A P_{II}-type Ca^{2+}-ATPase is essential for stress adaptation in Physcomitrella patens

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Transient cytosolic Ca^{2+} ([Ca^{2+}]_cyt) elevations are early events in plant signaling pathways including those related to abiotic stress. The restoration of [Ca^{2+}]_cyt to prestimulus levels involves ATP-driven Ca^{2+} pumps, but direct evidence for an essential role of a plant Ca^{2+}-ATPase in abiotic stress adaptation is missing. Here, we report on a stress-responsive Ca^{2+}-ATPase gene (PCA1) from the moss Physcomitrella patens. Functional analysis of PCA1 in a \textit{Ca}^{2+}-transport-deficient yeast mutant suggests that PCA1 encodes a \textit{P_{II}}-type Ca^{2+}-ATPase harboring an N-terminal autoinhibitory domain. In vivo localizations identified membranes of small vacuoles as the integration site for a PCA1-GFP fusion protein. PCA1 mRNA levels are up-regulated by dehydration, NaCl, and abscisic acid, and PCA1 loss-of-function mutants (\textit{\Delta}PCA1) exhibit an enhanced susceptibility to salt stress. The \textit{\Delta}PCA1 lines show sustained elevated [Ca^{2+}]_cyt in response to salt treatment in contrast to WT that shows transient Ca^{2+} elevations, indicating a direct role for PCA1 in the restoration of prestimulus [Ca^{2+}]_cyt. The altered Ca^{2+} response of the \textit{\Delta}PCA1 mutant lines correlates with altered expression levels of stress-induced genes, suggesting disturbance of a stress-associated signaling pathway. We propose that PCA1 is an essential component for abiotic stress adaptation in Physcomitrella involved in the generation of a specific salt-induced Ca^{2+} signature.

abiotic stress | calcium | signaling | targeted knockout

Ca^{2+} is an important second messenger in animals and plants, and changes in cytoplasmic free Ca^{2+} ([Ca^{2+}]_cyt) are early events in plant signaling pathways, including abiotic stress signaling (1–3). A recent study in \textit{Arabidopsis thaliana} identified genes whose expression was regulated by changes in [Ca^{2+}]_cyt, where stress-responsive genes were significantly overrepresented, substantiating the role of Ca^{2+} in stress signaling pathways (4).

Rising [Ca^{2+}]_cyt levels are caused by increased Ca^{2+} influx (1) or the release of Ca^{2+} from intracellular stores (5) through Ca^{2+}-permeable channels. [Ca^{2+}]_cyt is sensed by proteins that are activated upon Ca^{2+} binding such as Ca^{2+}-dependent protein kinases (CDPKs), or by proteins that undergo conformational changes such as calciuretin B-like (CBL) proteins to regulate downstream targets. The transcription of several CDPK genes is induced by abiotic stress (6–8), and \textit{Arabidopsis CPK4} and \textit{CPK11} loss-of-function mutants show pleiotropic abscisic acid (ABA)-insensitive phenotypes and enhanced salt sensitivity (7). Furthermore, \textit{Arabidopsis cplk3} and \textit{cplk6} mutants show impaired ABA- and Ca^{2+}-induced stomatal closing that is correlated with impaired guard cell ion channel regulation (9).

Likewise, CBL genes are up-regulated by abiotic stress factors, and CBL4 and CBL10 act as calcium sensors required for the acquisition of salt tolerance (10–12).

Influx of Ca^{2+} is countered by the removal of Ca^{2+} from the cytoplasm to reconstitute basal [Ca^{2+}]_cyt. The balance between Ca^{2+} influx and efflux determines the kinetic and temporal nature of the Ca^{2+} elevation. ATP-driven Ca^{2+} pumps (Ca^{2+}-ATPases) and transporters driven by electrochemical gradients, such as Ca^{2+}/H^{+} exchangers, play an important role in maintaining low [Ca^{2+}]_cyt (1).

