the plant journal
Society for Experimental Biology

The Plant Journal (2012) doi: 10.1111/j.1365-313X.2012.05058.x

PHO1 expression in guard cells mediates the stomatal response to abscisic acid in Arabidopsis

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SUMMARY

Stomatal opening and closing are driven by ion fluxes that cause changes in guard cell turgor and volume. This process is, in turn, regulated by environmental and hormonal signals, including light and the phytohormone abscisic acid (ABA). Here, we present genetic evidence that expression of *PHO1* in guard cells of *Arabidopsis thaliana* is required for full stomatal responses to ABA. *PHO1* is involved in the export of phosphate into the root xylem vessels and, as a result, the *pho1* mutant is characterized by low shoot phosphate levels. In leaves, *PHO1* was found expressed in guard cells and up-regulated following treatment with ABA. The *pho1* mutant was unaffected in production of reactive oxygen species following ABA treatment, and in stomatal movements in response to light cues, high extracellular calcium, auxin, and fusicoccin. However, stomatal movements in response to ABA treatment were severely impaired, both in terms of induction of closure and inhibition of opening. Micro-grafting a *pho1* shoot scion onto wild-type rootstock resulted in plants with normal shoot growth and phosphate content, but failed to restore normal stomatal response to ABA treatment. *PHO1* knockdown using RNA interference specifically in guard cells of wild-type plants caused a reduced stomatal response to ABA. In agreement, specific expression of *PHO1* in guard cells of *pho1* plants complemented the mutant guard cell phenotype and re-established ABA sensitivity, although full functional complementation was dependent on shoot phosphate sufficiency. Together, these data reveal an important role for phosphate and the action of *PHO1* in the stomatal response to ABA.

Keywords: Abscisic acid, guard cell, stomata, PHO1, phosphate, Arabidopsis.

INTRODUCTION

Plant stomatal pores are formed by two specialized epidermal cells known as guard cells. Guard cells integrate a myriad of complex environmental and hormonal signals to act as a gateway regulating gas exchange in leaf tissue and transpirational water loss (Hetherington and Woodward, 2003; Nilson and Assmann, 2007). Stomatal opening and closing is driven by fluctuations in guard cell turgor, which is in turn dependent on the concentrations of intracellular organic and inorganic ions. Ion concentrations are changed through transport across guard cell plasma membranes. Following an influx of potassium, chloride, and nitrate into guard cells, and the intracellular production of malate, water is drawn in by osmosis, increasing cell turgor and thereby opening the stomatal pore. Conversely, to induce closure of the stomatal pore, guard cell turgor is decreased following the release of potassium and anions into the apoplast (Roelfsema and Hedrich, 2005; Sirichandra *et al.*, 2009). The phytohormone abscisic acid (ABA) plays a key role in regulating stomatal movements in response to water stress. Abscisic acid activates guard cell anion efflux via R-type and S-type anion channels, and triggers potassium efflux via outward-rectifying K^+_{out} channels (Roelfsema *et al.*, 2012). Concurrently, ABA down-regulates the influx of potassium by inhibiting inward-rectifying K^+_{in} channels. Thus, the presence of ABA favors closure of the stomatal pore (Pandey *et al.*, 2007). In recent years, research efforts have focused on identifying the molecular components regulating changes in guard cell turgor through ion transport and

remobilization (Kim *et al.*, 2010). Substantial advances have been made, particularly in identifying the anion channels responsible for anion export in response to ABA. Specific examples include characterization of the S-type anion channel SLAC1 (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2009, 2010) and the recently described *SLAC1* homolog *SLAH3* (Geiger *et al.*, 2011), which are both involved in efflux of chloride and nitrate from guard cells, and the recent identification of *AtALMT12* as a malate- and sulfate-permeable guard cell R-type anion channel candidate (Meyer *et al.*, 2010).

Phosphate (PO_4^{3-}; Pi) is an essential plant macronutrient; it functions as a constituent of macromolecules, such as DNA and phospholipids, and is involved in many cellular processes, including enzyme regulation and control of signaling pathways through phosphorylation (Poirier and Bucher, 2002). The potential role of Pi as an anion in the response of guard cells to the environment has not yet been addressed. However, in *Vicia faba* it was reported that Pi accumulates at a higher level in guard cells compared with other leaf cell types, thus suggesting a potential role for Pi in stomatal physiology (Outlaw *et al.*, 1984).

In *Arabidopsis thaliana*, *PHO1* is primarily expressed in the root vascular tissues and is involved in the transfer of Pi into the apoplastic space of xylem vessels (Hamburger *et al.*, 2002). Consequently, the *pho1* mutant shows strong reduction of Pi loading into the xylem and displays a strong Pi-deficient phenotype in aerial parts of the plant, resulting in an overall reduction in shoot growth (Poirier *et al.*, 1991). Ectopic overexpression of *PHO1* in leaves (Stefanovic *et al.*, 2011) and in mesophyll protoplasts (Arpat *et al.*, 2012) showed that PHO1 mediates specific phosphate efflux from cells. Interestingly, in addition to phosphate transport, *PHO1* plays a role in the transduction of the long-distance Pi-deficiency response (Rouached *et al.*, 2011).

In the present study, we report that, aside from its known expression in root vascular tissue, *PHO1* is also expressed in guard cells and is induced by ABA treatment. We characterized the stomatal movements in *pho1*, and showed that whilst still able to respond to light/dark cycles, auxin, fusicoccin, and high extracellular calcium stimuli, stomata in *pho1* were severely impaired in their response to ABA. This impairment was not a simple pleiotropic consequence of Pi deficiency. Using guard cell-specific regulation of gene expression, we found that a knockdown of *PHO1* expression altered stomatal movements in response to ABA. Conversely, guard cell-specific expression of *PHO1* in the *pho1* mutant background restored stomatal responsiveness to ABA, although full complementation of the mutant phenotype was co-dependent on sufficiency of shoot Pi. Down-regulation of *PHO1* in guard cells did not alter the expression of ABA-induced marker genes, indicating that PHO1 does not affect the ABA signal transduction cascade at the transcriptional level. Together, these data reveal an important role for Pi and the expression of *PHO1* in the stomatal response to ABA.

RESULTS

In *A. thaliana* leaves, *PHO1* is expressed predominantly in guard cells compared with mesophyll cells, and expression is induced by ABA

We investigated *PHO1* expression in the leaves following ABA treatment. We used transgenic *A. thaliana* lines in which the *PHO1*-GUS fusion gene was expressed under the control of a 2.1-kb DNA fragment upstream of the *PHO1* start codon. Two-week-old rosettes were cut at the hypocotyl and floated in water with or without 10 μM ABA for 3 h prior to GUS staining. For both treatments, we observed GUS staining in the vasculature of the petiole and in leaf guard cells. However, the intensity and frequency of the guard cell staining was enhanced under ABA treatment (Figure 1a).

To assess guard cell-specific expression of *PHO1*, we performed quantitative real-time PCR (qRT-PCR) using RNA extracted from guard cell and mesophyll cell protoplast preparations. We assessed the relative purity of each protoplast preparation by measuring the expression of the known guard cell-specific gene *KAT1* (Schachtman *et al.*, 1992) and the mesophyll cell-specific gene *At4G26530* (Yang *et al.*, 2008) (Figure 1b). The *PHO1* transcript was approximately six times more abundant in guard cell preparations than in mesophyll cell protoplast preparations (Figure 1b). Interestingly, *PHO1* expression in guard cell protoplasts was nearly half of that of the guard cell-specific *KAT1*.

We also analyzed the *PHO1* expression in guard cell protoplasts that were subjected to ABA treatment. The level of PHO1 expression increased between 40 min and 6 h after addition of 10 μM ABA (Figure 1c, left side). *PHO1* expression was similarly induced after 4-h treatments with ABA concentrations ranging from 5 to 100 μM (Figure 1c, right side).

