Abscisic Acid Modulates Seed Germination via ABA INSENSITIVE5-Mediated PHOSPHATE1[1]

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The phytohormone abscisic acid (ABA) controls many developmental and physiological processes. Here, we report that PHOSPHATE1 (PHO1) participates in ABA-mediated seed germination and early seedling development. The transcription of *PHO1* was obviously enhanced during seed germination and early seedling development and repressed by exogenous ABA. The *pho1* mutants (*pho1-2*, *pho1-4*, and *pho1-5*) showed ABA-hypersensitive phenotypes, whereas the *PHO1*-overexpressing lines were ABA-insensitive during seed germination and early seedling development. The expression of *PHO1* was repressed in the *ABI5*-overexpressing line and elevated in the *abi5* mutant, and ABI5 can bind to the *PHO1* promoter in vitro and in vivo, indicating that ABI5 directly down-regulated *PHO1* expression. Disruption of *PHO1* abolished the ABA-insensitive germination phenotypes of *abi5* mutant, demonstrating that *PHO1* was epistatic to *ABI5*. Together, these data demonstrate that PHO1 is involved in ABA-mediated seed germination and early seedling development and transcriptionally regulated by ABI5.

Abscisic acid (ABA) is a key phytohormone that modulates plant growth and development as well as abiotic and biotic stress responses (Verslues and Zhu, 2007). ABA accumulates in the developing embryo and regulates seed development, seed maturation, and seed dormancy (Verslues and Zhu, 2007). ABA functions through complex signaling networks, some components of which have been identified. The core components include ABA receptors PYR/PYL/RCAR, protein phosphatases PP2Cs, protein kinases SnRK2s, and various transcription factors (Hauser et al., 2011; Rushton et al., 2012).

The ABI5, a bZIP transcription factor, is a key regulator in the ABA signaling pathway (Finkelstein and Lynch, 2000; Yu et al., 2015). The expression of *ABI5* is positively modulated by transcription factors ABI3 (Lopez-Molina et al., 2002) and HY5 (Chen et al., 2008); and down-regulated by WRKY40 (Shang et al., 2010), and RAV1 (Feng et al., 2014). ABI5 is also modulated at the post-translational level. The kinases SnRK2.2 (Fujii et al., 2007; Piskurewicz et al., 2008), SnRK2.3 (Fujii et al., 2007; Piskurewicz et al., 2008), BIN2 (Hu and Yu, 2014), and PKS5 (Zhou et al., 2015) phosphorylate ABI5 to control ABI5 activity. In contrast, the phosphatases FyPP1 and FyPP3 dephosphorylate and destabilize ABI5 (Dai et al., 2013). Protein sumoylation and ubiquitination also modulate ABI5 protein activity. The SUMO E3 ligase, SIZ1, sumoylates ABI5 and represses ABI5 function (Miura et al., 2009); and the E3 ligases, KEG (Liu and Stone, 2010) and CUL4 (Lee et al., 2010), regulate ABI5 degradation.

As a transcription factor, ABI5 functions mainly through regulating the expressions of its target genes. ABI5 can up-regulate expressions of two Arabidopsis (*Arabidopsis thaliana*) *LEA* (late embryogenesis abundant) genes, *AtEm1* and *AtEm6* (Carles et al., 2002), which encode ABA-inducible proteins that accumulate during seed maturation (Gaubier et al., 1993). ABI5 mediates the expressions of high-temperature-inducible genes, including *SOMNUS* (*SOM*) in response to high-temperature stress (Lim et al., 2013). In addition, ABI5 modulates early seed development by down-regulating the expression of *SHORT HYPOCOTYLUNDER BLUE1* (*SHB1*; Cheng et al., 2014).