According to their homology to animal counterparts, plant Ca^{2+}-ATPases are subgrouped into types IIA and IIB (13). The latter contain an N-terminal autoinhibitory domain that responds to Ca^{2+} signals by a Ca^{2+}-induced binding of calmodulin, resulting in the activation of the Ca^{2+} pump (14). Even though changes in [Ca^{2+}]_cyt are associated with abiotic stress signaling there is as yet only indirect evidence for a role of plant Ca^{2+}-ATPases in stress signaling based on the ABA-responsive expression of the \textit{Arabidopsis} genes \textit{ACA6} and \textit{ACA9} (15) and the acquisition of enhanced osmotolerance of a yeast strain overexpressing the \textit{Arabidopsis} \textit{ACA4} gene (16). However, transgenic approaches have shown that plant Ca^{2+}-ATPases are involved in other fundamental processes such as pollen tube growth, vegetative development, inflorescence architecture, and gibberellin signaling (17–19). These studies suggest similar fundamental functions of P-type Ca^{2+}-ATPases in plants and animals as the generation of KO mice revealed perturbations upon the targeted ablation of specific Ca^{2+}-ATPases including lethality, tumorigenesis, skin and muscle diseases, deafness, balance disorders, and male infertility (20). It is assumed that these defects rely on the role of animal Ca^{2+}-ATPases in the clearance of [Ca^{2+}]_cyt, making them critical factors in Ca^{2+}-mediated signaling cascades (21–23).

In bryophytes, changes in [Ca^{2+}]_cyt control developmental programs such as caulonema differentiation (24), protoplast division (25) and cytokinin-induced bud formation (26, 27). Changes in [Ca^{2+}]_cyt were also reported in response to abiotic stress including mechanical stimulation (28, 29), mechanorelocation of chloroplasts (30), UV-A light exposure (31), and cold (29). Thus, changes in [Ca^{2+}]_cyt occur in response to internal and external stimuli in bryophytes, but the constituents that control [Ca^{2+}]_cyt in mosses have not yet been identified.

Results

Isolation of PCA1 from \textit{Physcomitrella}. Cloning of a \textit{Ca}^{2+}-ATPase was initiated by using a partial 750-bp \textit{Physcomitrella} cDNA homologous to the \textit{C} terminal region of P-type \textit{Ca}^{2+}-ATPases (32). The full-length cDNA was isolated from a cDNA library and


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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ428945, AJ544769, At5g57110, At4g29900, AJ566740, AJ566742, Phya, 74335, and Phya, 79531).

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ATPase genes characteristic for this class of Ca\(^{2+}\) ATPases (14). Furthermore, a topology prediction (33) suggests ATPase (Fig. S1). The most striking motif is a calmodulin binding (Fig. S2).

suggesting a high conservation of the gene structure in land plants exons and the exon/intron borders are identical in 30 of 33 positions, mutant grows as well as WT, but growth is inhibited at low Ca\(^{2+}\). yeast Saccharomyces cerevisiae high Ca\(^{2+}\) the P-type Ca\(^{2+}\) ATPases (13). PCA1 also shares with the Arabidopsis Pm-type Ca\(^{2+}\)-ATPases genes ACA18 and ACA10. Physcomitrella PCA1 contains 34 exons and the exon/intron borders are identical in 30 of 33 positions, suggesting a high conservation of the gene structure in land plants (Fig. S2).

**PCA1 Complements a Ca\(^{2+}\) Transport-Deficient Yeast Mutant.** In the yeast Saccharomyces cerevisiae, Ca\(^{2+}\) homeostasis is maintained by the P-type Ca\(^{2+}\)-ATPases PMC1, PMR1, and the Ca\(^{2+}/H^+\) antiporter VCX1 (35) that is inhibited by calcineurin and is not active at low Ca\(^{2+}\) concentrations. In the yeast strain K616 (pmr1, pmc1, cnb1) PMC1, PMR1 and the calcineurin subunit CNB1 are deleted and maintenance of Ca\(^{2+}\) homeostasis relies solely on VCX1 activity. At physiological Ca\(^{2+}\) concentrations (≥ 1 mM Ca\(^{2+}\)) the mutant grows as well as WT, but growth is inhibited at low Ca\(^{2+}\) concentrations because of the inactivation of VCX1 (13) and complementation assays in the K616 yeast mutant background are used for the functional analysis of Ca\(^{2+}\)-ATPases (14, 16). We cloned the PCA1 ORF and a truncated PCA1 cDNA lacking the N-terminal autoinhibitory domain into the yeast expression vector pYES2 (Fig. L4 and Fig. S3). The protein derived from the latter construct should not require activation by Ca\(^{2+}\)/calmodulin and therefore was expected to be constitutively active. As a positive control, we used the yeast PMCI gene encoding a Ca\(^{2+}\)-ATPase (35). The expression of the cDNAs was controlled by the GAL1 promoter that is induced by galactose and repressed by glucose. At high Ca\(^{2+}\) concentrations, the strain K616 grew as well as the WT and the expression of any of the cDNAs had no effect on the growth rate (Fig. 1B). However, at low Ca\(^{2+}\) concentrations in the presence of galactose transformants harboring the truncated PCA1 or the control PMCI cDNA, but not those harboring the full-length PCA1 cDNA, were able to grow. In the presence of glucose that represses the GAL1 promoter only the WT grew. These results indicate that PCA1 displays Ca\(^{2+}\)-transporting activity in yeast. Moreover, the growth inhibition of transformants harboring the full-length PCA1 protein suggests that PCA1 presents a Pm-type Ca\(^{2+}\)-ATPase containing an N-terminal autoinhibitory domain.

