Vacuolar Ca^{2+}/H^+ Transport Activity Is Required for Systemic Phosphate Homeostasis Involving Shoot-to-Root Signaling in Arabidopsis[1][W][OA]

Tzu-Yin Liu, Kyaw Aung[2], Ching-Ying Tseng[3], Tzu-Yun Chang, Ying-Shin Chen, and Tzyy-Jen Chiou*

Agricultural Biotechnology Research Center (T.-Y.L., K.A., C.-Y.T., T.-Y.C., Y.-S.C, T.-J.C.) and Molecular and Biological Agricultural Sciences Program, Taiwan International Graduate Program (K.A., T.-J.C.), Academia Sinica, Taipei 115, Taiwan; and Department of Life Sciences, National Chung-Hsing University, Taichung 402, Taiwan (T.-J.C.)

Calcium ions (Ca^{2+}) and Ca^{2+}-related proteins mediate a wide array of downstream processes involved in plant responses to abiotic stresses. In Arabidopsis (*Arabidopsis thaliana*), disruption of the vacuolar Ca^{2+}/H^+ transporters CAX1 and CAX3 causes notable alterations in the shoot ionome, including phosphate (P_i) content. In this study, we showed that the *cax1/cax3* double mutant displays an elevated P_i level in shoots as a result of increased P_i uptake in a miR399/PHO2-independent signaling pathway. Microarray analysis of the *cax1/cax3* mutant suggests the regulatory function of CAX1 and CAX3 in suppressing the expression of a subset of shoot P_i starvation-responsive genes, including genes encoding the *PHT1;4* P_i transporter and two SPX domain-containing proteins, *SPX1* and *SPX3*. Moreover, although the expression of several *PHT1* genes and PHT1;1/2/3 proteins is not up-regulated in the root of *cax1/cax3*, results from reciprocal grafting experiments indicate that the *cax1/cax3* scion is responsible for high P_i accumulation in grafted plants and that the *pht1;1* rootstock is sufficient to moderately repress such P_i accumulation. Based on these findings, we propose that CAX1 and CAX3 mediate a shoot-derived signal that modulates the activity of the root P_i transporter system, likely in part via posttranslational regulation of PHT1;1 P_i transporters.

Transient increases in cytoplasmic calcium concentrations ($[Ca^{2+}]_{cyt}$) or the spatial and temporal dynamics of stimulus-induced alterations in $[Ca^{2+}]_{cyt}$ constitute a signal that mediates a wide array of downstream processes involved in plant responses to many developmental cues and environmental stresses (Knight, 2000; McAinsh and Pittman, 2009). The generation of such stimulus-specific Ca^{2+} signatures is associated with various Ca^{2+} channels, transporters, and pumps throughout the membrane system. In particular, tonoplast-localized Ca^{2+}/H^+ exchangers and Ca^{2+}-ATPase pumps play a key role in the sequestration of Ca^{2+} into the vacuole, the primary pool for Ca^{2+} buffering and release, and are assumed to participate in resetting the $[Ca^{2+}]_{cyt}$ following stimuli (Hirschi, 2004; McAinsh and Pittman, 2009).

In yeast, the vacuolar Ca^{2+}/H^+ exchanger VCX1, as a high-capacity and low-affinity Ca^{2+} transporter, functions to rapidly sequester cytosolic Ca^{2+} and supposedly attenuates the activation of Ca^{2+} signaling pathways, as the *vcx1*Δ strain displayed a transient and strongly elevated $[Ca^{2+}]_{cyt}$ followed by a slow and weak recovery from a Ca^{2+} shock (Miseta et al., 1999). In Arabidopsis (*Arabidopsis thaliana*), the cation/H^+ exchangers CAX1 (the ortholog of VCX1), CAX3, and CAX4 are phylogenetically grouped into type IA, whereas CAX2, CAX5, and CAX6 belong to type IB (Shigaki et al., 2006). However, only CAX1 to CAX4 have been functionally characterized to possess a vacuolar Ca^{2+}/H^+ exchange activity. Whereas CAX1 and CAX3 mediated specifically Ca^{2+} transport (Hirschi, 1999; Catala et al., 2003; Cheng et al., 2003; Mei et al., 2007; Zhao et al., 2008), CAX2 and CAX4 were documented to have high transport and selectivity for cadmium ions (Cd^{2+}) over Ca^{2+} in tonoplast vesicles (Hirschi et al., 2000; Cheng et al., 2002; Pittman et al., 2004; Koren'kov et al., 2007).

Knockout of *CAX1* in Arabidopsis increased the tolerance to high concentrations of various ions, Ca^{2+}-depleted conditions, and freezing after cold acclimation (Catala et al., 2003; Cheng et al., 2003). Conversely, transgenic tobacco plants overexpressing *CAX1* were hypersensitive to ion imbalance and cold shock and

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[2] Present address: Plant Biology Department, Michigan State University, East Lansing, MI 48824.

[3] Present address: School of Biological Sciences, University of Texas at Austin, Austin, TX 78712.

* Corresponding author; e-mail tjchiou@gate.sinica.edu.tw.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Tzyy-Jen Chiou (tjchiou@gate.sinica.edu.tw).

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exhibited Ca^{2+}-deficient symptoms in spite of increased accumulation of Ca^{2+} (Hirschi, 1999). Loss-of-function of *CAX3*, a close homolog of *CAX1*, increased the sensitivity to salt stress, lithium, and low pH (Zhao et al., 2008). While *cax1* and *cax3* single mutants displayed subtle phenotypes, the *cax1/cax3* double mutant showed stunted growth with chlorosis on the leaf tips and a drastic reduction in silique size and seed numbers (Cheng et al., 2005). In addition to a decreased activity of vacuolar Ca^{2+}/H^+ antiport, the V-ATPase and P-ATPase activities were reduced in *cax1/cax3* (Cheng et al., 2005; Zhao et al., 2008). Elemental analysis has also revealed that impairment of CAX1 and CAX3 caused dramatic alterations in the shoot ionome, namely elevated levels of phosphate (PO_4^{3-}; P_i), manganese (Mn^{2+}), and zinc (Zn^{2+}) and decreased Ca^{2+} and magnesium (Mg^{2+}) ion concentrations (Cheng et al., 2005). A recent study further showed that the leaf apoplastic free Ca^{2+} concentration ($[Ca^{2+}]$) of *cax1/cax3* was 3-fold greater than that of the wild type, accounting for the phenotypes of reduced cell wall extensibility, stomatal aperture, transpiration, CO_2 assimilation, and leaf growth rate (Conn et al., 2011b). Despite no evidence yet of a direct involvement of Ca^{2+} in P_i signaling, the observation of an increased accumulation of P_i in the *cax1/cax3* mutant has provided the first link between Ca^{2+} and P_i homeostasis and thus drew our attention and interest to investigate the potential role of CAX1 and CAX3 in P_i signaling.

Phosphorus (P), one of the mineral nutrients essential for plant survival and productivity, is a major structural constituent of fundamental macromolecules such as nucleic acids and phospholipids and is involved in energy transfer, metabolic regulation, and protein activation. However, most of the P in the soil is unavailable for plant uptake because of adsorption, precipitation, or conversion to organic forms (Marschner, 1995). As a result, plants constantly encounter P_i limitation and have developed a number of adaptive strategies to maintain P_i homeostasis, including enhancing acquisition of P_i, coordinating allocation of P_i among different organs, and remobilizing P_i from old to young tissues (Poirier and Bucher, 2002; Ticconi and Abel, 2004). Although many plant responses to P_i starvation have been extensively explored, the molecular mechanisms by which plants sense the P_i signal and elicit these responses remain largely unknown.

Identification and characterization of several mutants with aberrant responses to P_i starvation or with altered levels of P_i has advanced our understanding of the molecular components involved in P_i homeostasis (Lin et al., 2009; Chiou and Lin, 2011). For example, the Arabidopsis *pho2* mutant accumulated high levels of P_i in the shoot and showed symptoms of P_i toxicity as a result of increased P_i uptake and translocation of P_i from roots to shoots (Delhaize and Randall, 1995; Dong et al., 1998). The *PHO2* gene was identified to encode an E2 ubiquitin-conjugating enzyme (UBC24), whose expression during P_i deficiency is posttranscriptionally suppressed by a specific microRNA, miR399 (Fujii et al., 2005; Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006). In accordance with the inverse correlation between miR399 and *PHO2* mRNA levels, transgenic plants overexpressing miR399 phenocopied the *pho2* mutant and *PHO2* T-DNA knockout lines (Aung et al., 2006; Chiou et al., 2006). Reciprocal micrografting experiments further demonstrated that a *pho2* root genotype is sufficient and necessary for P_i accumulation in the shoot, whereas the shoot-to-root movement of mature miR399 is responsible for the degradation of *PHO2* mRNA in roots (Bari et al., 2006; Lin et al., 2008; Pant et al., 2008). Moreover, upregulation of miR399 by P_i deprivation is mediated by the PHOSPHATE STARVATION RESPONSE1 (PHR1) transcription factor, a key positive regulator of multiple P_i starvation-induced (PSi) genes (Rubio et al., 2001; Bari et al., 2006). These findings suggest that the P_i starvation signaling involving PHR1, miR399, and PHO2 is crucial for the maintenance of P_i homeostasis.

Recently, a unique gene family in Arabidopsis (*AtSPX1–AtSPX4*) exclusively harboring the SPX (for SYG1/Pho81/XPR1) domain was identified to be regulated by P_i starvation, in part through PHR1 (Hamburger et al., 2002; Wang et al., 2004; Duan et al., 2008). Overexpression of *SPX1* (At5g20150) increased the transcript levels of several PSi genes under both P_i-sufficient and P_i-deficient conditions, whereas RNA interference-mediated partial down-regulation of *SPX3* (At2g45130) led to aggravated P_i deficiency symptoms, altered P_i allocation, and enhanced expression of a subset of PSi genes (Duan et al., 2008). In rice (*Oryza sativa*), OsSPX1 (Os06g40120), the ortholog of SPX3, acts via a negative feedback loop to adjust the expression of several PSi genes under P_i-limited conditions (Wang et al., 2009; Liu et al., 2010). These findings revealed that the plant SPX domain-containing proteins are new players in the regulatory network of P_i signaling.