PHO1 functions in ABA induction of stomatal closure and ABA repression of stomatal opening

Stomatal movements are regulated by a number of environmental factors and hormonal signals. Therefore, to assess the function of *PHO1* in guard cells, we analyzed stomatal movements in the *pho1* mutant in response to different stimuli (Figure 2). We first analyzed stomatal movements in the allelic mutants *pho1.2* and *pho1.4* following treatment with ABA. Epidermal preparations of the wild type (WT), *pho1.2*, and *pho1.4* were subjected to light in order to induce stomatal opening, then treated with varying concentrations of ABA for 2 h to induce stomatal closing. In the absence of ABA, stomatal apertures in *pho1.2* and *pho1.4* were comparable to WT (Figure 2a; ABA 0 μM). However, following ABA treatment, stomatal apertures in *pho1.2* and *pho1.4* were consistently wider than observed in

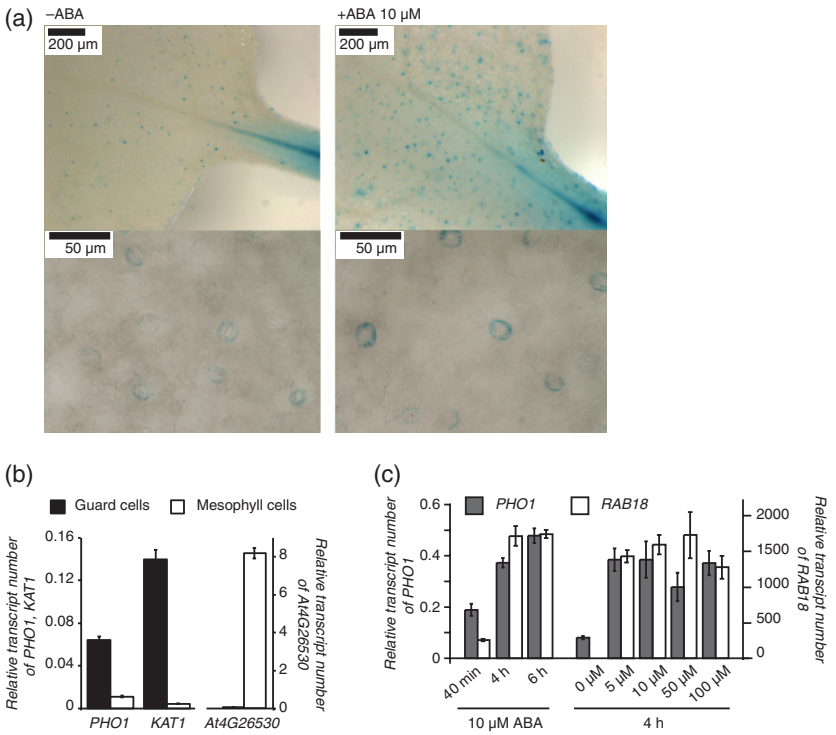

Figure 1. *PHO1* is preferentially expressed in guard cells compared with mesophyll cells, and expression is induced by ABA.
(a) Localization of PHO1::GUS translational fusion protein activity in leaves. Expression was under the control of 2.1 kb of the *PHO1* promoter region. Rosettes were cut at the hypocotyl and floated in water with or without 10 μM ABA for 3 h before GUS staining. In the leaf blade, GUS staining is visible in guard cells, and the intensity is increased under ABA treatment. Staining in vascular tissue of the leaf petiole can also be seen.
(b) Variation in *PHO1* transcript level between wild-type guard cell and mesophyll cell protoplasts preparations. The guard cell-specific gene *KAT1* and the mesophyll cell-specific gene *At4G26530* were also measured as controls. $n = 3$ biological replicates; average ± SE.
(c) Expression levels of *PHO1* in wild-type guard cell protoplasts following treatment with 10 μM ABA for 40 min to 6 h, and treatment with 0–100 μM ABA for 4 h. Expression levels of the known ABA-induced gene *RAB18* are also represented. $n = 3$ biological replicates; average ± SE.
Relative expression levels refers to transcript abundance of target genes, normalized against expression of the reference gene *At1G13320* (Czechowski *et al.*, 2005).

WT, even under high concentrations of ABA (Figure 2a), suggesting that ABA is unable to fully induce stomatal closure in *pho1*.

To further investigate stomatal movements in response to ABA, we analyzed ABA inhibition of stomatal opening in *pho1.2*. Epidermal peels of WT and *pho1.2* were prepared and incubated in the dark to ensure that stomata were closed. Interestingly, prior to treatment, stomata in *pho1.2* were fully closed and stomatal aperture widths were comparable to WT (Figure 2b; t0), indicating that the inability of ABA treatment to induce stomatal closure in *pho1* is not due to a mechanical incapacity of *pho1* stomata to close. Furthermore, no difference was observed in the morphology or size between the stomata of WT and the mutants (Figure S1 in Supporting Information). Wild-type and *pho1.2* epidermal peels were placed under light and treated with 10 μM ABA for 2 h. The ABA treatment effectively inhibited stomatal opening in WT. In contrast, stomata in *pho1.2* were unresponsive to ABA treatment and opened fully (Figure 2b), displaying final aperture widths similar to untreated WT stomata. This result suggests that ABA is also unable to repress stomatal opening in *pho1*.

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are known mediators of stomatal movements and are produced in response to ABA (Wang and Song, 2008). Therefore, it was of interest whether guard cells in *pho1* produced ROS in response to ABA. The H_2O_2-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFDA) was used to assess relative ROS levels in WT and *pho1.2* guard cells before and after ABA treatment. Wild-type and *pho1.2* epidermal peels were incubated in 50 μM DCFDA followed by 50 μM ABA. The DCFDA fluorescence increased in guard cells of both *pho1.2* and WT following ABA treatment (Figure 2c), suggesting that ROS production increased in *pho1* in response to ABA. This provides evidence that the impaired stomatal ABA response in *pho1* occurs downstream of ABA perception and ROS production. In agreement with this hypothesis, H_2O_2 treatment failed to induce stomatal closure in *pho1.2* (Figure 2d) compared with WT.

To assess whether impaired stomatal movements in *pho1* were specific to the ABA pathway, we tested whether stomata in *pho1.2* were responsive to other stimuli, such as blue light. Wild-type and *pho1.2* epidermal peels were maintained in the dark to promote stomatal closure,

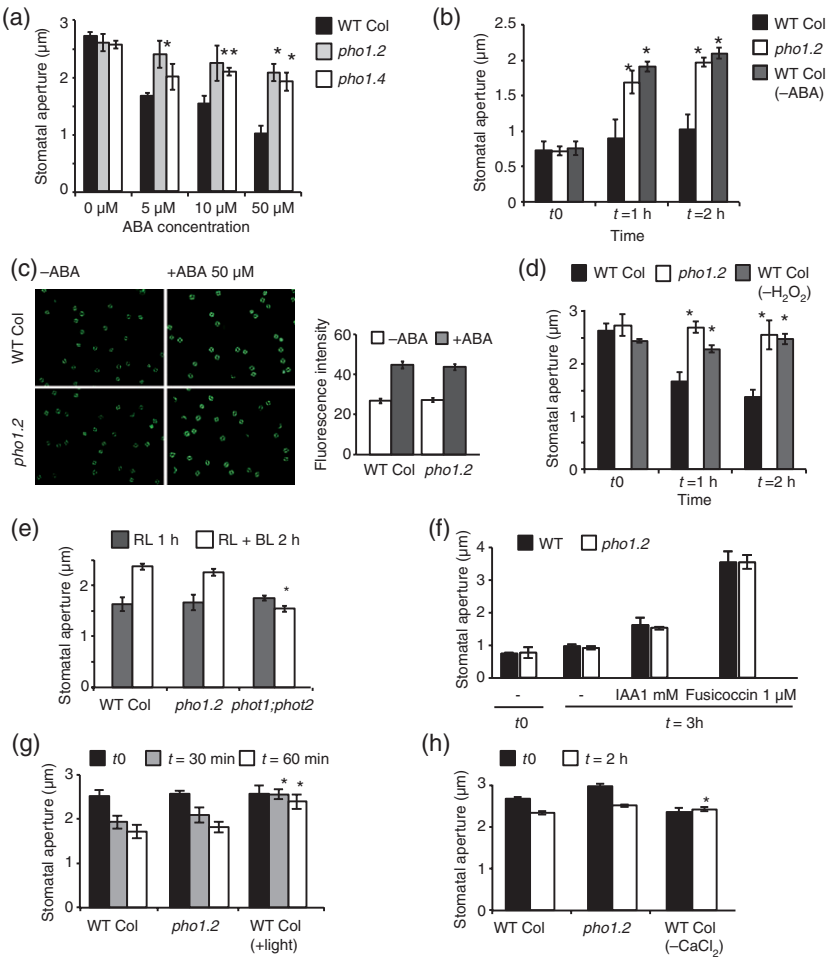

Figure 2. *pho1.2* guard cells are non-responsive to ABA and H_2O_2, but maintain production of reactive oxygen species (ROS) and normal stomatal movements under other treatments.