**PCA1 Is Localized to Small Vacuoles.** Exploring the intracellular localization of Ca\(^{2+}\)-ATPases may provide insights into the subcellular compartments that are involved in the maintenance of low [Ca\(^{2+}\)]cyt. Previous studies indicated a diverse localization of plant Pm-type Ca\(^{2+}\)-ATPases in the plasma membrane (17, 36), the endoplasmic reticulum (ER) (37), small vacuoles (16), and the central vacuole (38). To analyze the localization of PCA1, we fused the cDNA of the GFP to the 3’ end of the PCA1 coding region under the control of the cauliflower mosaic virus 35S promoter and transfected the construct into Physcomitrella protoplasts that were analyzed by confocal laser scanning microscopy 48 h after the transformation. GFP fluorescence could not be detected at the plasma membrane, the central vacuole, or structures of the ER. However, GFP accumulation was observed in small vacuoles (Fig. 2) and confocal sections indicated that the fusion protein resides in the membranes of these small vacuolar structures (Fig. 2B). Thus, the localization of PCA1 is identical to the reported localization of the Arabidopsis ACA4 Pm-type Ca\(^{2+}\)-ATPase (16).

**PCA1 Is Induced by Dehydration, Salt, and ABA.** To obtain indications for the function of PCA1 in the abiotic stress response, mRNA levels were analyzed in response to dehydration, salt, and ABA, which mediates stress-induced gene expression in mosses and seed plants (39). RNA gel blots indicated increased PCA1 mRNA levels in response to all stimuli (Fig. 3). During dehydration we observed elevated PCA1 mRNA levels after 4 h that did not change after 8 h. Upon NaCl treatment induction of PCA1 occurred after 1 h and transcript levels remained high up to 8 h. ABA caused a transient induction of the PCA1 gene rising up to 4 h, thereafter returning to the basal prestimulus level. From the elevated PCA1 mRNA levels in response to abiotic stress and ABA we assumed a functional role of PCA1 in abiotic stress adaptation.

**Generation of PCA1 KO Mutants.** In Physcomitrella, the integration of DNA into its genome by means of homologous recombination facilitates the generation of targeted gene KO lines (40). Southern

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** PCA1 complements the Ca\(^{2+}\) transport-deficient yeast mutant K616. (A) Arrangement of the yeast mutant strain K616 and WT strain K601 transformed with different expression constructs. (B) Growth of the transformed yeast strains at high (Upper) and low (Lower) Ca\(^{2+}\) concentrations in the presence of galactose (Right) or glucose (Left).

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** PCA1 resides in membranes of small vacuoles. (A) Overlay of the red chlorophyll and green GFP fluorescence of a protoplast transfected with a PCA1/GFP C-terminal fusion construct. (B) Confocal section of a transformed protoplast. (Scale bars: 25 μm.)