Although Ca^{2+} and Ca^{2+}-related proteins are indispensable messengers in the signal transduction of many stress responses, the role of Ca^{2+} in P_i signaling and the cross talk between Ca^{2+} and P_i homeostasis are barely understood. The association between loss of CAX1 and CAX3 activities and increased P_i accumulation as seen in *cax1/cax3* (Cheng et al., 2005) provides an opportunity to tackle this issue. It is also of interest to compare *cax1/cax3* and *pho2* mutants in terms of regulatory pathways, because both mutants show elevated shoot P_i concentrations. Here, our study of the *cax1/cax3* mutant suggests that vacuolar Ca^{2+}/H^+ transporters exert a negative regulation of P_i starvation responses, as revealed by suppression of the expression of a subset of shoot P_i starvation-responsive (PSR) genes and inhibition of P_i uptake activity in the root. Our results also suggest that the effects of CAX1/CAX3- and PHO2-mediated signaling pathways on the suppression of P_i uptake are different. Moreover, results from reciprocal grafting experiments demonstrate that CAX1 and CAX3 mediate a shoot-derived

signal that modulates the activity of the root P_i transporter system, likely in part via posttranslational regulation of PHT1;1.

RESULTS

cax1/cax3 Mutant Accumulates High Levels of P_i in Shoot and Displays Increased P_i Transport Activity

The shoot P_i concentration has been shown to increase by 66% in the *cax1/cax3* mutant but to remain unchanged in the single *cax1* and *cax3* mutants (Cheng et al., 2005). To confirm this finding, we grew *cax1*, *cax3*, and *cax1/cax3* along with the *pho2* mutant and wild-type controls in P_i-sufficient ($+P_i$) or P_i-deficient ($-P_i$) half-strength modified Hoagland hydroponic solution for P_i concentration measurement. Under both $+P_i$ and $-P_i$ conditions, the shoot and root P_i concentrations of *cax1* and *cax3* were similar to those of the wild type; by contrast, the *cax1/cax3* double mutant accumulated high levels of P_i in the shoot under both conditions and showed modestly increased P_i concentrations in the root under P_i deficiency (Fig. 1). Of note, *cax1/cax3* did not accumulate P_i in the shoot to a level as high as *pho2* under $+P_i$ conditions but maintained a comparable level of shoot P_i as *pho2* under $-P_i$ conditions (Fig. 1A).

We then performed P_i transport assays to determine whether P_i accumulation in *cax1/cax3* can be attributed to an enhanced P_i uptake rate. As expected, *cax1/cax3* exhibited a higher P_i uptake activity than wild-type plants, regardless of external P_i concentrations (Fig. 2, A and B). By contrast, *pho2* exhibited a higher P_i uptake activity than *cax1/cax3* when the P_i supply was adequate (Fig. 2A) but exhibited only a slightly enhanced P_i uptake activity as compared with the wild type when P_i was limited (Fig. 2B). Under $+P_i$ conditions, *pho2* but not *cax1/cax3* showed an increased shoot-to-root ratio of P_i distribution (Fig. 2C), indicating that the P_i translocation activity of *cax1/cax3* from roots to shoots was not changed even though the P_i uptake activity was increased. However, under $-P_i$ conditions, the shoot-to-root ratio of P_i distribution in *cax1/cax3* was increased (Fig. 2D). Taken together, *cax1/cax3* displayed increased shoot P_i accumulation, increased P_i uptake activity regardless of external P_i concentrations, and greater P_i translocation from roots to shoots when P_i was limited.

P_i Accumulation in *cax1/cax3* Mutant Increases in an Exogenous [Ca^{2+}]-Dependent Manner

It has been reported that *cax1/cax3* showed a higher sensitivity to Ca^{2+} stress when grown in medium supplemented with high [Ca^{2+}] (Cheng et al., 2005). To address whether the impaired cellular Ca^{2+} homeostasis due to the loss of CAX1 and CAX3 plays a direct role in enhancing P_i uptake activity, we examined the effect of exogenous Ca^{2+} on P_i accumulation of *cax1/cax3* by growing mutants in $+P_i$ hydroponic medium containing different concentrations of Ca^{2+} (0.05 mM, 0.25 mM, 1 mM, and 2 mM [Ca^{2+}]), contrasting with 2.5 mM [Ca^{2+}] in half-strength modified Hoagland solution. Interestingly, P_i accumulation in the shoot of *cax1/cax3* was increased in a [Ca^{2+}]-dependent manner (Fig. 3B) and displayed a negative correlation with the leaf size of mutants (Fig. 3A). Consistent with previous results showing that the growth defects of *cax1/cax3* mutant were suppressed by supplemented exogenous Mg^{2+} (Cheng et al., 2005), we also found that the exacerbated P_i accumulation in *cax1/cax3* mutants resulting from exogenous Ca^{2+} stress could be attenuated by supplementing high concentrations of Mg^{2+} (Fig. 3). These results suggest an antagonistic relationship between Ca^{2+} and Mg^{2+} regarding their interplay in P_i accumulation of *cax1/cax3*.

Cross-Regulation of *PHO2* and *CAX1/CAX3* Expression in *cax1/cax3* and *pho2* Mutants

To investigate the role of CAX1 and CAX3 in P_i signaling, we first used quantitative reverse transcrip-

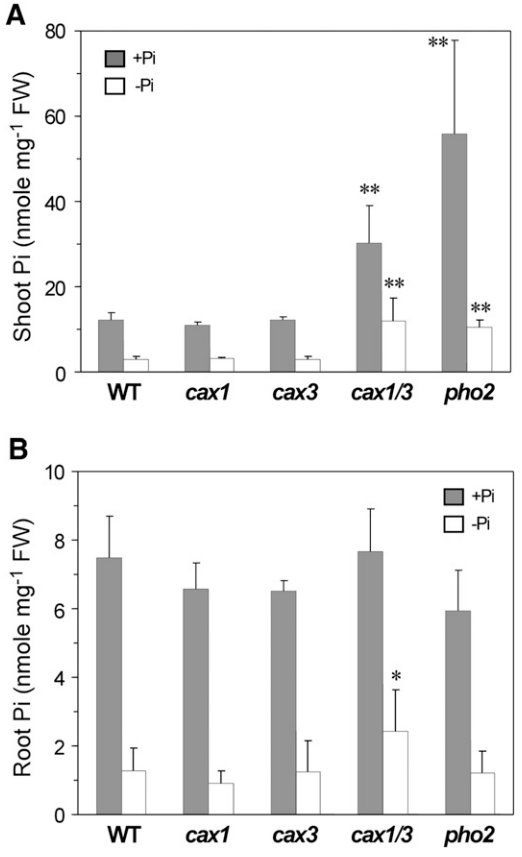

Figure 1. Analysis of P_i concentrations in the *cax1/cax3* mutant. The P_i concentrations are shown in the shoot (A) and root (B) of 23-d-old wild-type (WT), *cax1*, *cax3*, *cax1/cax3*, and *pho2* plants grown under $+P_i$ or $-P_i$ conditions. Values represent means + SD of three to five biological replicates. Data significantly different from the corresponding wild-type controls are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t* test). FW, Fresh weight.

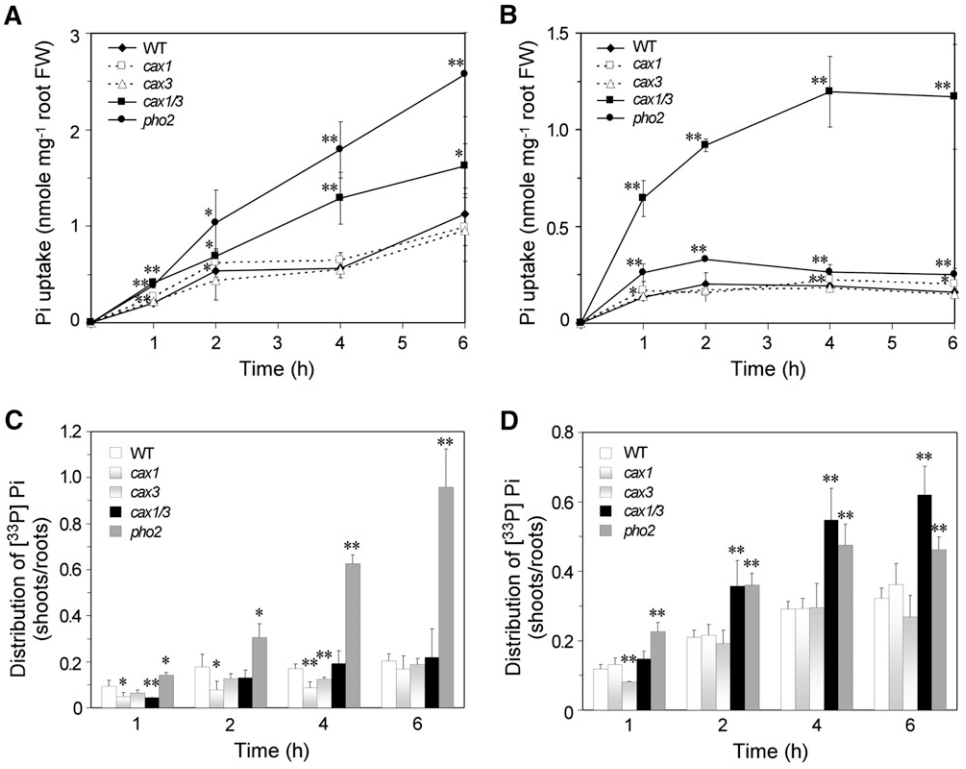

Figure 2. Analysis of P_i uptake rate and P_i translocation in the *cax1/cax3* mutant. A and B, The uptake rate of $[^{33}P]P_i$ in wild-type (WT), *cax1*, *cax3*, *cax1/cax3*, and *pho2* plants grown under $\pm P_i$ (250 μM KH_2PO_4; A) or $-P_i$ (10 μM KH_2PO_4; B) conditions. C and D, The shoot-to-root ratio of $[^{33}P]P_i$ accumulation during the transport period under $+P_i$ (C) or $-P_i$ (D) conditions. Values represent means ± SD of three biological replicates. Data significantly different from the corresponding wild-type controls are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t* test). FW, Fresh weight.

tion (qRT)-PCR to examine changes in the expression levels of *CAX1* and *CAX3* in wild-type plants subjected to P_i deficiency. In the shoot, the transcript level of *CAX1* was reduced by 60% after 5 d of P_i deprivation (Fig. 4A). However, no difference was observed in the expression level of *CAX3* between P_i-sufficient and P_i-deficient shoots (Fig. 4B). In the root, no significant change was found in the transcript level of *CAX1* in response to P_i starvation (Fig. 4A), while the *CAX3* transcript level was increased (Fig. 4B). Regulation of the expression of *CAX1* and *CAX3* by P_i deficiency implied the involvement of these genes in P_i starvation responses.