(a) Stomatal closure in wild type (WT), *pho1.2*, and *pho1.4*, as represented by decreasing stomatal apertures, following 2-h treatments with ABA concentrations varying from 0 to 50 µM. n = 3 independent experiments; average ± SE.

(b) Stomatal opening under light in WT and *pho1.2*, as represented by increasing stomatal apertures, following 0-, 1-, and 2-h treatments with 10 µM ABA. Stomatal opening of WT without ABA (WT Col –ABA) was also monitored. n = 3 independent experiments; average ± SE.

(c) Production of ROS in leaf epidermal stomata of WT and *pho1.2* following application of 50 µM ABA, as revealed by fluorescence levels of the fluorescent dye 2′,7′-dichlorofluorescein diacetate. (–ABA) pictures represent epidermis before addition of ABA; (+ABA) represent epidermis 3 min after the addition of ABA. Bar = 100 µm. Fluorescence intensity was also quantified (n = 45 stomata; average ± SE).

(d) Stomatal closure in WT and *pho1.2* following 0-, 1-, and 2-h treatments with 100 µM H_2O_2. The stomatal aperture of WT without H_2O_2 treatment (WT Col –H_2O_2) was also monitored. n = 3 independent experiments; average ± SE.

(e) Stomatal opening under blue light in WT and *pho1.2*. Stomatal apertures were measured following 1 h exposure to red light (RL 1 h), and again after a 2-h superimposition of blue light (RL + BL 2 h). The double mutant *phot1/phot2* was used as a control.

(f) Stomatal opening in the dark in WT and *pho1.2* following treatment with auxin (IAA) and fusicoccin. Pre-treatment (t0) measurements were of stomata maintained in the dark. n = 3 independent replicates; average ± SE.

(g) Stomatal closure in WT and *pho1.2* following 30 and 60 min of darkness. Stomatal aperture of WT kept under light (WT Col + light) was also monitored. n = 3 independent experiments; average ± SE.

(h) Stomatal closure in WT and *pho1.2* following treatment with 5 mM $CaCl_2$. Stomatal aperture of WT without $CaCl_2$ treatment (WT Col –$CaCl_2$) was also monitored. n = 3 independent experiments; average ± SE.

Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding WT control values ($P < 0.05$).

followed first by a 1-h exposure to strong red light (50 µE m^{-2} sec^{-1}) and then a 2-h blue light (10 µE m^{-2} sec^{-1}) superimposition. Interestingly, similar stomatal aperture widths were observed in both WT and *pho1.2* under red light (Figure 2e; RL 1 h) and red/blue light (Figure 2e; RL + BL 2 h) treatments. In contrast, the stomata of the phototropin double mutant *phot1*/*phot2* did not respond to blue light, as previously described (Kinoshita *et al.*, 2001). Furthermore, we examined stomatal opening in the dark following treatment with auxin or fusicoccin (Figure 2f), as well as stomatal closure following the transition from light to dark (Figure 2g) or high extracellular calcium treatment (Figure 2h). Again we observed similar responses in both WT and *pho1.2*.

Phosphate sufficiency does not restore the stomatal response of *pho1.2* to ABA

Phosphorus starvation induces a myriad of transcriptional, biochemical, and physiological effects, including adaptive changes in shoot growth, carbohydrate metabolism, and ion composition (Hammond and White, 2008). Such pleiotropic effects could potentially impair the stomatal response to ABA. Therefore, to determine whether the impaired ABA-induced stomatal movements observed in *pho1* were due to the absence of specific *PHO1* function in guard cells or to Pi deficiency alone, we conducted stomatal aperture assays on chimeric plants composed of a *pho1.2* scion micro-grafted with WT rootstock (*pho1.2*/WT) (Figure 3a). As previously described, WT rootstock restored root-to-shoot Pi transfer to *pho1.2* scions, resulting in WT levels of shoot Pi content and normal shoot growth (Stefanovic *et al.*, 2007) (Figure 3a). The stomata of plants composed of a WT scion micro-grafted to a WT rootstock (WT/WT) responded normally to ABA treatment (Figure 3b, c). However, stomatal movements in plants composed of a *pho1.2* scion micro-grafted to a WT rootstock (*pho1.2*/WT) remained unresponsive to ABA treatment; ABA failed to induce stomatal closure (Figure 3b) or repress stomatal opening (Figure 3c). Therefore, the impaired stomatal response to ABA in *pho1* cannot be attributed to Pi deficiency alone.

In addition, we quantified the rate of transpirational water loss from *pho1.2*/*pho1.2*, *pho1.2*/WT, and WT/WT micro-grafted plants by monitoring weight loss from excised rosettes over time (Figure 3d). The accumulation of ABA in shoots is a known response to water stress, and creates drought tolerance by reducing stomatal aperture. Mutants affected in ABA-induced stomatal closure typically have higher transpirational water loss, even under dehydration stress (Nilson and Assmann, 2007). In agreement with the impaired ABA-induced stomatal response described above for *pho1*, *pho1.2*/*pho1.2* self-grafted plants displayed more transpirational water loss than WT/WT self-grafted plants (Figure 3d). High-level transpirational water loss was also observed in chimeric *pho1.2*/WT plants, again indicating that the *pho1* guard cell phenotype is not due to phosphate deficiency alone.

A knockdown of *PHO1* expression in guard cells reduces stomatal responsiveness to ABA

To assess the importance of *PHO1* expression specifically in guard cells, we generated transgenic *A. thaliana* lines with guard cell-specific *PHO1* expression knockdown mediated by RNA interference (RNAi). The strong guard cell-specific promoter *pGC1*, described by Yang *et al.* (2008), was used to express inverted repeats of a *PHO1* gene-specific tag (*PHO1* gst) (Figure 4b) in wild-type plants. We isolated two independent transgenic lines, referred to as p*GC1::PHO1*R-NAi #6-6 and #10-3, with corresponding non-transgenic