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Expression analysis of PCA1 in response to ABA, dehydration, and NaCl. (Upper) PCA1 hybridization signals. (Lower) Ethidium bromide-stained rRNA bands (loading control).
Fig. 4. Generation and molecular analysis of ΔPCA1 KO lines. (A) (Top) Insertion of a nptII selection marker cassette into a HindIII restriction site in exon 32 of the PCA1 gene. (Middle) Resulting PCA1 KO construct. (Bottom) Genomic structure after integration of the PCA1 KO construct by homologous recombination. Primers used for the analysis of transgenic lines are indicated by arrows and roman numbers. Black box: nptII cassette; open boxes: exons sequences within the KO construct; gray boxes: exons flanking the KO construct. (B) PCR analysis to confirm 5' and 3' integration of the PCA1 KO construct. (Left) 5' Integration analysis with primers I and III. (Right) 3' Integration analysis with primers IV and V. PCR products obtained from ΔPCA1 KO lines 27 and 125 are indicated by arrows. (C) (Left) RT-PCR analysis with primers II and V (PCA1) and primers for the control gene EF1α. (Right) RNA gel blot hybridized with a PCA1 probe and ethidium bromide-stained RNA bands to indicate equal loading. (D) (Left) Genomic Southern blot from Physcomitrella WT DNA digested with the indicated restriction enzymes hybridized with a PCA1 probe (HindIII cuts once; PvuII and VspI do not cut). (Right) Genomic Southern blot with DNA from the ΔPCA1 mutants 27 and 125 digested with the indicated restriction enzymes and hybridized with the npt II selection marker cassette (XhoI cuts once; Sacl does not cut).

blot analysis revealed that PCA1 is a single-copy gene, thus representing a suitable target for a gene disruption approach (Fig. 4D). A PCA1 gene disruption construct was generated by inserting a nptII selection marker cassette into the 3' genomic region of PCA1 (Fig. 4A) and used for the transformation of Physcomitrella protoplasts. Regenerating plants were screened for a disrupted PCA1 locus by performing PCR on genomic DNA of transgenic lines with primers flanking the expected integration site in combination with primers derived from the 3'SS promoter and 3'SS terminator sequences present in the nptII cassette. PCR products of the expected size were amplified from independent transgenic lines demonstrating the disruption of the PCA1 genomic locus by precise 5' integration and 3' integration events. Two lines (ΔPCA127 and ΔPCA1125) were selected for further analysis (Fig. 4B). RT-PCR and RNA gel blot analyses did not detect a PCA1 transcript in the 2 ΔPCA1 mutant lines, but did in WT (Fig. 4C), verifying the generation of PCA1 loss-of-function mutants. To determine the number of integration sites of the PCA1 KO construct the 2 ΔPCA1 mutant lines were subjected to genomic Southern blot analysis using the nptII selection marker cassette as hybridization probe. The resulting hybridization patterns demonstrate 1 integration event of the nptII cassette in the mutant ΔPCA1125 and 2 integration events in the mutant ΔPCA127 (Fig. 4D). Furthermore, we analyzed both ΔPCA1 mutants by flow cytometry to exclude the generation of diploid lines by protoplast fusion during the transformation process. Both KO lines were shown to be haploid as was the WT (data not shown).

PCA1 KO Lines Display Decreased Salt Tolerance. To investigate phenotypic effects caused by the disruption of the PCA1 gene, the 2 ΔPCA1 mutant lines were compared with Physcomitrella WT plants on standard growth medium. We did not observe any difference in growth rate or developmental progression. Likewise, the addition of auxin or cytokinin that are known to cause changes in [Ca²⁺]cyt in moss (24–27) did not reveal any differences between WT and the ΔPCA1 mutants, suggesting that PCA1 is not involved in the regulation of development. Because Ca²⁺ is a second messenger in abiotic stress responses and PCA1 mRNA levels were stress-induced, we extended the functional studies of the ΔPCA1 mutants to growth experiments on medium supplemented with NaCl. Both ΔPCA1 mutant lines and WT plants were grown on medium containing 500 mM NaCl, and the salt tolerance was assessed by monitoring the degree of bleaching of the plants and the measurement of cell death (Fig. 5A–D). After 3 days, the 2 ΔPCA1 mutant lines showed enhanced bleaching compared with WT plants (Fig. 5B). Also, after 6 days the ΔPCA1 mutants were more susceptible to salt stress as indicated by partial bleaching of the majority of plants and complete bleaching of single plants, whereas most WT plants remained green and only a few plants showed partial bleaching (Fig. 5C). These apparent deviating phenotypes revealed significant differences between the WT and the 2 ΔPCA1 mutant lines. In addition, we determined cell death rates in the 2 ΔPCA1 mutants and WT plants by Evans blue staining that is indicative for loss of plasma membrane integrity (41). ΔPCA1 mutants and WT were grown in liquid medium containing 500 mM NaCl. According to the phenotypic analysis the 2 ΔPCA1 mutant lines displayed higher cell death rates upon NaCl treatment compared with WT plants (Fig. 5D). The different degree of chlorosis during the NaCl treatment in WT and the ΔPCA1 mutants should have a direct effect on photosynthesis rates that can be determined by chlorophyll fluorescence measurements (42). Liquid cultures of WT and the ΔPCA1125 mutant were grown at different NaCl concentrations (200, 300, 400, and 500 mM NaCl), and the maximum quantum yield of photosystem II was measured 12, 24, and 48 h after the beginning of the treatment. Both WT and the ΔPCA1125 mutant line showed a concentration and time-dependent decrease in the photosynthesis rate in the presence of elevated NaCl concentrations (Fig. 5E). Furthermore, the ΔPCA1125 mutant line exhibited lower maximum quantum yields compared with WT, which became most evident after 48 h of salt treatment. The reduced photosynthesis rates in the ΔPCA1 mutant in relation to WT correspond to the enhanced chlorosis observed in the phenotypic analysis, providing further evidence for the reduced salt tolerance of the ΔPCA1 mutant lines. Based on these results we infer that PCA1 is essential for salt stress adaptation in Physcomitrella.