Although *cax1/cax3* and *pho2* mutants showed distinct properties in terms of P_i uptake and translocation (Fig. 2), we wondered whether there is a cross talk between PHO2 and CAX1/CAX3-mediated P_i signaling pathways. Therefore, we examined the expression of *CAX1* and *CAX3* in *pho2* and of *PHO2* in *cax1/cax3*. Under both $+P_i$ and $-P_i$ conditions, the transcript levels of *CAX1* in the shoot and root of *pho2* were similar to that of wild-type plants (Fig. 4A). Under $+P_i$ conditions, the transcript levels of *CAX3* in the shoot and root were higher in *pho2* than in wild-type plants; however, under $-P_i$ conditions, the transcript level of *CAX3* was higher in the shoot but not in the root of *pho2* as compared with wild-type plants (Fig. 4B).

The complementary measurements revealed that the transcript level of *PHO2* in the shoot of *cax1/cax3* was higher than that of wild-type plants under both $+P_i$ and $-P_i$ conditions (Fig. 4C). As the *pho2* rootstock genotype has been shown sufficient for P_i accumulation in the scion (Bari et al., 2006; Lin et al., 2008), the role of PHO2 in the shoot and the implication of increased transcript levels of *PHO2* in the shoot of *cax1/cax3* remain to be resolved. On the other hand, the level of *PHO2* mRNA in the root of *cax1/cax3* was reduced by 36% under $+P_i$ conditions as compared with wild-type plants but was not as low as that detected in the root of wild-type plants under P_i deficiency (Fig. 4C). The mature miR399 was not detectable in the shoot or root of *cax1/cax3* under $+P_i$ conditions (Fig. 4D), indicating that the moderate reduction of *PHO2* mRNA level in the root of *cax1/cax3* under $+P_i$ conditions did not result from suppression by miR399. Furthermore, the *PHO2* transcripts were reduced to a similar level in the root of *cax1/cax3* and wild-type plants under $-P_i$ conditions (Fig. 4C), supporting the idea that the increased P_i uptake activity in the root of *cax1/cax3* under P_i deficiency (Fig. 2B) is unlikely mediated by a PHO2-dependent signaling pathway. Of interest, we also found that the induction of mature miR399s under P_i deficiency in the shoot of *cax1/cax3* was lower than that of wild-type controls; therefore, it is likely that miR399 induction is inhibited by the elevated concentrations of internal shoot P_i, as shown in Figure 1A. From these results, we conclude that the expression of *CAX1/CAX3* and *PHO2* appears to be cross-regulated, even though they function to suppress P_i uptake and regulate root-to-shoot P_i translocation in different modes.

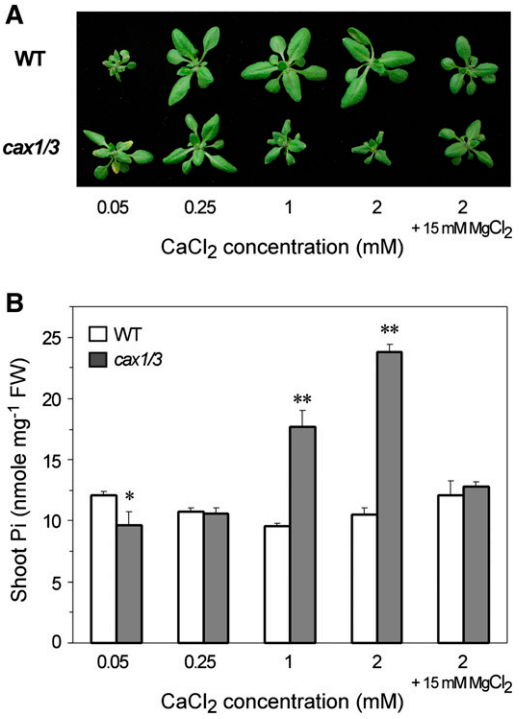

Figure 3. Analysis of *cax1/cax3* mutants grown under various exogenous Ca^{2+} concentrations. The phenotypes (A) and P_i concentrations in the shoot (B) are shown for 21-d-old wild-type (WT) and *cax1/cax3* plants grown under $+P_i$ (250 μM KH_2PO_4) conditions supplemented with 0.05, 0.25, 1, or 2 mM $CaCl_2$. An additional 15 mM $MgCl_2$ was added to the medium containing 2 mM $CaCl_2$. Values represent means + SD of three biological replicates. Data significantly different from the corresponding wild-type controls are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t* test). FW, Fresh weight.

Suppression of a Subset of Shoot PSR Genes by CAX1 and CAX3

To gain more insight into the molecular basis for P_i accumulation in *cax1/cax3*, we analyzed the expression profiles of *cax1/cax3* and ecotype Columbia (Col-0) wild-type plants grown in hydroponic solutions under $+P_i$ and $-P_i$ conditions using the Affymetrix ATH1 chip. The numbers of differentially expressed genes ($P \leq 0.01$, more than 2-fold change) were identified as follows: 854 and 1,047 under $+P_i$ and $-P_i$ conditions, respectively, between *cax1/cax3* and wild-type shoots; and 84 and 655 under $+P_i$ and $-P_i$ conditions, respectively, between *cax1/cax3* and wild-type roots (Supplemental Table S1, subgroups 1–4). We also determined the number of PSR genes with more than 2-fold change in expression ($P \leq 0.01$) between the $+P_i$ and $-P_i$ conditions (Supplemental Table S1, subgroups 5–8) and compared the expression of the PSR genes between *cax1/cax3* and wild-type plants. To evaluate the effects of our P_i starvation regimes on gene expression in wild-type plants, we plotted the fold change ratio of the differentially regulated shoot and root PSR genes from our microarray data against that reported by Morcuende et al. (2007), who subjected liquid culture-grown whole seedlings to P_i deprivation under continuous light. Despite different plant growth stages and growth conditions applied in these two studies, there is a clear positive correlation in the comparison as revealed by the r^2 value (Supplemental Fig. S1).

None of the wild-type shoot PSR genes (0 of 163) and only 2.6% (12 of 455) of the wild-type root PSR genes were regulated in the same fashion in *cax1/cax3* (Fig. 5A). Strikingly, in the shoot of *cax1/cax3* under $+P_i$ conditions, 22.1% (32 of 145) of the wild-type PSi genes were up-regulated and 22.2% (4 of 18) of the wild-type P_i starvation-repressed genes were down-regulated (Fig. 5B), indicating that one-fifth of the PSR genes are constitutively activated in the shoot of *cax1/cax3*. Among these 36 differentially expressed PSR genes (Table I), we further validated the up-regulation of several PSi genes, including genes implicated in P_i signaling, *SPX1* (At5g20150) and *SPX3* (At2g45130), and *PHT1;4* (At2g38940), a member of the P_i high-affinity transporter (*PHT1*) gene family, by qRT-PCR (Fig. 6, A–C; Supplemental Fig. S3). Although our microarray data did not reveal gene expression changes of other *PHT1* members, we were able to observe by qRT-PCR 4- to 5-fold increased transcript levels for *PHT1;1* (At5g43350) and *PHT1;3* (At5g43360) in the shoot of *cax1/cax3* under $+P_i$ conditions (data not shown). This discrepancy can be explained by the higher sensitivity of qRT-PCR, as the levels of *PHT1;1* and *PHT1;3* transcripts in the shoot were low. Up-regulation of these *PHT1* genes may reflect an increased sink demand for P_i, likely a downstream event of the CAX1/CAX3 P_i signaling cascade. When the expression of the PSR genes was examined in *cax1/cax3*, most of them, such as *SPX1*, *SPX3*, and *PHT1;4*, were less responsive to P_i starvation compared with wild-type plants (Fig. 6, A–C; Supplemental Table S1, subgroups 5 and 6). This may have resulted from their suppression by the high shoot P_i levels of *cax1/cax3* (Fig. 1A). Given that a significant proportion of PSR genes were constitutively activated in the shoot of *cax1/cax3*, we conclude that the function of CAX1 and CAX3 is required for the suppression of a discrete subset of shoot PSR genes under $+P_i$ conditions.

In contrast to the gene expression profile in the shoot of *cax1/cax3* under $+P_i$ conditions, only 2% (seven of 343) of the wild-type PSi genes and 1.8% (two of 112) of the wild-type P_i starvation-repressed genes were up-regulated and down-regulated, respectively, in the root of *cax1/cax3* under $+P_i$ conditions (Fig. 5B; Table I). These results seem contradictory to our speculation that the increased P_i uptake rate in *cax1/cax3* may be caused by up-regulation of *PHT1* genes in the root, as many members in the *PHT1* P_i transporter family are expressed preferentially in root epidermal or cortical cells and function in P_i acquisition (Muchhal and Raghothama, 1996; Mudge et al., 2002). Both results of RT-PCR (Fig. 6D) and qRT-PCR (data not shown) analyses showed that the transcript levels of *PHT1* genes were not increased under $+P_i$ or under $-P_i$