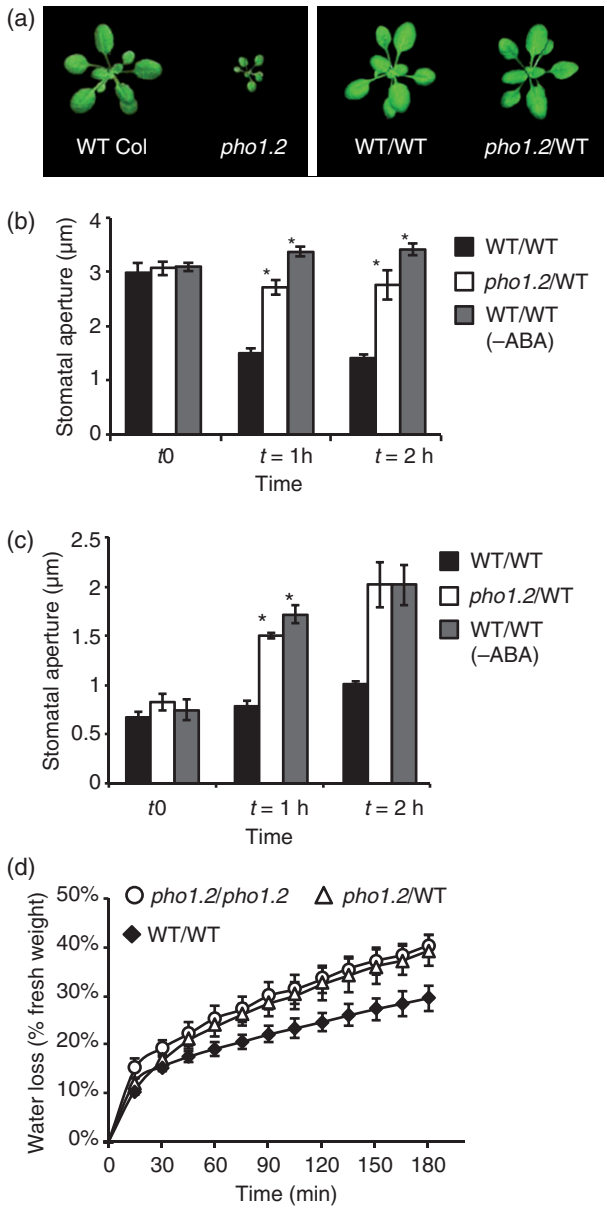

Figure 3. Stomata in *pho1.2* scion micro-grafted onto wild-type (WT) rootstock remain non-responsive to ABA, despite phosphate (Pi) sufficiency.
(a) Representative growth of 4-week-old WT, *pho1.2*, and chimeric plants composed of either WT or *pho1.2* scion micro-grafted onto WT rootstock (WT/WT and *pho1.2*/WT, respectively). Shoot growth of *pho1.2* scion with WT rootstock resembled growth of WT and the WT/WT micro-graft control, indicating restored Pi content in the *pho1.2* scion.
(b) Induction of stomatal closure by ABA in micro-grafted WT/WT and *pho 1.2*/WT, in response to 10 μM ABA, after 0, 1, and 2 h. $n = 3$ independent experiments; average ± SE.
(c) Repression of stomatal opening by ABA under light in micro-grafted WT/WT and *pho1.2*/WT, in response to 10 μM ABA, after 0, 1, and 2 h ($n = 3$ independent experiments, average ± SE). Stomatal closure and opening of WT/WT without ABA was also monitored as a control (WT/WT –ABA). Asterisks above the columns in (b) and (c) indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).
(d) Rate of water loss from detached rosettes of micro-grafts, measured as percentage of initial fresh weight after various time periods. $n = 3$ biological replicates; average ± SD.

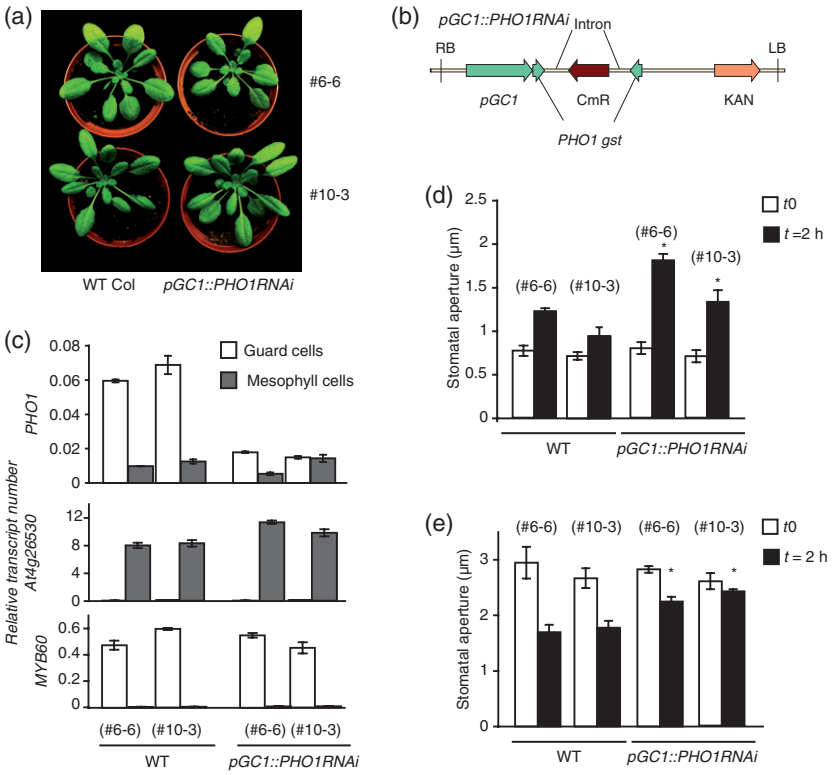

Figure 4. Guard cell-specific expression knockdown of *PHO1* through RNA interference (RNAi) reduces stomatal response to ABA.
(a) Representative growth of two independent 6-week-old transgenic lines with *PHO1* expression knocked down in guard cells through RNAi (p*GC1*::*PHO1*RNAi #6-6 and #10-3) and their corresponding non-transgenic segregants (NTS) [wild type (WT) Col #6-6 and #10-3].
(b) The T-DNA region containing expression construct p*GC1*::*PHO1*RNAi consisting of a spacer region flanked by *PHO1*'s gene sequence tag (GST) sense and antisense sequences. Expression is driven by the guard cell-specific promoter p*GC1*. LB, left border; RB, right border; *KAN*, plant resistance gene to kanamycin.
(c) Variation in *PHO1* transcript level between guard cell and mesophyll cell preparations of transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding NTS (WT #6-6 and #10-3, respectively), normalized against expression levels of the reference gene *At1G13320*. Transcripts of the mesophyll cell marker *At4G26530*, and the guard cell marker *MYB60* were also quantified as controls. $n = 3$ biological replicates; average ± SE.
(d) Repression of stomatal opening and (e) induction of stomatal closure by ABA, in WT and the guard cell RNAi lines p*GC1*::*PHO1*RNAi, following treatment with 10 µM ABA, after 0 and 2 h. $n = 3$ independent experiments; average ± SE.
Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (WT) control values ($P < 0.05$).

segregants (NTS) as controls (WT #6-6 and #10-3). At the whole-plant level there were no obvious morphological differences between the p*GC1::PHO1*RNAi lines and their respective NTS (Figure 4a). Quantification of *PHO1* transcripts in guard and mesophyll cell protoplasts revealed that guard cell-specific *PHO1* expression was reduced in lines expressing p*GC1::PHO1*RNAi, with 70 and 78% reductions in the *PHO1* transcript level observed for RNAi lines #6-6 and #10-3, respectively (Figure 4c). Specificity of the RNAi was assessed by measuring the transcript levels of the Arabidopsis *PHO1* homologs expressed in guard cells (Figure S2). The presence of the p*GC1::PHO1*RNAi construct did not consistently affect their expression levels. Differential expression of the mesophyll cell marker gene *At4G26530* and the guard cell marker gene *MYB60* confirmed the purity of the guard cell and mesophyll cell protoplast preparations.

Following confirmation of guard cell-specific *PHO1* expression knockdown, the p*GC1::PHO1*RNAi lines were assessed for their stomatal responsiveness to ABA. Abscisic acid-induced repression of stomatal opening was affected by p*GC1::PHO1*RNAi expression. In the dark, the p*GC1::PHO1*RNAi lines and their respective NTS possessed similar average stomatal apertures (Figure 4d; t0). However, following a 2-h light exposure in the presence of 10 µM ABA, repression of stomatal opening was seen in the NTS, but was altered in the two p*GC1::PHO1*RNAi lines. The p*GC1::PHO1*RNAi lines consistently displayed wider stomatal apertures than their respective NTS (Figure 4d; $t = 2$ h). ABA-induced stomatal closure was also compromised in p*GC1::PHO1*RNAi lines. Whilst average stomatal apertures were comparable between RNAi and WT lines prior to treatment (Figure 4e; t0), after 2 h of light exposure in the presence of 10 µM ABA, the average aperture width was higher in p*GC1::PHO1*RNAi lines than in their respective NTS (Figure 4e; $t = 2$ h). Combined, these results suggest that *PHO1* expression specifically in guard cells plays a fundamental role in ABA-mediated stomatal movements.