PHOSPHATE1 (PHO1) plays important roles in phosphate (Pi) homeostasis in Arabidopsis (Chiou and Lin, 2011). PHO1 contains two distinct domains: SPX and EXS (Wang et al., 2004). The SPX domain provides a basic binding surface for inositol polyphosphates (InsPs; Wild et al., 2016), and the EXS domain is essential for Pi export activity of PHO1 (Wege et al., 2016). PHO1 is mainly expressed in roots and functions in Pi transfer from roots to shoots (Poirier et al., 1991; Hamburger et al., 2002). In leaves, PHO1 mediates the stomatal response to ABA (Zimmerli et al., 2012).

In this study, we found that three *pho1* mutants displayed ABA-hypersensitive germination phenotypes,

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and the *PHO1*-overexpressing lines were ABA insensitive. ABI5 can bind to the *PHO1* promoter to downregulate *PHO1* expression, and disruption of *PHO1* abolished the ABA-insensitive germination phenotypes of the *abi5* mutant. Taken together, PHO1 is involved in ABA-mediated seed germination and early seedling development, and transcriptionally regulated by ABI5.

RESULTS

Disruption or Mutation of *PHO1* Enhances, and Overexpression of *PHO1* Reduces, ABA Sensitivity during Seed Germination and Early Seedling Development

Arabidopsis PHO1 plays an important role in Pi translocation from roots to shoots (Poirier et al., 1991; Hamburger et al., 2002) and, consistent with the role of PHO1 in Pi transfer, *PHO1* is mainly expressed in the root vascular system (Hamburger et al., 2002). From public microarray data, we found that the transcription level of *PHO1* was relatively high in seeds and enhanced after imbibition. This led us to investigate whether PHO1 was involved in the ABA-mediated seed germination. Quantitative real-time PCR (qRT-PCR) results showed that the transcript level of *PHO1* was elevated during seed germination and early seedling development, and this elevation was obviously repressed by exogenous ABA (Fig. 1A). The GUS staining of the *ProPHO1:GUS* line (Chen et al., 2009) showed that *PHO1* expression was enhanced during seed germination and repressed by exogenous ABA (Fig. 1B). Similar to previous reports, *PHO1* was mainly expressed in roots of seedlings (Fig. 1B; Hamburger et al., 2002; Chen et al., 2009); interestingly, in imbibed seeds and the early germination stage, *PHO1* was mainly expressed in cotyledons (Fig. 1B). The transcriptional response of *PHO1* to ABA was also tested at the same development stage. The imbibed wild-type seeds were germinated and grown on Murashige and Skoog (MS) medium for 1.5 d and then treated with or without exogenous ABA. As shown in Figure 1C, the transcript level of *PHO1* was reduced when treated with exogenous ABA.

To determine the function of PHO1 in seed germination, three *PHO1* alleles with point mutations were used in this study: *pho1-2*, *pho1-4*, and *pho1-5* (Hamburger et al., 2002; Liu et al., 2012). Similar to previous reports, the aerial parts of *pho1* mutants were much smaller than wild-type plants when grown in soil (Supplemental Fig. S1A; Hamburger et al., 2002; Liu et al., 2012). The phosphorus (P) contents of *pho1* seeds were much lower than that of wild-type seeds (Poirier et al., 1991), and the *pho1* mutants showed obviously reduced seed germination rates compared with wild-type plants (spray with water/-ABA; Supplemental Fig. S1B), indicating that the phosphorus deficiency in seeds repressed the seed germination. In order to enhance the seed P content, we supplemented leaves with high Pi. Each plant was foliar sprayed every 4 d with