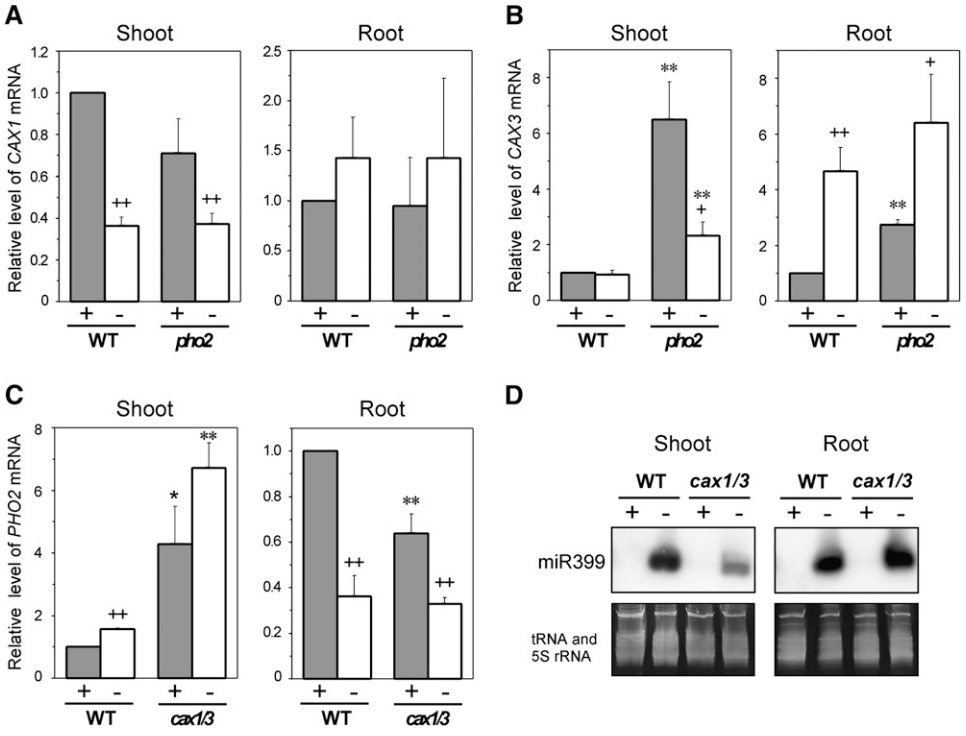

Figure 4. P_i starvation regulation of *CAX1*, *CAX3*, *PHO2*, and miR399 expression in the *cax1/cax3* and *pho2* mutants. A and B, qRT-PCR analysis of *CAX1* (A) and *CAX3* (B) expression in the shoot and root of wild-type (WT) and *pho2* plants. C and D, qRT-PCR analysis of *PHO2* expression (C) and small RNA gel-blot analysis of mature miR399 (D) in the shoot and root of wild-type and *cax1/cax3* plants. Plants were hydroponically grown under $+P_i$ (+) or $-P_i$ (−) conditions. The value for each gene is presented as the fold change relative to the expression in wild-type plants under $+P_i$ conditions. tRNA and 5S rRNA were used as loading controls for small RNA. Error bars represent SE of biological replicates from three independent experiments. Data significantly different from the corresponding controls are indicated (mutant versus the wild type, * $P < 0.05$, ** $P < 0.01$; P_i sufficient versus P_i deficient, $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$; Student's *t* test).

conditions in the root of *cax1/cax3* as compared with the wild-type controls, indicating that a posttranscriptional regulation of the *PHT1* or other unidentified P_i transporters accounts for enhanced P_i transport activity in *cax1/cax3*.

Since the P_i-replete *pht1;1* mutant showed reduced P_i uptake activity, PHT1;1 was suggested to play a primary role in P_i acquisition under $+P_i$ conditions (Shin et al., 2004). Thus, we next examined whether PHT1;1 is up-regulated at the protein level in *cax1/cax3*. Because of the high homology in protein sequence (94%–98% identity) among PHT1;1, PHT1;2, and PHT1;3, we raised an antibody against all three proteins. While the levels of PHT1;1/2/3 were increased in wild-type seedlings in response to low P_i availability, they were greatly decreased in the $+P_i$ but not $-P_i$ root of the *pht1;1* knockout mutant (Fig. 7A). This validated the specificity of this antibody against PHT1;1/2/3 and supported the conclusion that PHT1;1 is the major P_i transporter accountable for P_i acquisition under $+P_i$ conditions (Shin et al., 2004). Surprisingly, we did not observe much difference in the protein level of PHT1;1/2/3 in the $+P_i$ root between *cax1/cax3* and the wild type (Fig. 7B). Taken together, the enhanced P_i uptake activity in *cax1/cax3* does not result from an up-regulation of PHT1;1/2/3 at the protein level.

Shoot-Derived Signals Are Responsible for the High Accumulation of P_i in the *cax1/cax3* Mutant

Since our microarray data revealed the activation of about 22% of PSR genes in the shoot of *cax1/cax3* under $+P_i$ conditions, we asked next whether a shoot-derived P_i starvation signal mediates the enhanced P_i uptake activity resulting in P_i accumulation of *cax1/cax3*. To address this issue, we performed reciprocal micrografting between *cax1/cax3* and wild-type plants. When grown under $+P_i$ conditions, the grafted plants with *cax1/cax3* scions and wild-type rootstocks resembled the phenotype of P_i toxicity and stunted growth seen in the *cax1/cax3* mutant, whereas the grafted plants with wild-type scions and *cax1/cax3* rootstocks showed a wild-type phenotype (Fig. 8A). In accordance with the phenotype, while *cax1/cax3* scions grafted on wild-type rootstocks exhibited high shoot P_i levels (Fig. 8B) and P_i uptake activity (data not shown) as *cax1/cax3* self-grafts, wild-type scions grafted

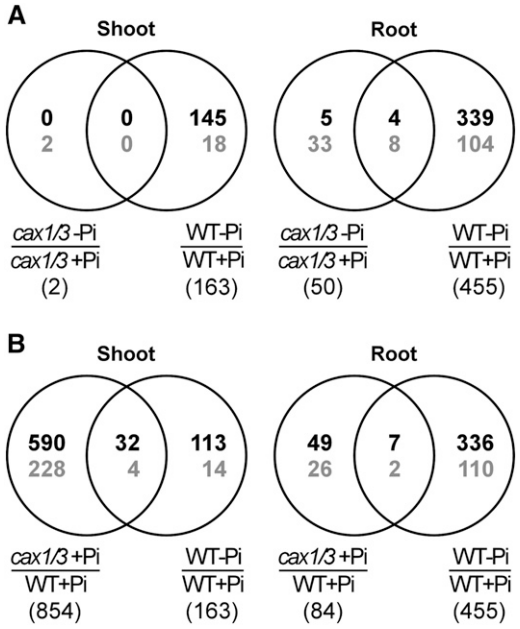

Figure 5. The number of genes differentially expressed in *cax1/cax3* and wild-type (WT) plants under P_i-sufficient and P_i-deficient conditions. A, Overlap of the PSR genes between *cax1/cax3* and wild-type plants. B, Overlap of wild-type PSR genes and genes differentially expressed between *cax1/cax3* and wild-type plants under $+P_i$ conditions. Numbers designate the genes with significantly differential expression ($P \leq 0.01$ and more than 2-fold change) between the indicated data sets derived from microarray analysis. The total number of genes in each data set is shown in parentheses. The numbers of induced and repressed genes are indicated in black and gray, respectively.

on *cax1/cax3* rootstocks behaved in a similar way as wild-type self-grafts (Fig. 8B). Although the protein amount of PHT1;1/2/3 was not increased in the $+P_i$ root of *cax1/cax3*, we checked whether a posttranslational regulation of PHT1;1 is involved in activating P_i uptake of *cax1/cax3* by grafting *cax1/cax3* scions on *pht1;1* rootstocks. The *pht1;1* self-grafts showed slightly but significantly reduced shoot P_i levels compared with the grafted plants with *pht1;1* scions and *cax1/cax3* rootstocks (Fig. 8C). Likewise, the shoot P_i level of the grafted plants with *cax1/cax3* scions and *pht1;1* rootstocks was also modestly reduced compared with *cax1/cax3* self-grafts (Fig. 8C), suggesting that lack of PHT1;1 in the rootstock is sufficient to partially repress P_i accumulation in the *cax1/cax3* scion.

To further elucidate the systemic role of CAX1 and CAX3 in P_i signaling, split-root experiments were carried out, in which one half of the root system of a single plant was grown in $+P_i$ medium and the other half in $-P_i$ medium (designated as $+/-P_i$ plants). Plants grown in $+P_i$ and $-P_i$ media were included as controls. As expected, in the $+P_i$ and $-P_i$ controls, the expression of *SPX1*, *PHT1;4*, and *At4* was increased in $+P_i$ *cax1/cax3* shoots as compared with the wild type, but their induction by P_i deficiency in *cax1/cax3* was not as high as in the wild type in both roots and shoots (Supplemental Fig. S2). The expression level of these PSi genes in the shoot of $+/-P_i$ *cax1/cax3* and wild-type plants showed an intermediate level between their own $+P_i$ and $-P_i$ controls (Supplemental Fig. S2, A, C, and E), reflecting the intermediate shoot P_i levels of the $+/-P_i$ plants (data not shown). In agreement with earlier observations of systemic down-regulation of several PSi genes in the $-P_i$ root half of the split-root system (Burleigh and Harrison, 1999; Franco-Zorrilla et al., 2005; Thibaud et al., 2010), we found that the induction of *SPX1*, *PHT1;4*, and *At4* in the $-P_i$ root half was suppressed in both $+/-P_i$ wild-type and $+/-P_i$ *cax1/cax3* plants in a similar fashion (Supplemental Fig. S2, B, D, and F). This suggests that the systemic P_i signal transmitting from the $+P_i$ root half via the shoot to the $-P_i$ root half is not impaired in *cax1/cax3*.

Taken together, our results here suggest that the *cax1/cax3* scion genotype is responsible for high levels of P_i accumulation as a result of increased P_i uptake in the wild-type rootstocks and that a signal originating from *cax1/cax3* shoots may move across grafting junctions to activate the P_i transport activity in roots, likely in part through posttranslational up-regulation of PHT1;1. By contrast, CAX1 and CAX3 appear not to be involved in the generation of the putative systemic suppressor in $+P_i$ roots and its long-distance movement.