ΔPCA1 Mutants Fail to Restore Resting Ca²⁺ Levels After Salt Stress Treatments and Display Disturbed Expression of Stress-Induced Genes. To test a direct role of PCA1 in salt stress adaptation, we measured changes in [Ca²⁺]cyt in response to NaCl in Physcomitrella WT and the mutant line ΔPCA1125. [Ca²⁺]cyt was measured ratiometrically after biologic delivery of the Ca²⁺-sensitive dye Oregon Green 488 BAPTA dextran and the Ca²⁺-insensitive

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mutant lines were comparable to those in WT with the exception of slightly increased mRNA levels of *PpCOR-TMC-AP3* in WT plants in response to ABA. However, 3 h after the ABA and NaCl treatment transcript levels were significantly lower in the Δ*PCA1* mutants compared with WT (Fig. 6 C and D and Table S2). In *Arabidopsis*, it was suggested that ABA-responsive elements (ABRE) within promoter regions mediate Ca\(^{2+}\)-responsive gene expression (4). Also in *Physcomitrella*, ABREs are functional cis-elements conferring stress-induced gene expression (44, 45). To test whether ABREs are present in the genes *PpCOR47* and *PpCOR-TMC-AP3*, we analyzed their promoter regions. In fact, we identified putative ABREs in the promoter regions of both genes, suggesting that these elements may transmit changes in [Ca\(^{2+}\)]\(_{cyt}\) in *Physcomitrella* (Fig. 6E). According to the empirical cumulative distribution of all ABRE matches in the 1.5-kb upstream regions of all 27,962 *Physcomitrella* V1.2 gene models (46) (Fig. S5), the overrepresentation of ABREs was found to be significant \(P(x > c) = 0.00021; P(x > g) = 7 \times 10^{-06}\). The promoter of *PCA1*, which is transiently induced by ABA, does not harbor ABREs, suggesting that the expression might be controlled by a different pathway. We further analyzed whether the application of exogenous Ca\(^{2+}\) is able to mimic the stress induction of *PpCOR47* and *PpCOR-TMC-AP3*. WT plants were grown in medium with 100 mM CaCl\(_2\), which corresponds to the basal [Ca\(^{2+}\)]\(_{cyt}\) and subsequently CaCl\(_2\) was added at concentrations of 1 and 5 mM. For both genes, we did not detect considerable changes in mRNA steady-state levels in response to applied Ca\(^{2+}\), which may resemble the stress-induced expression pattern of the genes in response to NaCl or ABA (Fig. 6). Thus, application of exogenous Ca\(^{2+}\) is not sufficient to activate the stress signaling pathway(s) regulating *PpCO47* and *PpCOR-TMC-AP3*. Taken together, the perturbation of the salt-induced Ca\(^{2+}\) signal and the deregulation of stress-induced genes in the Δ*PCA1* mutant lines suggest a function of *PCA1* in stress signaling.