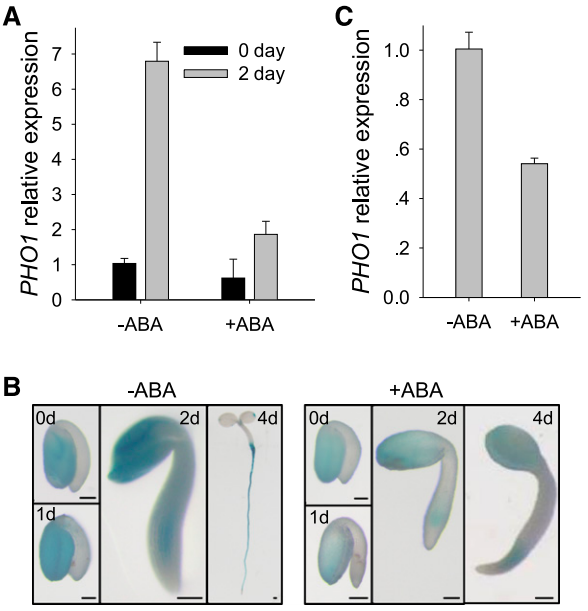

Figure 1. Expression pattern of *PHO1* during seed germination and early seedling development. A, qRT-PCR assay of *PHO1* expression. Wild-type imbibed seeds were transferred to MS medium (-ABA) or MS medium supplemented with 0.5 μM ABA (+ABA) and then harvested at the indicated time for RNA extraction. Data are shown as mean ± SE ($n = 3$). B, GUS-staining assay of *ProPHO1:GUS* line. The imbibed seeds were germinated on MS medium (-ABA) or MS medium supplemented with 0.5 μM ABA (+ABA) and then harvested at the indicated time for GUS staining. Bars = 100 μm. C, qRT-PCR assay of *PHO1* expression. The imbibed wild-type seeds were germinated and grown on MS medium for 1.5 d and then treated with or without 20 μM ABA for 12 h. Data are shown as mean ± SE ($n = 3$).

600 μL of 1/8 MS solution containing 3 mM Pi, and all genotypes were foliar sprayed during the growth and grown in the same green house at the same time. The *abi5* mutant and wild-type plants showed no obvious difference in plant growth and seed germination rate with or without foliar spraying of high Pi (Supplemental Figs. S1A and S1B), indicating that the foliar spray with high Pi did not cause additional secondary stress. Different from the *abi5* mutant and wild-type plants, supplementing leaves with high external Pi helped the *pho1* mutants to grow larger and healthier (Supplemental Fig. S1A). In addition, after foliar spray with high Pi, the seed P contents and seed germination rates of *pho1* mutants were obviously enhanced, similar to those of *abi5* mutant and wild-type plants (spray with Pi/-ABA; Supplemental Figs. S1B and S1C). Then the germination phenotypes were observed in the seeds that were harvested from the plants sprayed with high Pi. These three *pho1* mutants were similar to wild-type plants when germinated and grown on MS medium (0 μM ABA; Fig. 2A); however, they showed ABA-hypersensitive phenotypes when germinated and grown on MS medium including 0.3 or 0.5 μM exogenous ABA (Fig. 2A). The germination rate and cotyledon-greening percentage were further tested. In

