yeast protein in plants can modulate ion homeostasis in much the same way as it does in yeast, even if no homologs of such a protein are normally present in plants. This might have important implications for plant biotechnological applications. Our study further shows that the plant plasma membrane H^+-ATPase is a primary target for the heterologously expressed calcineurin and illustrates the importance of the H^+-ATPase for ion homeostasis, cell growth and salt tolerance.

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