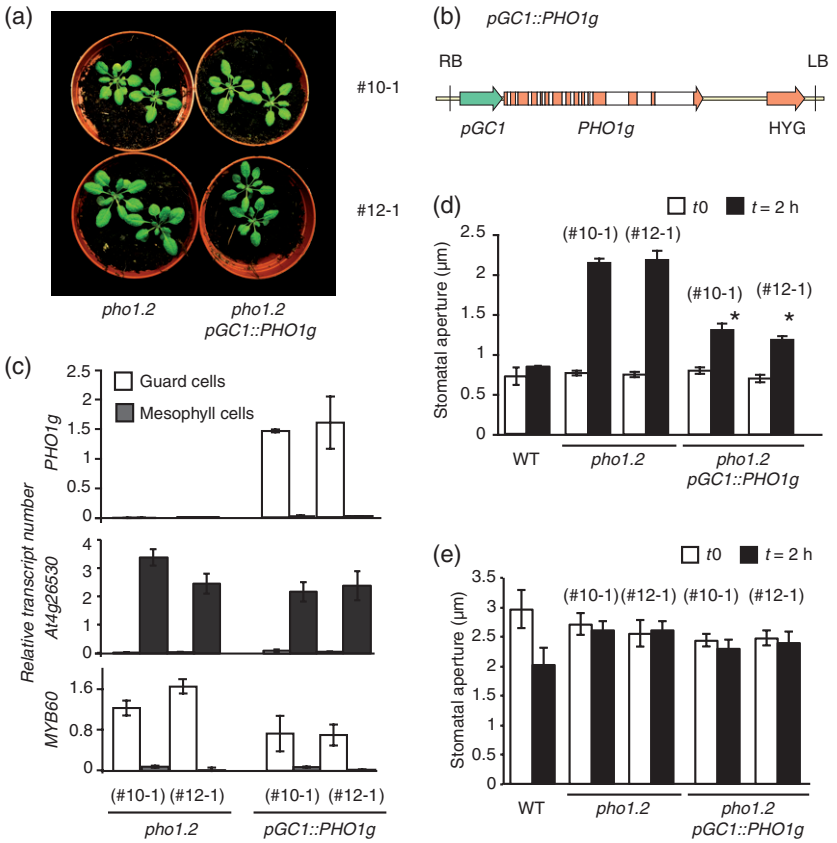

Figure 5. Guard cell-specific complementation of *pho1.2* partially restores stomatal responsiveness to ABA.
(a) Representative growth of two independent 6-week-old *pho1.2* transgenic lines with *PHO1* complementation in guard cells (*pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1) and their corresponding non-transgenic segregants (NTS) (*pho1.2* #10-1 and #12-1).
(b) The T-DNA region containing the expression construct, p*GC1*::*PHO1*g, consisting of the *PHO1* genomic sequence (*PHO1g*) under the control of the guard cell promoter p*GC1*. LB, left border; RB, right border; *HYG*, plant resistance gene to hygromycin.
(c) Guard cell and mesophyll cell transcript levels of the transgene *PHO1g*, in the guard cell complemented lines *pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1, and their corresponding NTS (*pho1.2* #10-1, and #12-1), normalized against expression levels of the reference gene *At1G13320*. Primers were chosen to hybridize to *PHO1*'s second to last exon and to a sequence specific to pMDC32. Transcript levels of the mesophyll cell marker *At4G26530* and the guard cell marker *MYB60* were also quantified as controls. $n = 3$ biological replicates; average ± SE.
(d) Repression of stomatal opening and (e) induction of stomatal closure by ABA, in WT, the *pho1.2* guard cell-complemented lines *pho1.2* p*GC1*::*PHO1*g #10-1, and #12-1, and their corresponding NTS (*pho1.2* #10-1, and #12-1), in response to 10 μM ABA, after 0 and 2 h. $n > 3$ independent experiments; average ± SE.
Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (*pho1.2*) control values ($P < 0.05$).

Guard cell-specific complementation of *pho1.2* restores ABA-induced repression of stomatal opening, but shoot phosphate sufficiency is required for stomatal closure

In parallel with guard cell-specific *PHO1* expression knockdown, we attempted to generate a WT stomatal phenotype in *pho1.2* through guard cell-specific complementation. For this, *pho1.2* was transformed with the full-length genomic sequence of *PHO1* (*PHO1g*) with expression governed by the guard cell-specific promoter p*GC1* (Figure 5b). Two independent transgenic lines were isolated, referred to as p*GC1*::*PHO1g* #10-1 and #12-1, along with their respective NTS as controls (*pho1.2* #10-1 and #12-1). Similar to the observations with guard cell-specific *PHO1* expression knockdown, at the whole-plant level p*GC1*::*PHO1g* lines displayed morphologies that were comparable to their NTS counterparts (Figure 5a). Quantitative RT-PCR was used to verify *PHO1g* expression in guard cell preparations of the p*GC1*::*PHO1*g lines (Figure 5c). No *PHO1g* transcripts were detected in their mesophyll cells, indicating a strong guard cell-specific *PHO1* complementation. Again, differential expression of the marker genes *At4G26530* and *MYB60* confirmed the purity of the guard cell and mesophyll cell protoplast preparations.

We determined the stomatal responsiveness to ABA in the p*GC1*::*PHO1*g lines compared with their NTS controls with stomatal aperture assays as described above. The ABA-induced repression of stomatal opening was markedly improved in guard cell-complemented p*GC1*::*PHO1*g lines (Figure 5d). Similar to the earlier observations in *pho1.2*, wider stomatal apertures were apparent in *pho1.2* NTS controls following a 2-h light exposure in the presence of

10 μM ABA (Figure 5d; t = 2 h). Under the same conditions, stomatal apertures in the p*GC1*::*PHO1g* lines were narrower (Figure 5d; t = 2 h), indicating restoration of stomatal responsiveness to ABA in these lines. Surprisingly, ABA-induced stomatal closure was not restored following guard cell-specific *PHO1* complementation. Stomatal aperture width was maintained in the complemented p*GC1*::*PHO1g* lines and their corresponding *pho1.2* NTS following 2 h of light treatment in the presence of 10 μM ABA (Figure 5e; t = 2 h), indicating that stomata failed to close in response to ABA treatment in all lines. It is thus apparent that guard cell-specific *PHO1* complementation is sufficient to restore ABA-induced repression of stomatal opening, but is not sufficient to restore ABA-induced stomatal closure.

It was then postulated that the inability of guard cell-specific *PHO1* complementation to restore ABA-induced stomatal closure was due to the unavailability of Pi to the guard cells or to the pleiotropic consequences of shoot Pi deficiency. To assess this, Pi was provided through incubation of the epidermal peels in potassium phosphate buffer [30 mM KH_2PO_4, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5] instead of potassium chloride. After a 2-h incubation under light, 10 μM ABA was added to the buffer. While stomata of WT plants responded to the ABA treatment, the use of potassium phosphate did not improve the stomatal closure response of both the guard cell-specific *PHO1*-complemented *pho1.2* and their respective NTS (Figure 6a).

To alleviate the effects of long-term shoot Pi deficiency, shoot Pi sufficiency was then restored in each transgenic line by creating chimeric plants composed of guard cell-specific *PHO1*-complemented *pho1.2* scion micro-grafted onto WT rootstock (*pGC1::PHO1g* #10-1 and #12-1/WT). Chimeric plants composed of NTS scion micro-grafted onto WT rootstock (*pho1.2* #10-1 and #12-1/WT) were generated for comparison. Self-grafted plants composed of WT scion and WT rootstock (WT/WT) were used again as a control. Following a 2-h incubation with 10 μM ABA, stomatal closure was assessed in all micro-grafted plants (Figure 6b). In the *pho1.2* NTS controls, stomata in *pho1.2* NTS/WT micro-grafts failed to close in response to ABA treatment. However, in contrast to what was observed for p*GC1*::*PHO1g* lines, stomata in *pGC1::PHO1g*/WT micro-grafts were responsive to ABA treatment and a reduction in stomatal aperture width was seen. This indicates that a restoration of ABA-induced stomatal closure in *pho1* is dependent on both guard cell-specific *PHO1* expression and shoot Pi sufficiency during plant development.