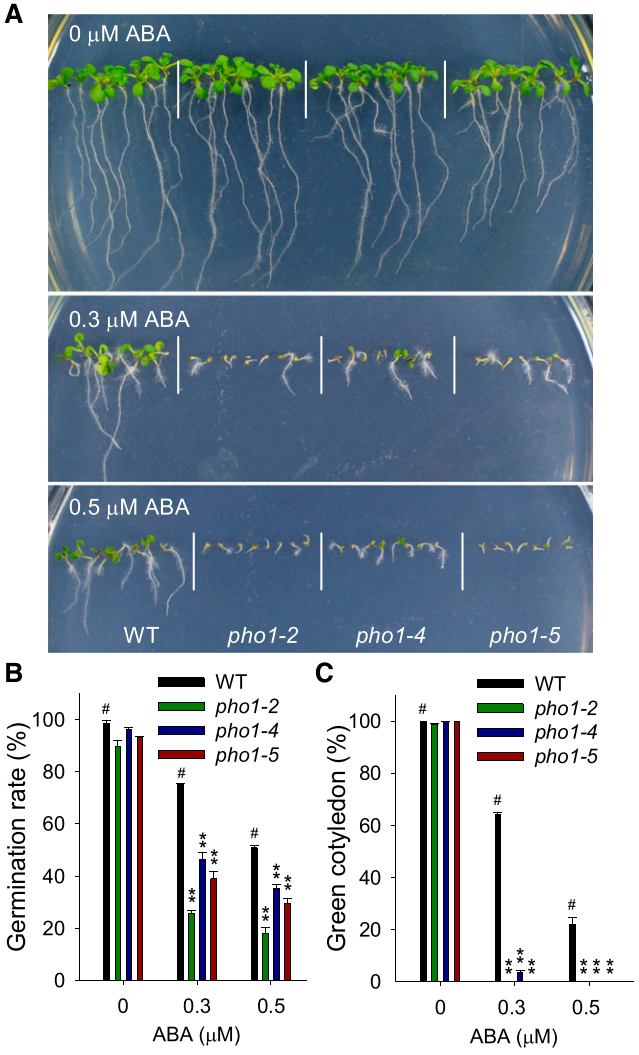

Figure 2. The *pho1* mutants are hypersensitive to exogenous ABA. A, Phenotypic comparison. Imbibed seeds were germinated and grown on MS medium with 0, 0.3, or 0.5 μM ABA for 10 d. B, Germination rate measurement. Imbibed seeds were transferred to MS medium with 0, 0.3, or 0.5 μM ABA for 3 d, and then the seed germination rates were calculated. Data are shown as mean ± SE (n = 3). C, Cotyledon-greening analysis. Imbibed seeds were transferred to MS medium with 0, 0.3, or 0.5 μM ABA for 7 d before determining cotyledon-greening percentages. Data are shown as mean ± SE (n = 3). Asterisks indicate significant differences compared with corresponding wild-type plants (#): $^{**}P < 0.01$.

the absence of ABA, the *pho1* mutants showed no obvious difference with wild-type plants (Fig. 2, B and C; Supplemental Fig. S1B); when germinated and grown on MS medium containing 0.3 or 0.5 μM ABA, the *pho1* mutants were germinated more slowly (Fig. 2B; Supplemental Fig. S1B) and displayed significantly reduced cotyledon-greening percentages (Fig. 2C) compared with wild-type plants. The seed germination phenotype of the *PHO1*-underexpressing line (B3; Supplemental Fig. S2A; Rouached et al., 2011) was also tested. There were no obvious differences between the B3 line and wild-type plants when germinated and grown on MS medium (0 μM ABA), whereas the B3 line, similar to the *pho1* mutants, showed ABA-sensitive germination phenotypes under exogenous ABA (Supplemental Fig. S2B).

Two *PHO1*-overexpressing lines (*PHO1pro:PHO1-12* and *PHO1pro:PHO1-15*), which had tissue-specific overexpression of *PHO1* (Liu et al., 2012), were also used to study the physiological function of PHO1 in seed germination (Fig. 3A). When germinated and grown on MS medium without ABA (0 μM ABA), *PHO1*-overexpressing lines had no obvious difference with wild-type plants (Fig. 3, B and C). In the presence of ABA, the *PHO1*-overexpressing lines showed ABA-insensitive phenotypes and increased germination rates compared with wild-type plants (Fig. 3, B and C). Together, these data demonstrate that PHO1 is involved in ABA-mediated seed germination and early seedling development.

Transcription Factor ABI5 Directly Down-Regulates *PHO1* Expression

Promoter sequence analysis showed that there are two ACGT motifs within the *PHO1* promoter (Fig. 4A). Previous reports showed that transcription factor ABI5 can bind to the ACGT motif in vitro (Carles et al., 2002) and that the *abi5* mutant was extremely insensitive to exogenous ABA during seed germination and early seedling development (Piskurewicz et al., 2008). We hypothesized that transcription factor ABI5 negatively regulated *PHO1* expression. The qRT-PCR results showed that the transcript level of *PHO1* was significantly repressed in the *ABI5*-overexpressing line (*35S:ABI5*; Bu et al., 2009) relative to wild-type plants (Fig. 4B). Transient expression experiments in *Nicotiana benthamiana* leaves also showed that ABI5 repressed *PHO1* promoter activity (Fig. 4C). In addition, *PHO1* expression was significantly enhanced in the *abi5* mutant in the presence of ABA (Fig. 4D). These data indicate that ABI5 negatively modulates *PHO1* expression.