DISCUSSION

CAX1- and CAX3-Mediated Regulation of Leaf Calcium Ion Homeostasis Is Required for Systemic P_i Homeostasis

To maintain the $[Ca^{2+}]_{cyt}$ levels in the micromolar range (Marty, 1999), plant cells transport Ca^{2+} out of the cytoplasm across the plasma membrane or into various organelles such as endoplasmic reticulum, chloroplast, and vacuole (Sze et al., 2000). CAX1 and CAX3 are tonoplast-localized Ca^{2+}/H^+ antiporters that mediate the sequestration of Ca^{2+} into the vacuole (Cheng et al., 2003, 2005). Knockout or overexpression of *CAX1* or *CAX3* alone in planta has been shown to result in perturbations in ion homeostasis and altered responses to salinity and cold stresses, while loss of both CAX1 and CAX3 led to a severe reduction in growth, leaf tip and flower necrosis, and a pronounced sensitivity to exogenous Ca^{2+} and other ions (Hirschi, 1999; Catala et al., 2003; Cheng et al., 2003, 2005; Mei et al., 2007; Zhao et al., 2009). Intriguingly, alterations in transport properties resulting from overexpression of both CAXs in yeast could not be recapitulated by high-level expression of either transporter individually (Zhao et al., 2009). It was postulated that the differential stress sensitivities of *cax* mutants are due to specific responses by CAX1 or CAX3 to individual stresses or to distinct transport properties conferred by hetero-CAX complexes formed by CAX1 and CAX3 (Cheng et al., 2005; Zhao et al., 2009). If we suppose that loss of the putative CAX1-CAX3 heteromer is

Table I. *Misregulated PSR genes in cax1/cax3*

AGI[a]	Fold Change in Expression		Gene Description
	$-P_i$:$+P_i$ in the Wild Type	*cax1/cax3*:Wild Type under $+P_i$	
Shoots			
At5g20790	88.4	5.6	Unknown protein
At1g73010	62.9	17.1	Pyrophosphate-specific phosphatase 1 (PPsPase1)
At2g38940/At3g54700	29.1	11.4	Phosphate transporter (PHT1;4/PHT1;7)
At5g20150	27.6	10.9	SPX domain-containing protein (SPX1)
At3g47420	20.7	9.9	Glycerol-3-phosphate transporter
At3g17790	18.5	12.1	Acid phosphatase type 5 (ACP5)
At1g23140	14.8	4.0	C2 domain-containing protein
At3g05630	13.6	4.9	Phospholipase Dζ2 (PLDζ2)
At1g17710	13.1	3.2	Putative phosphatase
At2g45130	10.1	6.9	SPX domain-containing protein (SPX3)
At4g31240	8.6	7.8	Protein kinase C-like zinc finger protein
At5g20400	4.6	2.3	Oxoglutarate/iron-dependent oxygenase
At3g11670	3.9	2.1	Digalactosyldiacylglycerol synthase (DGD1)
At1g61800	3.8	22.8	Glc-6-P/phosphate-translocator (GPT2)
At4g33550	3.4	2.7	Lipid transfer protein
At4g19880	3.1	2.1	Glutathione *S*-transferase-related protein
At4g35750	3.0	2.1	ρ-GTPase-activating protein-related protein
At5g44240	2.9	2.7	ATPase, Ca^{2+}-transporting (ALA2)
At2g46680	2.7	11.0	Homeobox-Leu zipper protein 7 (HB-7)/HD-ZIP transcription factor 7
At3g20250	2.7	3.3	RNA-binding protein
At3g56400	2.7	2.6	WRKY family transcription factor (WRKY70)
At1g72890	2.7	5.0	Similar to disease resistance protein
At1g80130	2.6	2.1	Unknown protein
At2g36790/At2g36800	2.5	2.3	Putative glucosyl transferase
At1g22070	2.4	2.0	bZIP transcription factor (TGA3/AtbZIP22)
At5g51070	2.3	2.0	ATP-dependent Clp protease ATP-binding subunit (ClpD), (ERD1)
Atcg00780	2.3	3.4	Ribosomal protein L14 (rpl14)
At1g65660	2.3	2.3	Zinc knuckle (CCHC-type) family protein; putative step II splicing factor
At1g72830	2.2	3.0	CCAAT-binding transcription factor B subunit (CBF-B/NF-YA)
At1g22930	2.2	3.0	Unknown protein
At2g30500	2.1	4.9	Kinase-interacting family protein
At2g17290	2.0	3.9	Ca^{2+}-dependent protein kinase isoform 6 (CPK6)
At2g19970	0.43	0.48	Putative pathogenesis-related protein
At5g49560	0.42	0.44	Putative methyltransferase family protein
At4g16563	0.4	0.05	Putative aspartyl protease family protein
At1g10550	0.3	0.11	Xylogucan:xyloglusyl transferase 33 (XTH33)
Roots			
At2g30540	11.9	2.1	Putative glutaredoxin
At2g21900	4.0	2.2	WRKY family transcription factor (WRKY59)
At4g01390	4.0	3.7	TRAF-like family protein
At2g18050	3.4	3.7	Histone H1-3 (HIS1-3)
At5g13580	3.2	2.1	ABC transporter-like protein
At1g28480	2.6	2.4	GRX480, a member of the glutaredoxin family
At4g14060	2.2	2.6	Major latex protein (MLP)-related protein
At5g59305	0.3	0.3	Unknown protein
At4g25100	0.3	0.3	Fe superoxide dismutase (FSD1)

[a]Arabidopsis Genome Initiative number. Genes validated by qRT-PCR are underlined.

responsible for the enhanced P_i uptake in *cax1/cax3*, then lack of either CAX1 or CAX3 should have also rendered an increased P_i uptake activity. However, our findings here suggest a functional redundancy of CAX1 and CAX3 regarding their role in regulating P_i uptake, because neither the *cax1* nor the *cax3* single mutant exhibits an increased uptake rate of P_i. Similarly, no significant change in total leaf [Ca^{2+}] in either the *cax1* or the *cax3* single mutant was observed (Cheng et al., 2005). Thus, one possible explanation for the impaired P_i homeostasis in *cax1/cax3* is that disruption of both vacuolar Ca^{2+} transporters leads to

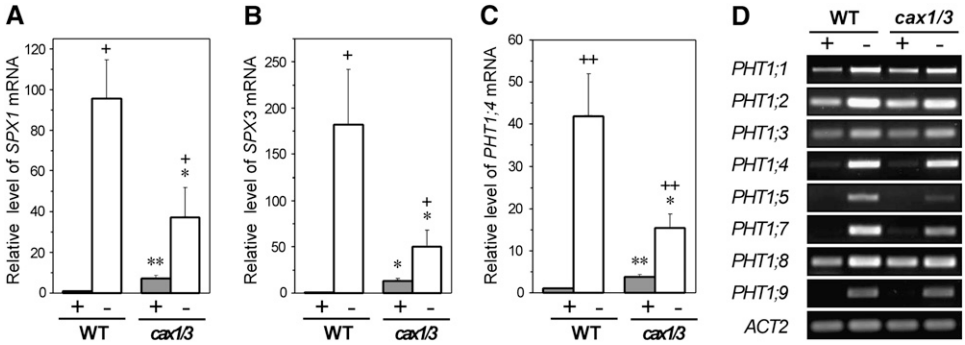

Figure 6. Gene expression of *SPX1*, *SPX3*, and *PHT1;4* P_i transporter in the *cax1/cax3* mutant. A to C, qRT-PCR analysis of *SPX1* (A), *SPX3* (B), and *PHT1;4* (C) in the shoot of wild-type (WT) and *cax1/cax3* plants under $+P_i$ (+) or $-P_i$ (−) conditions. The value for each gene is presented as the fold change relative to the expression of wild-type plants under $+P_i$ conditions. Error bars represent SE of biological replicates from three independent experiments. Data significantly different from the corresponding controls are as indicated (mutant versus the wild type, * $P < 0.05$, ** $P < 0.01$; P_i sufficient versus P_i deficient, $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$; Student's *t* test). D, RT-PCR analysis of members in the *PHT1* gene family in the root of wild-type and *cax1/cax3* plants under $+P_i$ (+) or $-P_i$ (−) conditions.

elevated levels of $[Ca^{2+}]_{cyt}$ and/or aberrant subcellular compartmentation of $[Ca^{2+}]$ due to the inability to move excess cytosolic Ca^{2+} into the vacuole. Although approximately 50% of wild-type vacuolar Ca^{2+}/H^+ transport activity was reported for *cax1/cax3* following pretreatment with exogenous Ca^{2+} (Cheng et al., 2005), whether the $[Ca^{2+}]_{cyt}$ level is indeed increased in *cax1/cax3* has not yet been demonstrated.

Recently, Conn et al. (2011b) convincingly demonstrated that the severe phenotypes of *cax1/cax3*, such as reduced leaf growth rate, mainly result from the increased leaf apoplastic free $[Ca^{2+}]$ rather than from the reduced vacuolar $[Ca^{2+}]$ of mesophyll cells. Their findings prompted us to suspect that the abrogated cellular Ca^{2+} homeostasis in the leaf of *cax1/cax3* brings about misregulation of P_i homeostasis. As qRT-PCR analysis revealed that the transcript level of *CAX1* in shoot was decreased upon P_i limitation, it is likely that the leaf vacuolar sequestration of Ca^{2+} by CAX1 is down-regulated under P_i deficiency (Fig. 4A). We thus surmised that simultaneous loss of *CAX1* and *CAX3*, a gene ectopically expressed in leaves upon knockout of CAX1 (Cheng et al., 2003, 2005), may mimic the demands on reduction of the shoot vacuolar Ca^{2+}/H^+ transport activity under P_i deficiency, accompanied by activation of a subset of shoot PSR genes (Fig. 5B). By contrast, since *CAX4* is primarily expressed in root tissues and up-regulated in the *cax1* mutant (Cheng et al., 2003), the increased expression of *CAX4* may compensate for the functional role of CAX1 and CAX3 in the root of *cax1/cax3*, hinting at why activation of the PSR genes was not observed in the root of *cax1/cax3* (Fig. 5B). In fact, overexpression of *CAX4* was able to partially suppress the *cax1* defect in vacuolar Ca^{2+}/H^+ transport (Zhao et al., 2009).

Notably, *cax1/cax3* displays 47% and 20% reductions, respectively, in V-ATPase and P-ATPase activities (Cheng et al., 2005; Zhao et al., 2008). As these H^+-ATPases generate a pH gradient across membranes that provides the driving force for the H^+-coupled transporters and contributes to the maintenance of the cytosolic pH homeostasis, whether the complex interaction of H^+-ATPases with other transporters and/or the resulting impaired pH homeostasis is associated with the activation of P_i transporters remains obscure. It is important to note that the *vha-a2/vha-a3* double mutant, which lacks the tonoplast V-ATPase, was shown to contain reduced Ca^{2+} levels in leaves and to display symptoms of Ca^{2+} deficiency similar to *cax1/cax3* (Krebs et al., 2010). Moreover, it has been demonstrated that cytosolic Ca^{2+} homeostasis is a constitutive function of the yeast V-ATPase. Cellular responses to a brief Ca^{2+} challenge were affected not only by an acute loss of V-ATPase activity (in temperature-sensitive *vma* mutants or in wild-type cells treated with a V-ATPase inhibitor) but also by a permanent loss of V-ATPase activity in a *vma* deletion mutant (Förster and Kane, 2000). In the future, it would be interesting to determine whether V-ATPase contributes to the impaired P_i homeostasis of the *cax1/cax3* mutant through the misregulation of Ca^{2+} homeostasis.