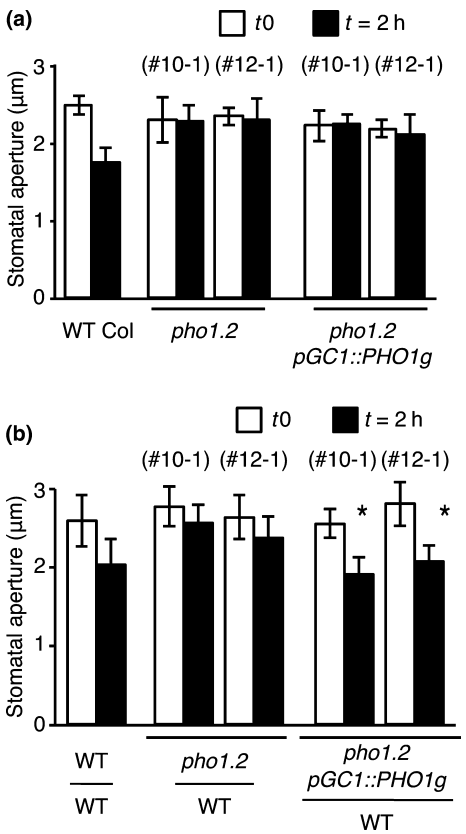

Figure 6. Complete responsiveness of guard cell complemented *pho1.2* is co-dependent on shoot phosphate (Pi) sufficiency during plant development.
(a) Induction of stomatal closure under light in the wild type (WT), the *pho1.2* guard cell-complemented lines *pho1.2* p*GC1*::*PHO1*g #10-1, and #12-1, and their corresponding non-transgenic segregants (NTS) (*pho1.2* #10-1 and #12-1), after 0 and 2 h of treatment with 10 μM ABA in potassium phosphate buffer composed of 30 mM KH_2PO_4, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5. n = 3 independent experiments; average ± SE.
(b) Induction of stomatal closure under light following treatment with 10 μM ABA, after 0 and 2 h, on Pi sufficient micro-grafts composed of either WT, *pho1.2* NTS, or *pho1.2* p*GC1*::*PHO1*g scion micro-grafted onto WT rootstock (WT/WT, *pho1.2*/WT, and *pho1.2* p*GC1*::*PHO1*g/WT, respectively). n = 5 independent experiments; average ± SE.
Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (*pho1.2*) control values (P < 0.05).

Expression profiling of ABA-induced marker genes shows normal transcriptional response to ABA when *PHO1* is down-regulated in guard cells

We investigated the effect of *PHO1* expression in guard cells on the transcriptional response of some ABA-responsive genes. Since Pi deficiency in the mutant *pho1* induces major transcriptional changes, we chose to compare the transcript-level responses to ABA in the Pi-sufficient transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding NTS. One hour after the beginning of the light cycle, 6-week-old plants were sprayed with 50 μM ABA or control buffer and incubated under light for 4 h. Then RNA was extracted from enriched guard cell preparations. We measured the following ABA marker genes by qRT-PCR: *ABI2*, *RD20*, *KIN2*, *LTP4*, *LEA6* (ABA-induction markers), and *MYB60* (ABA-repression marker). Overall, the transcript-level changes induced by the ABA treatment were similar between the p*GC1*::*PHO1*RNAi lines and their respective NTS (Figure 7).

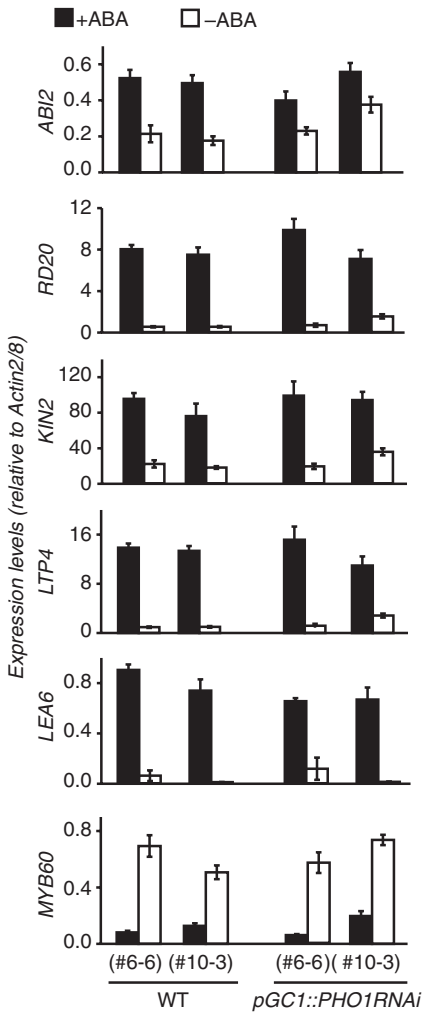

Figure 7. *PHO1* down-expression in guard cells does not affect the transcriptional response of marker genes to ABA.
Transcript levels of known ABA-responsive marker genes in enriched guard cell preparations of the transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding non-transgenic segregants [wild type (WT) #6-6 and #10-3], following a 4-h treatment with or without 50 μM ABA, normalized against the mean expression levels of the reference genes *Actin 2* and *8*. $n = 4$ biological replicates; average ± SE.

DISCUSSION

In agreement with its main role in transferring Pi to the root xylem vessel, *PHO1* is primarily expressed in the root vascular cylinder; however, weak *PHO1* expression has also been detected in leaves (Hamburger *et al.*, 2002; Ribot *et al.*, 2008; Stefanovic *et al.*, 2011). The present study shows that in the leaf blade, *PHO1* is predominantly expressed in guard cells, while expression also occurs in the vascular cylinder of the leaf petiole. The level of *PHO1* expression in stomata was only slightly lower than that of *KAT1,* a well-characterized potassium channel involved in ABA signaling. Ribot *et al.* (2008) observed that a long-term (2-day) treatment with 10 μM ABA repressed *PHO1* in whole seedlings, the majority of the repression most likely occurring in the root vascular cylinder, the tissue with the highest *PHO1* expression. In this study we examined more precisely the transcriptional response of *PHO1* to short-term (up to 6 h) ABA in guard cells. We show that, in stomata, *PHO1* expression increases rapidly following ABA application. These results are in agreement with microarray analyses that presented overviews of ABA-induced and repressed genes in guard cell and mesophyll cell protoplasts (Leonhardt *et al.*, 2004; Yang *et al.*, 2008).

The specific expression of *PHO1* in guard cells suggested its potential involvement in stomatal movements. Interestingly, the mutant *pho1* presented a WT aperture under white light, in the dark, and under red and blue light treatments. Red light triggers stomatal opening through phytochrome B (Wang *et al.*, 2010). Blue light signals activation of the plasma membrane H^+-ATPase and inhibition of S-type anion channels. The resulting membrane hyperpolarization activates uptake of K^+ via the voltage-gated inward-rectifying K^+ channels (Shimazaki *et al.*, 2007; Roelfsema *et al.*, 2012). Our results reveal that the stomata in *pho1* are functional in these pathways and can fully open and close in response to light cues. The functionality of *pho1* stomata was further confirmed by their WT-like response following treatments with the phytohormone auxin and the fungal toxin fusicoccin, which are known inducers of stomatal opening that act through the activation of the H^+-ATPases (Kinoshita and Shimazaki, 2001; Acharya and Assmann, 2009). In addition, we observed that the stomata of *pho1* were able to close like the WT during transition to the dark and to high extracellular calcium loads. These results suggest that *pho1* does not present structural problems preventing normal stomatal movements under a variety of stimuli.