An EMSA was conducted to test whether ABI5 bound to *PHO1* promoter. The ABI5 recombinant protein can bind to P1 and P2 fragments within the *PHO1* promoter, and these bindings were effectively reduced by unlabeled competitors (Cold Probe; Fig. 4E). When the ACGT motif in the P2 fragment was mutated to TTTT (Fig. 4A), the ABI5 recombinant protein cannot bind to the mutated P2 fragment (Fig. 4E). These data indicate that ABI5 protein binds to *PHO1* promoter in vitro. Consistent with previous report, the ABI5 protein was accumulated in wild-type plants under exogenous ABA treatment and was not detected in the *abi5* mutant (Fig. 4F; Piskurewicz et al., 2008). Then a chromatin immunoprecipitation (ChIP) experiment was conducted in the *abi5* mutant and wild-type plants under exogenous ABA treatment. The ChIP results showed that the chromatin immunoprecipitated with the anti-ABI5 antibody was enriched in the P2 fragment of the *PHO1* promoter, and ABI5 enrichment in the P2

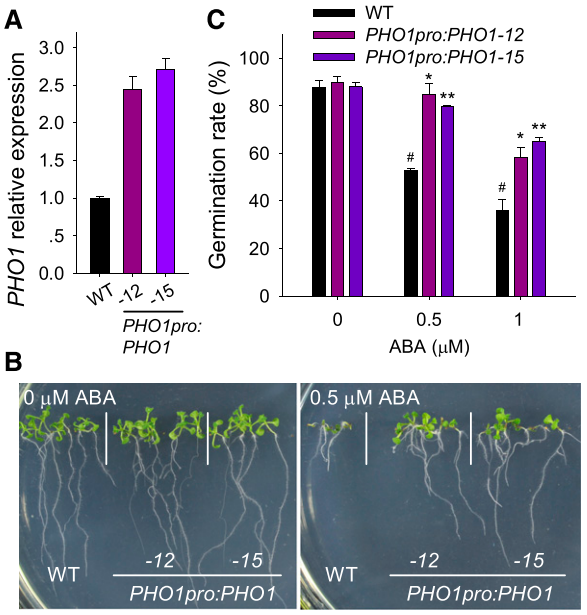

Figure 3. The *PHO1*-overexpressing lines are insensitive to exogenous ABA during seed germination. A, qRT-PCR assay of *PHO1* expression. The *PHO1*-overexpressing lines (*PHO1pro:PHO1-12* and *PHO1pro:PHO1-15*) and wild-type plants were germinated and grown on MS medium for 1.5 d and then harvested for RNA extraction. Data are shown as mean ± SE (n = 3). B, Phenotypic comparison. Imbibed seeds were germinated and grown on MS medium with 0 or 0.5 μM ABA for 10 d. C, Germination rate measurement. Imbibed seeds were transferred to MS medium with 0, 0.5, or 1 μM ABA for 3 d, and then the seed-germination rates were calculated. Data are shown as mean ± SE (n = 3). Asterisks indicate significant differences compared with corresponding wild-type plants (#): $^*P < 0.05$ and $^{**}P < 0.01$.

fragment was not detected in the *abi5* mutant (Fig. 4G). The P1 fragment of the *PHO1* promoter and the exon region of the *ACTIN* gene did not show any detectable binding by ABI5 (Fig. 4G). Then the mutated *PHO1* promoter was generated by removing the ACGT motif within the P2 fragment and named mProPHO1 (Supplemental Fig. S3). The transient expression experiment in *N. benthamiana* leaves showed that the ABI5 could not regulate the activity of the mutated *PHO1* promoter (Fig. 4C). These data demonstrate that transcription factor ABI5 down-regulates *PHO1* expression by binding to the *PHO1* promoter.