**Discussion**

The stimulus-dependent generation of [Ca\(^{2+}\)]\(_{cyt}\) transients has been intensively studied (47), but experimental data on the proteins that restore [Ca\(^{2+}\)]\(_{cyt}\) are scarce. Ca\(^{2+}\)-ATPases are thought to be the major components regulating [Ca\(^{2+}\)]\(_{cyt}\) homeostasis. *Physcomitrella* PCA1 contains all characteristic motifs of plant P\(_{i}\)\_H\(_{i}\)_type Ca\(^{2+}\)-ATPases, and its Ca\(^{2+}\)-transport activity was substantiated by a yeast complementation where PCA1 displayed an identical mode of action already described for other plant P\(_{i}\)\_H\(_{i}\)_type Ca\(^{2+}\)-ATPases (14, 16, 17).

To date, there are only 2 reported *Arabidopsis* P\(_{i}\)\_H\(_{i}\)_type Ca\(^{2+}\)-ATPase gene KO lines, where the disruption of *ACA19* caused partial male sterility, and disruption of *ACA10* led to defects in vegetative growth and inflorescence architecture (17, 19). Nevertheless, the expression pattern of other Ca\(^{2+}\)-ATPase genes in *Arabidopsis* suggested a function of Ca\(^{2+}\)-ATPases in abiotic stress-related pathways (15). Likewise, *PCA1* is regulated by an ABA-dependent stress signaling pathway. Moreover, the reduced tolerance of the Δ*PCA1* mutants to salt provides genetic evidence for its role in salt stress adaptation, and the reduced mRNA levels of stress-induced genes in response to salt and ABA suggest that they are controlled by a signaling pathway requiring PCA1 activity. Evidence that plant Ca\(^{2+}\)-ATPases are positive regulators of signaling pathways was obtained from the overexpression of a gibberellin-responsive Ca\(^{2+}\)-ATPase in rice aleurone cells that caused induction of a gibberellin-responsive \(\alpha\)-amylase gene in the absence of gibberellin (18).

The Δ*PCA1* mutants fail to generate a salt-induced transient Ca\(^{2+}\) peak and exhibit elevated [Ca\(^{2+}\)]\(_{cyt}\). One could expect that the elevated [Ca\(^{2+}\)]\(_{cyt}\) leads to an enhanced Ca\(^{2+}\) response and finally results in elevated transcript levels of genes controlled by this pathway. Actually, loss of *PCA1* resulted in decreased transcript levels of stress-responsive genes. We therefore propose that PCA1 is required to restore the [Ca\(^{2+}\)]\(_{cyt}\) for the generation of a stimulus-
specific transient increase in [Ca$^{2+}$]$_{cyt}$ (3, 47) that is required for the transmission of the initial stress signal. Substantial evidence that Ca$^{2+}$-ATPases are in fact key regulators of [Ca$^{2+}$]$_{cyt}$ was provided in animal cells where Ca$^{2+}$-ATPases are indispensable for the restoration of basal [Ca$^{2+}$]$_{cyt}$ and required for proper signal transmission (21–23). It is plausible that altered Ca$^{2+}$ signatures affect the activation of Ca$^{2+}$ sensor proteins, resulting in perturbed downstream signaling steps.

PCA1 was localized in membranes of small vacuolar compartments, which is consistent with the localization of the Arabidopsis P$\text{H}+$-type Ca$^{2+}$-ATPase ACA4 (16). The additional subcellular localization sites of plant P$\text{H}+$-type Ca$^{2+}$-ATPases in the plasma membrane (17, 36), the ER (37), and the central vacuole (38) point to functional diversities within the plasma membrane and the endomembrane system in relation to Ca$^{2+}$+ homeostasis and Ca$^{2+}$ signaling. Besides Ca$^{2+}$-ATPases, Ca$^{2+}$/H$^{+}$ exchangers control Ca$^{2+}$ homeostasis under stress conditions as Arabidopsis T-DNA mutants of the CAX1 Ca$^{2+}$/H$^{+}$ antiporter displayed an enhanced freezing tolerance that was correlated with an elevated expression of CBF/DREB1 genes and their corresponding targets (48). Furthermore, Arabidopsis cax1 and cax3 mutants display an increased sensitivity to ABA and sugar during seed germination and an increased tolerance to ethylene (49). CAX1 and CAX3 were also found to play a role in plant growth and nutrient acquisition (50). Furthermore, it was suggested that CAX3 is the predominant Ca$^{2+}$/H$^{+}$ antiporter in Arabidopsis under salt stress conditions, whereas CAX1 is essentially inactive, which is correlated with their expression as CAX3 is up-regulated by salt but CAX1 is not (49).