In contrast, the mutant *pho1* exhibited impaired stomatal responses to ABA, both for stomatal closure and inhibition of stomatal opening. Following ABA treatment, increased production of ROS was observed in the guard cells of both *pho1* and WT, indicating that the early perception of ABA was unaltered in *pho1* and suggesting that PHO1 is involved further downstream in the ABA response pathway. Both micro-grafting experiments between *pho1* and WT, as well as experiments involving the down-regulation of *PHO1* expression in WT plants through RNAi, demonstrated that the impaired response of stomata to ABA in plants with abolished or reduced expression of *PHO1* occurred even in Pi-sufficient leaves. Repression of stomatal opening by ABA was not affected by Pi deficiency in the *pho1.2* plants with a guard cell-specific expression of *PHO1*; however, induction of stomatal closure by ABA was inhibited in this low-Pi situation. Incubation of the epidermal peels in Pi buffer was not sufficient to restore the induction of stomatal closure, suggesting that the inhibition is not simply due to a lack of available Pi anions. In contrast, using micro-grafting to

restore Pi sufficiency in the leaves of these lines enabled the induction of stomatal closure by ABA, indicating that the Pi starvation response inhibited this stomatal response. Thus, while ABA inhibition of stomatal opening is dependent on guard cell *PHO1* expression and independent of the leaf Pi status, ABA induction of stomatal closure is dependent on both leaf Pi sufficiency and guard cell expression of *PHO1.* The effect of Pi deficiency on ABA-induced stomatal closure could be explained as an indirect effect of low cellular Pi on metabolism. For example, long-term Pi starvation affects photosynthesis and carbon assimilation, triggering the accumulation of starch and sucrose in the leaves (Hammond and White, 2008). Similarly modified carbohydrate metabolism in Pi-deprived guard cells may lead to the accumulation of osmotically active sugars that could potentially counteract stomatal closure following the application of ABA in the light.

Abscisic acid mediates stomatal closure through repression of H^+-ATPases and K^+ influx, as well as activation of K^+ and anion efflux (Sirichandra *et al.*, 2009; Roelfsema *et al.*, 2012). Recent studies have identified the genes encoding anion channels, including *SLAC1*, *SLAH3*, and *ALMT12* (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Meyer *et al.*, 2010; Geiger *et al.*, 2011). Although both malate and chloride have long been recognized as two important anions mediating stomatal movement, other anions are also involved. For example, nitrate import into guard cells via NRT1.1 was shown to influence stomatal opening (Guo *et al.*, 2003). Furthermore, anion channels contributing to stomatal closure exhibit a higher permeability to nitrate than chloride (Schmidt and Schroeder, 1994) and the anion channels SLAC1 and SLAH3 both exhibit significantly higher conductance for nitrate than chloride (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2011). PHO1 is involved in export of Pi into the xylem (Poirier *et al.*, 1991; Hamburger *et al.*, 2002), and overexpression of *PHO1* in leaves (Stefanovic *et al.*, 2011) or in isolated leaf mesophyll protoplasts (Arpat *et al.*, 2012) leads to specific efflux of Pi into the perfusion medium. It is thus likely that *PHO1* expression in guard cells is also associated with Pi efflux. One hypothesis is that Pi efflux mediated by PHO1 may, together with Cl, NO_3^-, and $malate^{2-}$, participate in the depolarization of the plasma membrane and down-regulation of osmotic pressure during stomatal closure mediated by ABA. Down-regulation of *PHO1* in guard cells did not alter the expression of ABA-induced marker genes, indicating that PHO1 does not affect the ABA signal transduction cascade at the transcriptional level.

The possible role of Pi as an anion for guard cell function in Arabidopsis has not been fully addressed in the literature. Early studies described an accumulation of phosphorus in the epidermis of *Lupinus luteus* (Treeby *et al.*, 1987), a more specific accumulation of Pi in guard cells of *V. faba* (Outlaw *et al.*, 1984), and an accumulation of phosphorus in *Thlaspi montanum* stomata (Heath *et al.*, 1997), suggesting a potential biological significance of Pi in stomatal function. Phosphorus did not show large repartition changes in closed versus open stomata of *V. faba* and *Commelina communis* (Garrec *et al.*, 1983), but this finding did not exclude smaller variations. Further investigation will be required to characterize the potential existence of Pi fluxes in guard cells during the ABA response, as well as the possible role of PHO1 in that activity. This will contribute to our understanding of the role of PHO1 in guard cells, either in Pi transport or as a component of the signaling pathway involved during ABA induction and maintenance of stomatal closure.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana WT and mutant plants were all from the Columbia ecotype (WT Col). *pho1.2* and *pho1.4* have previously been described by Poirier *et al.* (1991), Delhaize and Randall (1995) and Hamburger *et al.* (2002). *phot1/phot2* was kindly supplied by Dr K. Shimazaki (Kyushu University, Japan).

Micro-grafting experiments

Plant grafting using collars was performed as previously described by Turnbull *et al.* (2002).

Stomatal aperture bioassays

Seed was sown directly onto potting compost and vernalized for 2 days at 4°C. Plants were grown for 4–6 weeks at 18°C, 60% relative humidity, 10-h light/14-h dark, 100 μE m^{-2} sec^{-1}. The youngest fully expanded leaves were excised in the dark 1 h before the beginning of the light cycle, and epidermal peels were prepared immediately: the abaxial epidermis was fixed to a microscope slide using liquid medical adhesive B (VM 355-1; Ulrich Swiss, http://www.ulrich-swiss.ch), and the resulting epidermal peels were floated in Petri dishes containing 50 ml of incubation buffer (KCl 30 mM, MES-KOH 10 mM pH 6.5, unless otherwise noted) for 30 min in the dark before being subjected to light (100 μE m^{-2} sec^{-1}, 25°C) and chemical treatments. For stomatal closure experiments, the epidermal peels were exposed to light for 2 h prior to any further treatments in order to trigger stomatal opening. Either ABA, H_2O_2 or $CaCl_2$ was then added to the incubation buffer up to the indicated concentrations. The ABA stock was prepared in ethanol.

Analysis of stomatal opening under blue light was conducted as follows: epidermal peels were prepared before the beginning of the light cycle, floated in incubation buffer and kept in the dark for 2 h to promote stomatal closure. Strong red light (50 μE m^{-2} sec^{-1}) was applied for 1 h using a red Plexiglas filter (GS Rot 501; Röhm Schweiz GmbH, http://www.roehmschweiz.ch). Initial stomatal apertures were recorded before blue light (10 μE m^{-2} sec^{-1}), from a second light source with a blue Plexiglas filter (GS Blau 612; Röhm Schweiz GmbH), was superimposed onto the red light. Blue/red light treatment was continued for 2 h, after which final stomatal apertures were measured.

For the measurement of stomatal apertures in the dark and under indole-3-acetic acid (IAA) or fusicoccin treatments, epidermal peels were prepared as above. After the initial stomatal apertures had been recorded, epidermal peels were floated in darkness for an additional 3 h in incubation buffer containing 1 mM IAA or 1 μM fusicoccin (Sigma, http://www.sigmaaldrich.com/), and final stomatal

apertures were measured. The IAA stock solution (100 mM) was prepared in water and the fusicoccin stock solution (1 mM) was prepared in ethanol.

All stomatal apertures were observed under an optical microscope before digital images were taken and subsequently used to measure aperture width in IMAGEJ (National Institutes of Health, http://rsbweb.nih.gov/ij/). Approximately 40 stomatal apertures were measured for each independent experiment, time point, treatment, and genotype. The significance of aperture fold change differences (stomatal aperture at t2/stomatal aperture at t0) between genotypes was assessed by non-coupled one-tailed Student's *t*-test analysis. Values of $P < 0.05$ were considered statistically significant.

For stomatal aperture assays on transgenic lines (WT *pGC1::PHO1RNAi* and *pho1.2 pGC1::PHO1g*), results were compared with replicated experiments where each genotype was given a blind treatment. In an effort to reduce sampling error, the same area of the epidermal peel (approximately 1 mm^2, defined with a marker on the microscope slide) was used for initial (t0) and post-treatment (t = 2 h) measurement of stomatal aperture.

Detection of ROS production

The production of ROS in response to ABA treatment in guard cells was observed using DCFDA (Sigma) according to Zhang *et al.* (2009). Epidermal peels were prepared, floated in incubation buffer (KCl 30 mM, MES-KOH 10 mM pH 6.5) and exposed to light for 2 h to induce stomatal opening. Epidermal peels were then placed in incubation buffer containing 50 μM DCFDA for 10 min, followed by 5 min of washes. Digital images were taken (−ABA) before epidermal peels were exposed to ABA 50 μM for 3 min and digital images taken again (+ABA). Observation of fluorescence was performed at an excitation at 430–510 nm and emission at 475–575 nm. Fluorescence intensity was quantified as mean pixel intensity.