Disruption of *PHO1* Abolishes ABA Insensitivity of *abi5* Mutant during Seed Germination and Early Seedling Development

The genetic relationship between *ABI5* and *PHO1* was further analyzed by crossing *pho1-2* with *abi5* to produce the *abi5 pho1* double mutant (Fig. 5, A and B). When germinated and grown on MS medium (0 μM ABA), all genotypes showed similar phenotypes (Fig. 5C). When germinated and grown on MS medium containing 0.5 μM ABA, the *abi5* mutant showed ABA-insensitive phenotypes, whereas the *abi5 pho1* double mutant was ABA hypersensitive, similar to the *pho1-2* mutant (Fig. 5C). Consistent with the phenotypes, when germinated and grown on MS medium with 0.5 μM ABA added, the *abi5 pho1* double mutant, similar to the *pho1-2* mutant, had much lower cotyledon-greening percentages than wild-type plants, and the *abi5* mutant had a higher cotyledon-greening percentage (Fig. 5D). In addition, in the presence of exogenous ABA, the *abi5 pho1* double mutant, similar to the *pho1-2* mutant, showed significantly slower and the *abi5* mutant displayed increased germination rates compared with wild-type plants (Fig. 5E). These results demonstrate that *PHO1* is epistatic to *ABI5* in ABA-mediated seed germination and early seedling development.

DISCUSSION

PHO1 Is an Important Target of ABI5 in Modulating Seed Germination and Early Seedling Development

The transcription factor ABI5 is an important regulator in the ABA signaling pathway. ABI5 confers enhanced responses to exogenous ABA during seed germination: the *abi5* mutant shows obvious ABA-insensitive phenotypes (Piskurewicz et al., 2008), whereas *ABI5*-overexpressing lines are ABA sensitive (Piskurewicz et al., 2008; Bu et al., 2009). As a transcription factor, ABI5 modulates seed germination mainly through regulating the expressions of its target genes. Two Arabidopsis *LEA* genes, *AtEm1* and *AtEm6*, were reported to be target genes of ABI5 (Carles et al., 2002). The expressions of *AtEm1* and *AtEm6* are decreased in the *abi5* mutant (Carles et al., 2002; Feng et al., 2014), and ABI5 can bind to the promoters of *AtEm1* and *AtEm6 in vitro* (Carles et al., 2002) and to the *AtEm6* promoter in vivo (Lopez-Molina et al., 2002), demonstrating that ABI5 directly regulates the expressions of *AtEm1* and *AtEm6*. The *atem6-1* mutant shows premature seed dehydration and maturation at the distal end of siliques (Manfre et al., 2006). A later report demonstrated that ABI5 could activate *SOM* expression at high temperature (Lim et al., 2013). The *som* mutants show higher germination rates than wild-type plants at high temperature (Lim et al., 2013). Recently, ABI5 was reported to directly down-regulate *SHB1* expression to modulate early seed development (Cheng et al., 2014).

Although several target genes of ABI5 have been reported, the mutants of these target genes did not show ABA-dependent germination phenotypes. In this study, the *PHO1* expression was enhanced during seed germination, and this enhancement was repressed by exogenous ABA (Fig. 1), suggesting that *PHO1* was regulated at the transcriptional level during seed germination. The *pho1* mutants and *PHO1*-underexpressing line showed ABA-hypersensitive phenotypes, whereas the *PHO1*-overexpressing lines were ABA insensitive during seed germination and early seedling development (Figs. 2 and 3; Supplemental Fig. S2), and ABI5 can