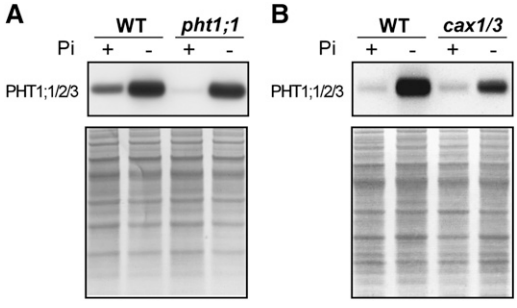

Figure 7. Analysis of PHT1;1/2/3 protein in the root of *pht1;1* seedlings (A) and *cax1/cax3* hydroponically grown plants (B) under $+P_i$ (+) and $-P_i$ (−) conditions. The bottom panels show the protein staining on the membrane. WT, Wild type.

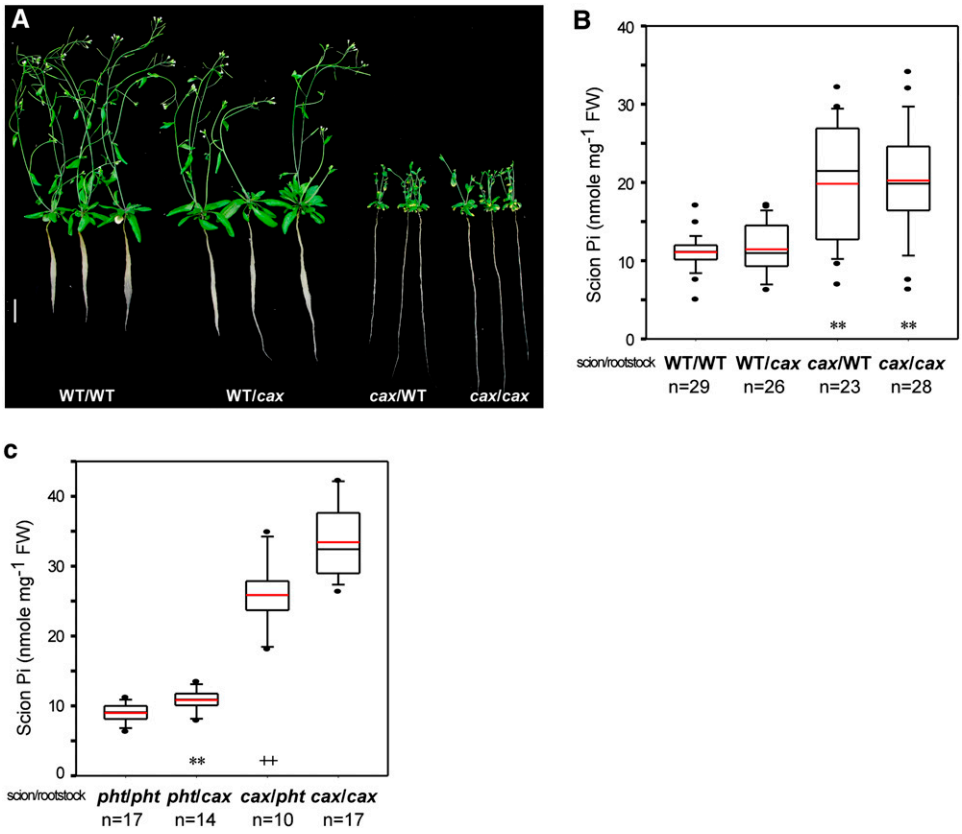

Figure 8. Analysis of reciprocal grafting of wild-type (WT), *pht1;1*, and *cax1/cax3* plants. Shown are the phenotypes (A; bar = 1 cm) and the scion P_i concentrations of 6-week-old grafted plants between wild-type and *cax1/cax3* plants (B) and between *pht1;1* and *cax1/cax3* plants (C). The P_i concentrations are presented as box plots. The boundaries of the boxes indicate the 25th and 75th percentiles. The median and the mean are marked by black and red lines, respectively, within the box. Error bars above and below the box indicate the 90th and 10th percentiles. Each individual plant outside the 10th and 90th percentiles is displayed as a single dot. In B, mean values significantly different from the wild-type self-grafts are indicated by asterisks (** $P < 0.01$; Student's *t* test). In C, mean values showing significant differences between the *pht1;1* self-grafts and the grafts with the *pht1;1* scion and the *cax1/cax3* rootstock (** $P < 0.01$; Student's *t* test) and between the *cax1/cax3* self-grafts and the grafts with *cax1/cax3* scion and *pht1;1* rootstock ($^{++}$ $P < 0.01$; Student's *t* test) are indicated by asterisks and crosses, respectively. n, The total number of grafted plants.

Besides high P_i accumulation, disturbance of other ion homeostases, such as increased levels of Mn^{2+} and Zn^{2+} and decreased levels of Mg^{2+}, has also been reported for *cax1/cax3* (Cheng et al., 2005). Although an interplay between these various ions within *cax1/cax3* cannot be excluded from participating in P_i signaling, our results that P_i accumulation of *cax1/cax3* was exacerbated upon exogenous supplement of Ca^{2+} support the hypothesis that leaf Ca^{2+} homeostasis is directly involved in P_i signaling. It is unclear why the growth retardation and P_i accumulation of *cax1/cax3* can be alleviated when high concentrations of Mg^2 are added to growth medium. However, it was argued that with a supplement of Mg^{2+} to growth medium, more Mg^{2+} is sequestered to the vacuole from the cytoplasm to compensate for the reduced vacuolar [Ca^{2+}] in *cax1/cax3* (Conn et al., 2011a).

Intriguingly, it is known that Ca^{2+} tends to precipitate with P_i, rendering the soil P unavailable for plant acquisition (Hinsinger, 2001). This aspect is noteworthy, considering that the excess cytosolic Ca^{2+} in the shoot of *cax1/cax3* may potentially decrease the available shoot P_i, which may alternatively but not perfectly explain why only a subset of the shoot PSR genes are activated in *cax1/cax3* under $+P_i$ conditions. However, x-ray microanalysis showed that Arabidopsis Col-0 plants preferentially accumulate Ca in the vacuoles of mesophyll cells but P within vacuoles of the epidermis and bundle sheath (Conn et al., 2011b). Cell-specific compartmentation of these two elements makes this possibility unlikely.

Loss of Function of *CAX1* and *CAX3* Disturbs the Regulation of PSR and Calcium-Related Gene Expression in the Shoot

Under $+P_i$ conditions, one-fifth of the PSR genes are constitutively activated in the shoot of *cax1/cax3*,

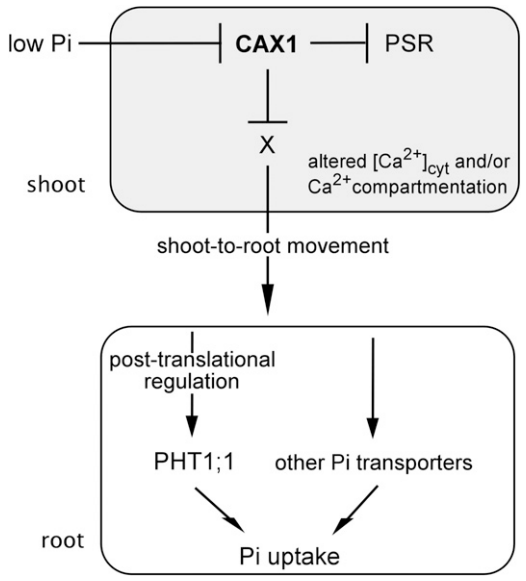

Figure 9. Working hypothesis of the CAX1/CAX3-mediated P_i signaling pathway in Arabidopsis. When P_i is limited, repression of shoot CAX1 leads to abrogated cellular Ca^{2+} homeostasis involving altered $[Ca^{2+}]_{cyt}$ and/or misregulated compartmentation of Ca^{2+}, which initiates the activation of a subset of shoot PSR genes. A shoot-born signal (X) is thereby generated and moves toward the root to activate the P_i transporter system, including PHT1;1, at the posttranslational level.

including acid phosphatase type 5 (*ACP5*; At3g17790), digalactosyldiacylglycerol synthase (*DGD1*; At3g11670), phospholipase Dζ2 (*PLDζ2*; At3g05630), *SPX1*, and *SPX3* (Table I). ACP5 has been proposed to be involved in P_i mobilization (del Pozo et al., 1999), whereas DGD1 and PLDζ2 participate in the biosynthesis of nonphosphorus lipids during P_i-limited growth (Härtel et al., 2000; Cruz-Ramírez et al., 2006). Loss of PLDζ1 and PLDζ2 reduces primary root elongation under low-P_i conditions (Li et al., 2006), but their roles in the shoot remain unknown. Nucleus-localized SPX1 was suggested to be involved in transcriptional activation of genes related to P_i mobilization and scavenging of reactive oxygen species in response to P_i starvation (Duan et al., 2008). By contrast, SPX3 was shown to localize to intracellular compartments. *SPX3* RNA interference lines exhibited higher total P and P_i contents yet stronger induction of several PSi genes, including *SPX1*, suggesting that SPX3 negatively regulates P_i starvation signaling (Duan et al., 2008). Up-regulation of both genes in the shoot of *cax1/cax3* under $+P_i$ conditions may reflect the activation of the SPX1/SPX3-mediated P_i signaling pathway. Moreover, a group of genes encoding Ca^{2+}-ATPases, including *ACA2* (At4g37640), *ACA10* (At4g29900), *ACA11* (At3g57330), *ACA12* (At3g63380), and *ACA13* (At3g22910), were up-regulated in the shoot but not in the root of *cax1/cax3* (Supplemental Table S2), implying that Ca^{2+}-ATPases are activated to balance the decreased Ca^{2+} efflux in the shoot due to the dysfunction of CAX1 and CAX3. Therefore, it seems that the disturbed cellular Ca^{2+} homeostasis and misregulated expression of Ca^{2+}-related proteins caused by lack of CAX1 and CAX3, rather than CAX1 and CAX3 per se, are involved in transcriptional regulation of PSR genes.