β-Glucuronidase staining

Transgenic *A. thaliana pho1-4* plants expressing PHO1 fused to the β-glucuronidase (*uidA*) gene was done by cloning the *PHO1* promoter (2.1 kb upstream of the transcription start) along with the complete *PHO1* open reading frame into the pMDC163 vector (Curtis and Grossniklaus, 2003). Plants were grown in pots for 2 weeks at 18°C, 60% relative humidity, 10-h light/14-h dark, 100 μE m^{-2} sec^{-1}. Three hours after the beginning of the light cycle, rosettes were cut at the hypocotyl and floated in water or 10 μM ABA for 3 h. The GUS staining was conducted as previously described (Hamburger *et al.* (2002).

Water loss measurements

Whole rosettes of 6-week-old plants were detached from their roots by cutting with a clean razor blade and were weighed immediately. Rosettes were placed on chromatography paper (3MM Whatman, http://www.whatman.com/) and put back under light at 18°C, 60% relative humidity. Fresh weight was measured at the indicated time.

Guard-cell-specific *PHO1* RNAi

A 203-bp region localized in the 3′ untranslated region of *PHO1* (gene sequence tag GST CATMA3c57344, Sclep *et al.*, 2007) was PCR amplified using the primers *PHO1gst-F* and *PHO1gst-R* (Table S1a). This fragment, flanked by *att*B sites, was inserted between *att*P sites of the entry vector pDONR201 and subsequently cloned by Gateway LR recombination into a pB7GWIWG2(II) RNAi binary destination vector (Karimi *et al.*, 2002). In parallel, the 1.2-kb guard cell promoter *pGC1* of *At1g22690* (Yang *et al.*, 2008) was PCR amplified using primers *pGC1-F1* and *pGC1-R1* (Table S1a). The CaMV 35S cassette was excised from vector pART7 (Gleave, 1992) with *Sac*I/*Xho*I double digestion and replaced with *pGC1*. The *PHO1* gene sequence tag (GST) sense/intron/antisense construct in pB7GWIWG2(II) (*PHO1*RNAi) was PCR amplified with flanking *Xho*I sites and inserted into the *Xho*I site of pART7, creating pART7-*pGC1::PHO1*RNAi. The final *pGC1*::*PHO1*RNAi::ocs3′ expression cassette was excised with Bsp120I and inserted into the *Not*I site of binary vector pGreenII-KAN (kanamycin selective marker in plants; Hellens *et al.*, 2000). This binary vector was used to transform the *Agrobacterium tumefaciens* strain (GV3101) already containing the helper plasmid pSOUP. The resulting *A. tumefaciens* strain was used to transform *A. thaliana* (Columbia) by the floral dip method according to Clough and Bent (1998). Transformants were selected on half-strength MS medium containing 40 μg ml^{-1} kanamycin (Sigma) and 1% w/v sucrose, according to Harrison *et al.* (2006). Independent T_1 transformants were verified by PCR using primers specific to *pGC1*::*PHO1*RNAi::ocs3′, and an appropriate 3:1 resistance to sensitivity segregation ratio was confirmed in the T_2 generation. A number of homozygous transgenic lines were identified in the T_3 generation and, based on reduced *PHO1* mRNA levels in guard cell protoplasts as detected by qRT-PCR, two were selected for future stomatal assays (*pGC1::PHO1*RNAi #6-6 and #10-3) The NTS sharing the same T_1 parental plant were isolated simultaneously for both transgenic lines (WT #6-6 and #10-3).

Guard-cell-specific complementation of *pho1.2*

The 1.2 kb guard cell promoter *pGC1* of *At1g22690* (Yang *et al.*, 2008) was PCR amplified using primers *pGC1-F2* and *pGC1-R2* (Table S1a). The CaMV 35S cassette within vector pMDC32 (Curtis and Grossniklaus, 2003) was excised with *Hin*dII/*Asc*I double digestion and replaced with *pGC1*. The full-length *PHO1* genomic sequence (*PHO1g*, 5.4 kb) was PCR amplified using primers *PHO1g-F* and *PHO1g-R*, flanked with *att*B sites, inserted between *att*P sites of the entry vector pDONR201 and cloned by Gateway LR recombination into binary vector *pGC1*-pMDC32. As described above, this binary vector was introduced into the *A. tumefaciens* strain GV3101 which was then used to transform *A. thaliana pho1.2* as per the floral dip method. Transformants were selected on half-strength MS medium containing 60 μg ml^{-1} hygromycin (Sigma) and 1% w/v sucrose, according to Harrison *et al.* (2006). Again, independent T_1 transformants were verified by PCR and an appropriate 3:1 segregation ratio was confirmed in the T_2 generation. Homozygous transgenic lines *pho1.2 pGC1::PHO1g* #10-1 and #12-1 and their NTS counterparts *pho1.2* #10-1 and #12-1 were identified in the T_3 generation. These transgenic lines were selected for future stomatal assays based on *PHO1g* mRNA expression in guard cell protoplasts as verified by qRT-PCR analysis.

Quantitative RT-PCR on protoplasts

Guard cell and mesophyll cell protoplasts were isolated according to Pandey *et al.* (2002). For ABA treatment on protoplasts, guard cell protoplasts were re-suspended in 50 ml of basic medium (Pandey *et al.*, 2002) to which 50 μl of 0, 5, 10, 50, or 100 mM ABA was added. Replicated treatments were set up allowing analysis immediately following the addition of ABA and after 4 and 6 h incubations in the dark at room temperature (20–22°C). Protoplasts were collected by duplicate 15-min centrifugations at 350 ***g*** in 1.5-ml tubes and all but 100 μl of supernatant was removed from the pellet. Total RNA was extracted using 1 ml TRIZOL reagent (Invitrogen, http://www.invitrogen.com/) following the manufacturer's instructions. Glycogen (Roche, http://www.roche.com/) was used as an RNA carrier.

Total RNA was treated with DNase (Qiagen, http://www.qiagen.com/), and purified using a RNAeasy Minelute Cleanup Kit (Qiagen). Reverse transcription was performed using Moloney Murine

Leukemia Virus (M-MLV) reverse transcriptase (Promega, http://www.promega.com/).

Quantitative RT-PCR analysis was performed using SyberGreen mix and the reference dye ROX (ABgene, http://www.abgene.com/) in a Stratagene MPx3000 instrument. Default thermal cycle settings with a dissociation step were used. All amplification plots were analyzed using MxPro QPCR software (Stratagene, http://www.genomics.agilent.com/) with an *Rn* threshold of 0.1 to obtain *Ct* values. Transcript abundance was estimated as per the standard curve method (Rutledge and Cote, 2003) observing default settings. Transcript abundance of target genes was normalized against expression of the reference gene *At1G13320* (Czechowski *et al.*, 2005). The mesophyll cell-specific gene *At4G26530* was chosen according to microarray data described by Yang *et al.* (2008). The primers used for qRT-PCR are listed in Table S1b.

Quantitative RT-PCR on enriched guard cell preparations

Six-week-old plants were sprayed 1 h after the beginning of the light cycle with ±50 μM ABA and incubated under light for 4 h. Enriched guard cell fractions were promptly prepared by the 'blender method' as described (Geiger *et al.*, 2011). The RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), subjected to DNA digestion with RNase-free DNase (Fermentas, http://www.fermentas.de); first-strand cDNA was prepared by use of M-MLV-RT (Promega). All procedures were carried out according to the manufacturers' protocols. Quantitative RT-PCR was performed in an Eppendorf realplex^2 (http://www.eppendorf.de). Transcript numbers were normalized to molecules of actin2/8 as described elsewhere (Ivashikina *et al.*, 2003). Primers used for qRT-PCR are listed in Table S1b.

ACKNOWLEDGEMENTS

The authors are very grateful to Enrico Martinoia (University of Zurich), Nathalie Leonhardt (CEA, Cadarache), and Peter Ache (University of Würzburg) for helpful discussions. This research was funded from a FNS grant (3100A0-122493) to YP. The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Guard cell morphology of *pho1*.

Figure S2. Transcript levels of *PHO1* homologs in the p*GC1::PHO1*RNAi lines.

Table S1. List of primers used for cloning and quantitative real-time PCR analysis.

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