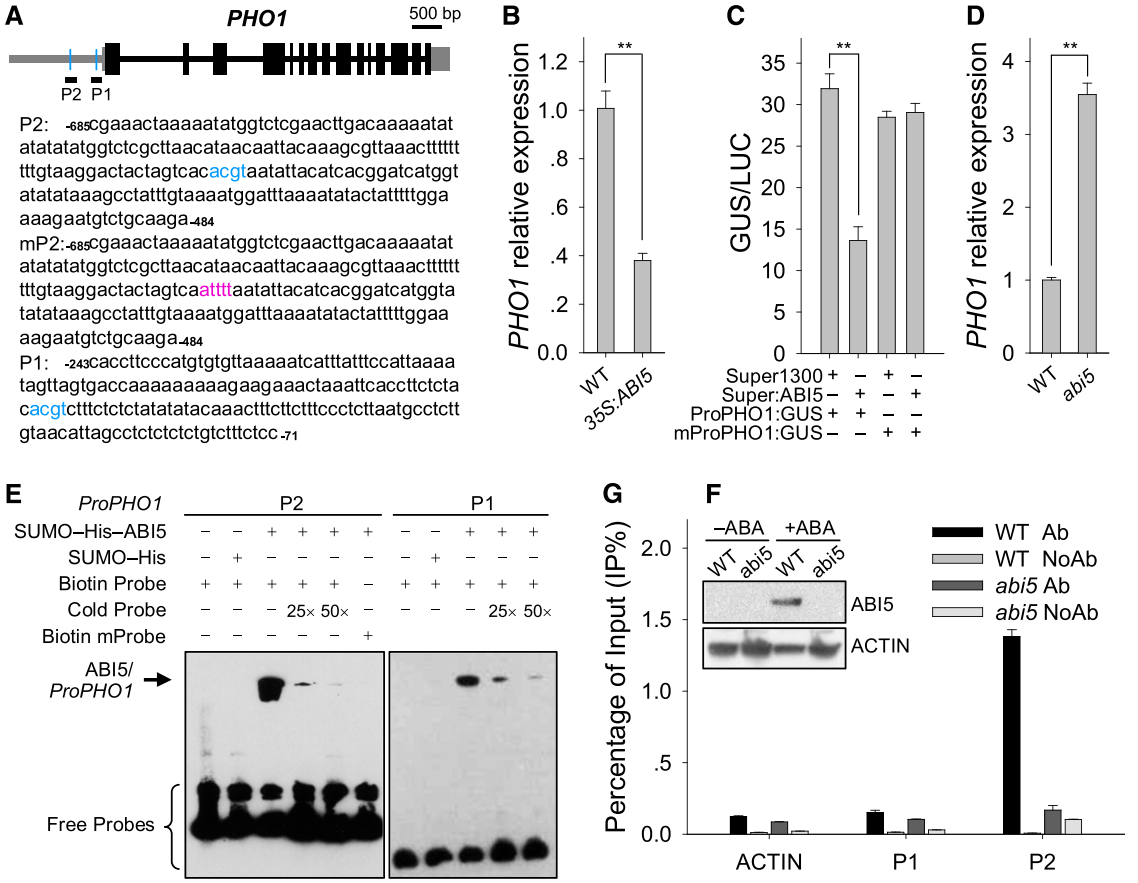

Figure 4. ABI5 directly represses *PHO1* expression. A, Schematic representation of *PHO1* locus. The *PHO1* putative promoter is indicated by a gray line showing the relative positions of the ACGT motifs (blue lines) and its transcribed sequence is indicated by black boxes (exons) and gray boxes (untranslated regions). The adenine (A) of the translational initiation codon ATG is assigned as position +1, and the numbers of *PHO1* promoter fragments are counted based on this number. P1 and P2 indicate PCR fragments for EMSA and ChIP-qPCR experiments. And the normal and mutated ACGT motifs in the P1 and P2 fragments were indicated by blue and burgundy letters, separately. B, qRT-PCR analysis of *PHO1* expression. The *ABI5*-overexpressing line (*35S:ABI5*) and wild-type plant were stratified at 4°C for 3 d and then harvested for RNA extraction. Data are shown as mean ± SE ($n = 3$). C, Transient co-overexpression of *Super:ABI5* and *ProPHO1:GUS* or *mProPHO1:GUS* in *N. benthamiana* leaves. Data are shown as mean ± SE ($n = 3$). D, qRT-PCR analysis of *PHO1* expression in the *abi5* mutant and wild-type plants germinated and grown on MS medium with 0.5 μM ABA for 1.5 d. Asterisks in B to D indicate significant differences: $^{**}P < 0.01$. E, EMSA of ABI5 binding to *PHO1* promoter in vitro. F, Immunoblot analysis of ABI5 protein. The imbibed seeds were transferred to MS medium with or without 0.5 μM ABA for 1.5 d and then harvested for immunoblot analysis using anti-ABI5 antibody. ACTIN was used as the loading control. G, ChIP-qPCR assay of ABI5 binding to *PHO1* promoter in vivo. The *abi5* mutant and wild-type plant were germinated and grown on MS medium with 0.5 μM ABA for 1.5 d and then harvested for ChIP-qPCR assay using anti-ABI5 antibody. Data are shown as mean ± SE ($n = 3$).

bind to *PHO1* promoter to repress *PHO1* expression (Fig. 4). More importantly, disruption of *PHO1* can rescue the ABA-insensitive germination phenotypes of *abi5* mutant (Fig. 5). These data demonstrate that *PHO1* is a key target of ABI5 functioning in ABA-mediated seed germination.