Common and Distinct P_i Signaling Pathways Mediated by PHO2 and CAX

Because both *pho2* and *cax1/cax3* mutants displayed an increased level of shoot P_i, it is tempting to know whether PHO2 and CAX1/CAX3 act in the same P_i signaling pathway. Down-regulation of *PHO2* in the roots results in increased uptake and root-to-shoot translocation of P_i; however, the role of *PHO2* in the shoots is unclear (Lin et al., 2008). Here, we observed that the *PHO2* transcript level was greatly increased in the shoot of *cax1/cax3* under $+P_i$ and $-P_i$ conditions, whereas the level in the root of *cax1/cax3* under $+P_i$ conditions was slightly reduced but still higher than that in the $-P_i$ root of wild-type plants (Fig. 4C). Given that, under P_i deficiency, the level of *PHO2* transcript in the root of *cax1/cax3* was as low as that in the root of wild-type plants (Fig. 4C), we argue that *PHO2* is not involved in enhancing the P_i uptake activity in *cax1/cax3* under $-P_i$ conditions. Although both *CAX1/CAX3* and *PHO2* inhibited the root P_i uptake activity, they seem to mediate different pathways leading to P_i accumulation. First, in contrast to *pho2*, which exhibits a dramatically increased P_i uptake activity only when P_i remains adequate, *cax1/cax3* displayed an enhanced P_i uptake activity under both $+P_i$ and $-P_i$ conditions (Fig. 2, A and B). Notably, although the increase of P_i uptake activity of *cax1/cax3* under $-P_i$ conditions was striking, the underlying mechanism is not clear at present. Second, unlike the *pho2* mutant, the *cax1/cax3* mutant had a similar shoot-to-root ratio of P_i distribution as the wild type under $+P_i$ conditions (Fig. 2C). Third, up-regulation of miR399, which acts upstream of the PHO2-dependent P_i signaling pathway, was not involved in the moderate reduction of *PHO2* mRNA level in the root of *cax1/cax3* under $+P_i$ conditions (Fig. 4, C and D). These lines of evidence suggest that CAX1/CAX3 and miR399-mediated PHO2 P_i signaling pathways are two distinct pathways attributed to the consequences of P_i uptake and accumulation.

In the *pho2* mutant, the expression levels of *PHT1;8* and *PHT1;9* were shown to be increased under $+P_i$ conditions and assumed to contribute to the establishment of high P_i in the shoot of *pho2* (Aung et al., 2006; Bari et al., 2006). However, in the root of *cax1/cax3* under both $+P_i$ and $-P_i$ conditions, no up-regulation of *PHT1* genes at the transcript level was observed (Fig. 6D). Although the protein level of PHT1;1/2/3 was not increased (Fig. 7B), our results from grafting demonstrate that PHT1;1 is partially responsible for P_i accumulation in the *cax1/cax3* scion (Fig. 8C), indicating that a posttranslational regulation of PHT1;1 may be involved. Several consensus sites for *N*-linked glycosylation and phosphorylation have been predicted in PHT1;1 (Muchhal et al., 1996). Indeed, PHT1;1/2

have been identified in phosphoproteomics of the Arabidopsis plasma membrane, and a phosphorylation site was detected in its C-terminal peptide (Nühse et al., 2004; Hem et al., 2007). Furthermore, as we did not examine the subcellular localization of PHT1;1 in *cax1/cax3*, changes in the membrane distribution of PHT1;1 cannot be excluded.

CAX1/CAX3-Mediated Shoot-to-Root P_i Signaling

A paradigm for systemic regulation of P_i homeostasis has been recently established. The miR399 generated in shoots after the onset of P_i starvation serves as a long-distance signal to activate P_i transport systems by suppressing *PHO2* expression in roots (Lin et al., 2008; Pant et al., 2008). Our results obtained in split-root experiments indicate that CAX1 and CAX3 do not mediate the generation and movement of systemic suppressors from the $+P_i$ root half to the $-P_i$ root half. By contrast, the results of reciprocal grafting experiments clearly suggest the involvement of CAX1 and CAX3 in shoot-to-root P_i signaling. We hypothesize that abrogation of CAX1 and CAX3 in the *cax1/cax3* mutant may relieve the repression of shoot PSR genes through alteration of $[Ca^{2+}]_{cyt}$ and/or misregulated compartmentation of Ca^{2+}, thereby triggering a systemic signal that moves from shoots to roots to activate the PHT1 P_i transporters or other unidentified P_i transport systems (Fig. 9). Because mature miR399 was not observed in the shoot and root of *cax1/cax3* under $+P_i$ conditions, it is clear that miR399 is not up-regulated to serve as a systemic signal traveling to roots in the CAX1/CAX3-mediated P_i signaling pathway. Several molecules, including hormones, sugars, nutrients themselves or their metabolites, and small RNAs, have been suggested as systemic signals in the long-distance signaling of nutrient status (Liu et al., 2009). In the future, it will be interesting to identify such a shoot-derived signal and the molecular components involved in the up-regulation of the P_i transport system and to establish how those PSR genes misregulated in *cax1/cax3* can modulate the P_i transport activity.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of Arabidopsis (*Arabidopsis thaliana*) *cax1*, *cax3*, and *cax1/cax3* were kindly provided as gifts by Drs. Kendal Hirschi and Ning-Hui Cheng (Baylor College of Medicine). Seeds of the wild type (Col-0) and the *pht1;1* T-DNA line (SALK_088586) were obtained from the Arabidopsis Biological Resource Center. The $+P_i$ and $-P_i$ media were supplemented with 250 μM KH_2PO_4 and 0 μM KH_2PO_4, respectively, unless specified otherwise. For hydroponic growth, 9-d-old seedlings grown on agar plates with half-modified Hoagland nutrient solution containing 250 μM KH_2PO_4 and 1% Suc solidified with 0.8% agar were transferred to the same nutrient solution containing 250 μM KH_2PO_4 without Suc for 8 to 10 d. A 5-d treatment of P_i starvation was initiated by replacing 250 μM KH_2PO_4 with P_i-free medium. For plants grown in the medium supplemented with Ca^{2+} and Mg^{2+}, 9-d-old seedlings grown on agar plates were transferred to hydroponic medium supplemented with various concentrations of Ca^{2+} and Mg^{2+} as indicated and grown for another 12 d. All plants were grown under a 16-h-light/8-h-dark cycle.

Grafting of Arabidopsis Plants

Hypocotyl reciprocal grafting was performed as described previously with minor modifications (Lin et al., 2008). Briefly, micrografting was conducted with 8-d-old seedlings, which were then incubated vertically in the dark for 1 d before being transferred to the culture room under dim light for another 2 d. Two weeks after micrografting, plants were transferred to hydroponic culture and grown for another 2 weeks before sample collection. Lack of contamination of adventitious roots in grafted plants was confirmed by genotyping using PCR.

Affymetrix ATH1 Array Hybridization and Data Analysis

Transcriptomic analyses of plants were conducted using Affymetrix ATH1 arrays. Wild-type and *cax1/cax3* plants grown in hydroponic cultures under $+P_i$ and $-P_i$ conditions (see above) were harvested for RNA isolation. Two independent biological replicates were performed. Ten micrograms of total RNA (see below) was used for cDNA synthesis, labeled by in vitro transcription, and followed by fragmentation according to the manufacturer's recommendations (GeneChip Expression Analysis Technical Manual, Revision 5; Affymetrix). The labeled samples were hybridized to the ATH1 array at 45°C for 16.5 h. Washing and staining were done on a Fluidics Station-450, and the ATH1 array was scanned using the Affymetrix GeneChip Scanner 7G. The results were quantified and analyzed using MicroArray Suite 5.0 software (Affymetrix). The obtained data were normalized using Robust Multichip Average (Irizarry et al., 2003), and the statistical significance of differential expression was determined by Limma analysis (Smyth, 2004).

P_i Concentration and P_i Uptake Analysis

P_i concentration and uptake activity were determined as described (Chiou et al., 2006). To assay the P_i uptake, 4-week-old plants grown under $+P_i$ or $-P_i$ conditions were transferred to medium containing 250 μM KH_2PO_4 ($+P_i$) or 10 μM KH_2PO_4 ($-P_i$) for the measurement of $[^{33}P]P_i$ uptake.

RNA Isolation, RT-PCR, and qRT-PCR

Total RNA from hydroponic samples was isolated by the use of TRIzol reagent (Invitrogen) and treated with DNase I (Ambion) before qRT-PCR to eliminate genomic DNA contamination. cDNA was synthesized from 0.5 to 1 μg of total RNA by use of Moloney murine leukemia virus reverse transcriptase (Promega) with oligo(dT) primer. RT-PCR conditions and sequences of primers used in our study were identical to those listed in supplemental table S1 of Aung et al. (2006). Sequences of additional primers are listed in Supplemental Table S3. qRT-PCR was performed using the Power SYBR Green PCR Master Mix kit (Applied Biosystems) on a 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. Relative expression levels were normalized to that of an internal control, *UBQ10*.

Immunoblot Analyses

For extraction of total protein, 10-d-old seedlings of wild-type and *pht1;1* plants with or without 5-d treatment of P_i deficiency were ground in liquid nitrogen and dissolved in protein lysis buffer (2% SDS, 60 mM Tris-HCl [pH 8.5], 2.5% glycerol, 0.13 mM EDTA, and 1× complete protease inhibitor [Roche]). Twenty micrograms of total protein was loaded onto the SDS-PAGE apparatus for each sample.

Hydroponically grown *cax1/cax3* and wild-type plants were harvested for total membrane protein extraction. One milligram of root tissues was ground with an ice-cold mortar and pestle and dissolved in 3 mL of membrane extraction buffer (330 mM Suc, 50 mM Tris [pH 7.5], 10 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor [Roche]). The extracts were then collected and centrifuged at 2,000*g* for 10 min at 4°C. Supernatants were collected and centrifuged at 400,000*g* for another 40 min at 4°C. Pellets were dissolved in membrane extraction buffer and collected as total membrane proteins. Twenty micrograms of total membrane protein was loaded onto the SDS-PAGE apparatus for each sample. Polyclonal rabbit antibodies were raised and affinity purified

against an internal fragment of PHT1;1 corresponding to amino acid residues 266 to 285 (ELEERVEDDVKDPRQNYGLF). The final concentration of 20 to 100 ng mL^{-1} affinity-purified antibodies was used for immunoblot analysis.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *UBQ10* (At4g05320), *PHT1;1* (At5g43350), *PHT1;2* (At5g43370), *PHT1;3* (At5g43360), *PHT1;4* (At2g38940), *PHT1;5* (At2g32830), *PHT1;7* (At3g54700), *PHT1;8* (At1g20860), *PHT1;9* (At1g76430), *PHO2* (At2g33770), *CAX1* (At2g38170), *CAX3* (At3g51860), *At4* (At5g03545), *SPX1* (At5g20150), and *SPX3* (At2g45130).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of 163 and 455 differentially expressed genes (more than 2-fold change) in wild-type shoots and roots under $-P_i$ conditions, respectively, with those reported by Morcuende et al. (2007).