Compared with elevated mRNA levels of stress-responsive genes in the Arabidopsis cax1 mutant, the reduced expression of stress-responsive genes in the Physcomitrella PCA1 mutants suggests that the action of Ca$^{2+}$/H$^{+}$ antiporters and Ca$^{2+}$ pumps lead to different responses in signaling pathways. In addition, the disruption of the Arabidopsis genes ACA9 and ACA10 (17, 19) and the deletion of the Physcomitrella PCA1 cause distinct phenotypes. However, the alterations are found in completely different biological pathways, namely developmental programs in Arabidopsis and abiotic stress signaling in Physcomitrella, suggesting that plant Ca$^{2+}$-ATPases have distinct biological functions.

Materials and Methods

Plant Material. P. patens was cultured as described (39, 51). Detailed descriptions of the phenotypic analysis, treatments with ABA and NaCl, and the application of exogenous Ca$^{2+}$ are provided in SI Text.
Molecular Cloning and Analysis of Transgenic Physcomitrella patens. A detailed description of the DNA constructs used in this study and the molecular analyses of the generated transgenic Physcomitrella patens are provided in SI Text.

Yeast Transformation. The transformation of yeast cells was performed according to Ausbel et al. (52). Transformed cells were plated onto SD-LAH plates that were supplemented with 2% glucose/10 mM CaCl₂, 2% glucose/10 mM EGTA, 2% galactose/10 mM CaCl₂, or 2% galactose/10 mM EGTA.

RNA and DNA Blot Hybridization. RNA blot hybridization was carried out as described (39) with the following radioactively labeled cDNA probes: PcPcOR47, PcPcOR-TMC-AP3 (39), the homolog to ribosomal protein L21 (32) amplified from cDNA of the primers 5'-GGTTGCTATGGTGTTG CGC-3' and 5'-GCGTCCGCAATGAGGTCG-3', and a 346 bp DNA fragment amplified with the primers 5'-TTGGCGATTGGCTTATAAGCTG-3' and 5'-ACCAGTAAAAA CAACAGCTAATG-3'. Signal intensities were quantified by using the Quantity One software package (BioRad). PcPcOR47 and PcPcOR-TMC-AP3 transcript levels were normalized to the constitutive control L21 mRNA. Mean values from 3 biological replicates and standard errors were calculated. Genomic DNA was digested with the indicated restriction enzymes, blotted, and hybridized with a PAC1 cDNA fragment (analysis of PAC1 gene copy number) or the npitl selection marker cassette present in the PAC1 gene disruption construct (analysis of integrations in the PAC1 mutation).

26. Holden AM, et al. (2007) Measuring of Cytosolic Calcium, [Ca²⁺]cyt, was measured ratiometrically after bioilistic delivery of the Ca²⁺-sensitive dye Oregon Green 488 BAPTA dextran and the Ca²⁺-insensitive Cascade Blue dextran into Physcomitrella cells as described (43). A detailed description of the method is provided in SI Text.

Promoter Analysis. Regions (1.5 kb) upstream of the translation start of the PcPcOR47 and PcPcOR-TMC-AP3 genes (V1.2 (46) were extracted and screened for overrepresented motifs by the Gibbs sampling algorithm implemented in AlignACE (53). The resulting overrepresented motifs were compared with the ABRE consensus sequence of seed plants (4, 54). Significance was tested by using the empirical cumulative distribution of all ABRE matches in the 1.5-kb upstream regions of all 27,962 V1.2 gene models, and probability values [P > x₀] for the occurrence of the observed ABRE frequencies (x₀) in the genes PcPcOR47 and PcPcOR-TMC-AP3 were calculated.

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Correction

PLANT BIOLOGY

The authors note that Hauke Holtorf should be added to the author line between Daniel Lang and Colin Brownlee. Hauke Holtorf should be credited with designing research and analyzing data. The corrected author line, affiliation line, and author contributions footnote appear below. The online version has been corrected.

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