Supplemental Figure S2. qRT-PCR analysis of *SPX1* (A and B), *PHT1;4* (C and D) and *At4* (E and F) expression in the shoot (A, C, and E) and root (B, D, and F) of split-root wild-type and *cax1/cax3* plants.

Supplemental Figure S3. qRT-PCR analysis of several PSR genes in the shoot of wild-type and *cax1/cax3* plants under $+P_i$ or $-P_i$ conditions.

Supplemental Table S1. Genes significantly differentially expressed ($P \leq 0.01$, more than 2-fold change) in data sets derived from microarray analysis.

Supplemental Table S2. Ca^{2+}-related genes that are differentially expressed in the shoot and root of *cax1/cax3* under P_i-sufficient conditions.

Supplemental Table S3. Sequences of qRT-PCR primers used in this study.

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LITERATURE CITED

Aung K, Lin SI, Wu CC, Huang YT, Su CL, Chiou TJ (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol **141:** 1000–1011

Bari R, Datt Pant B, Stitt M, Scheible WR (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol **141:** 988–999

Burleigh SH, Harrison MJ (1999) The down-regulation of *Mt4*-like genes by phosphate fertilization occurs systemically and involves phosphate translocation to the shoots. Plant Physiol **119:** 241–248

Catala R, Santos E, Alonso JM, Ecker JR, Martinez-Zapater JM, Salinas J (2003) Mutations in the Ca^{2+}/H^+ transporter CAX1 increase *CBF/DREB1* expression and the cold-acclimation response in *Arabidopsis*. Plant Cell **15:** 2940–2951

Cheng NH, Pittman JK, Barkla BJ, Shigaki T, Hirschi KD (2003) The *Arabidopsis cax1* mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters. Plant Cell **15:** 347–364

Cheng NH, Pittman JK, Shigaki T, Hirschi KD (2002) Characterization of CAX4, an Arabidopsis H^+/cation antiporter. Plant Physiol **128:** 1245–1254

Cheng NH, Pittman JK, Shigaki T, Lachmansingh J, LeClere S, Lahner B, Salt DE, Hirschi KD (2005) Functional association of Arabidopsis CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol **138:** 2048–2060

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su CL (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. Plant Cell **18:** 412–421

Chiou T-J, Lin S-I (2011) Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol **62:** 185–206

Conn SJ, Conn V, Tyerman SD, Kaiser BN, Leigh RA, Gilliham M (2011a) Magnesium transporters, MGT2/MRS2-1 and MGT3/MRS2-5, are important for magnesium partitioning within *Arabidopsis thaliana* mesophyll vacuoles. New Phytol **190:** 583–594

Conn SJ, Gilliham M, Athman A, Schreiber AW, Baumann U, Moller I, Cheng N-H, Stancombe MA, Hirschi KD, Webb AAR, et al (2011b) Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. Plant Cell **23:** 240–257

Cruz-Ramírez A, Oropeza-Aburto A, Razo-Hernández F, Ramírez-Chávez E, Herrera-Estrella L (2006) Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in *Arabidopsis* roots. Proc Natl Acad Sci USA **103:** 6765–6770

Delhaize E, Randall PJ (1995) Characterization of a phosphate-accumulator mutant of *Arabidopsis thaliana*. Plant Physiol **107:** 207–213

del Pozo JC, Allona I, Rubio V, Leyva A, de la Peña A, Aragoncillo C, Paz-Ares J (1999) A type 5 acid phosphatase gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J **19:** 579–589

Dong B, Rengel Z, Delhaize E (1998) Uptake and translocation of phosphate by *pho2* mutant and wild-type seedlings of *Arabidopsis thaliana*. Planta **205:** 251–256

Duan K, Yi K, Dang L, Huang H, Wu W, Wu P (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J **54:** 965–975

Förster C, Kane PM (2000) Cytosolic Ca^{2+} homeostasis is a constitutive function of the V-ATPase in *Saccharomyces cerevisiae*. J Biol Chem **275:** 38245–38253

Franco-Zorrilla JM, Martín AC, Leyva A, Paz-Ares J (2005) Interaction between phosphate-starvation, sugar, and cytokinin signaling in *Arabidopsis* and the roles of cytokinin receptors CRE1/AHK4 and AHK3. Plant Physiol **138:** 847–857

Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. Curr Biol **15:** 2038–2043

Hamburger D, Rezzonico E, MacDonald-Comber Petétot J, Somerville C, Poirier Y (2002) Identification and characterization of the *Arabidopsis PHO1* gene involved in phosphate loading to the xylem. Plant Cell **14:** 889–902

Härtel H, Dormann P, Benning C (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. Proc Natl Acad Sci USA **97:** 10649–10654

Hem S, Rofidal V, Sommerer N, Rossignol M (2007) Novel subsets of the Arabidopsis plasmalemma phosphoproteome identify phosphorylation sites in secondary active transporters. Biochem Biophys Res Commun **363:** 375–380

Hinsinger P (2001) Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil **237:** 173–195

Hirschi KD (1999) Expression of *Arabidopsis CAX1* in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell **11:** 2113–2122

Hirschi KD (2004) The calcium conundrum: both versatile nutrient and specific signal. Plant Physiol **136:** 2438–2442

Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000) Expression of Arabidopsis *CAX2* in tobacco: altered metal accumulation and increased manganese tolerance. Plant Physiol **124:** 125–133

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249–264

Knight H (2000) Calcium signaling during abiotic stress in plants. Int Rev Cytol **195:** 269–324

Koren'kov V, Park S, Cheng NH, Sreevidya C, Lachmansingh J, Morris J, Hirschi KD, Wagner GJ (2007) Enhanced Cd^{2+}-selective root-tonoplast-transport in tobaccos expressing Arabidopsis cation exchangers. Planta **225:** 403–411

Krebs M, Beyhl D, Görlich E, Al-Rasheid KA, Marten I, Stierhof YD,

Hedrich R, Schumacher K (2010) Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. Proc Natl Acad Sci USA **107:** 3251–3256

Li MY, Qin CB, Welti R, Wang XM (2006) Double knockouts of phospholipases Dζ1 and Dζ2 in Arabidopsis affect root elongation during phosphate-limited growth but do not affect root hair patterning. Plant Physiol **140:** 761–770

Lin SI, Chiang SF, Lin WY, Chen JW, Tseng CY, Wu PC, Chiou TJ (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiol **147:** 732–746

Lin WY, Lin SI, Chiou TJ (2009) Molecular regulators of phosphate homeostasis in plants. J Exp Bot **60:** 1427–1438

Liu F, Wang Z, Ren H, Shen C, Li Y, Ling HQ, Wu C, Lian X, Wu P (2010) OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of OsPT2 and phosphate homeostasis in shoots of rice. Plant J **62:** 508–517

Liu TY, Chang CY, Chiou TJ (2009) The long-distance signaling of mineral macronutrients. Curr Opin Plant Biol **12:** 312–319

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London

Marty F (1999) Plant vacuoles. Plant Cell **11:** 587–600

McAinsh MR, Pittman JK (2009) Shaping the calcium signature. New Phytol **181:** 275–294

Mei H, Zhao J, Pittman JK, Lachmansingh J, Park S, Hirschi KD (2007) *In planta* regulation of the Arabidopsis Ca^{2+}/H^{+} antiporter CAX1. J Exp Bot **58:** 3419–3427

Miseta A, Kellermayer R, Aiello DP, Fu L, Bedwell DM (1999) The vacuolar Ca^{2+}/H^{+} exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S. cerevisiae*. FEBS Lett **451:** 132–136

Morcuende R, Bari R, Gibon Y, Zheng WM, Pant BD, Bläsing O, Usadel B, Czechowski T, Udvardi MK, Stitt M, et al (2007) Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. Plant Cell Environ **30:** 85–112

Muchhal US, Pardo JM, Raghothama KG (1996) Phosphate transporters from the higher plant Arabidopsis thaliana. Proc Natl Acad Sci USA **93:** 10519–10523

Muchhal US, Raghothama KG (1996) Cloning and characterization of two high affinity phosphate transporter homologues in *Arabidopsis thaliana*. Plant Physiol **111:** 435

Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. Plant J **31:** 341–353

Nühse TS, Stensballe A, Jensen ON, Peck SC (2004) Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. Plant Cell **16:** 2394–2405

Pant BD, Buhtz A, Kehr J, Scheible WR (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J **53:** 731–738

Pittman JK, Shigaki T, Marshall JL, Morris JL, Cheng NH, Hirschi KD (2004) Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter. Plant Mol Biol **56:** 959–971

Poirier Y, Bucher M (2002) Phosphate transport and homeostasis in *Arabidopsis*. The Arabidopsis Book **1:** e0024, doi/10.1199/tab.0024

Rubio V, Linhares F, Solano R, Martín AC, Iglesias J, Leyva A, Paz-Ares J (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev **15:** 2122–2133

Shigaki T, Rees I, Nakhleh L, Hirschi KD (2006) Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. J Mol Evol **63:** 815–825

Shin H, Shin HS, Dewbre GR, Harrison MJ (2004) Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J **39:** 629–642

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol **3:** Article 3

Sze H, Liang F, Hwang I, Curran AC, Harper JF (2000) Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. Annu Rev Plant Physiol Plant Mol Biol **51:** 433–462

Thibaud M-C, Arrighi J-F, Bayle V, Chiarenza S, Creff A, Bustos R, Paz-Ares J, Poirier Y, Nussaume L (2010) Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. Plant J **64:** 775–789

Ticconi CA, Abel S (2004) Short on phosphate: plant surveillance and countermeasures. Trends Plant Sci **9:** 548–555

Wang C, Ying S, Huang HJ, Li K, Wu P, Shou HX (2009) Involvement of *OsSPX1* in phosphate homeostasis in rice. Plant J **57:** 895–904

Wang Y, Ribot C, Rezzonico E, Poirier Y (2004) Structure and expression profile of the Arabidopsis *PHO1* gene family indicates a broad role in inorganic phosphate homeostasis. Plant Physiol **135:** 400–411

Zhao J, Barkla BJ, Marshall J, Pittman JK, Hirschi KD (2008) The Arabidopsis *cax3* mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H^{+}-ATPase activity. Planta **227:** 659–669

Zhao J, Shigaki T, Mei H, Guo YQ, Cheng NH, Hirschi KD (2009) Interaction between Arabidopsis Ca^{2+}/H^{+} exchangers CAX1 and CAX3. J Biol Chem **284:** 4605–4615