Both SHB1 and PHO1 contain SPX and EXS domains (Wang et al., 2004; Kang and Ni, 2006) and belong to the PHO1 family (Wang et al., 2004; Secco et al., 2012). The Arabidopsis PHO1 family contains 11 members, named PHO1 and PHO1;H1-PHO1;H10 (Wang et al., 2004; Secco et al., 2012), with PHO1;H4 also named SHB1 (Secco et al., 2012). The expression of *SHB1* was also repressed by exogenous ABA (Cheng et al., 2014). The transcript level of *SHB1* was reduced in the *ABI5*-overexpressing line, and ABI5 can bind to the ACGT motif within the *SHB1* promoter (Cheng et al., 2014), indicating that *SHB1* was a target of ABI5. Although both PHO1 and SHB1 belonged to the PHO1 family and were transcriptionally regulated by the transcription factor ABI5, the functions of PHO1 and SHBI were different. SHB1 could mediate early seed development, and the *shb1* mutant displayed smaller seed size compared with wild-type seeds (Cheng et al., 2014). Whereas the *pho1* mutants showed ABA-dependent germination phenotypes (Fig. 2) and could rescue the

ABA-insensitive germination phenotypes of *abi5* mutant (Fig. 5), indicating that transcription factor ABI5 mediated ABA-dependent seed germination, at least mostly, through regulating the *PHO1* expression.

PHO1 Plays Important Roles in Pi Homeostasis and ABA Signaling

The *pho1* mutant was first reported to be deficient in Pi transfer from roots to shoots, with less shoot Pi content and reduced shoot growth (Poirier et al., 1991). *PHO1* is expressed in the root vascular system and hypocotyl of Arabidopsis seedlings (Hamburger et al., 2002), consistent with its role in Pi translocation from roots to shoots. In addition to Pi transfer from roots to shoots, ectopic expression of *PHO1* in leaves also mediates Pi efflux out of leaf cells (Stefanovic et al., 2011; Arpat et al., 2012). Arabidopsis is a dicotyledonous plant, and the major food reserves are typically stored in the cotyledons. The massive mobilization of reserves that occurs after germination provides nutrients to the growing seedling. Different from the seedling stage, *PHO1* was mainly expressed in cotyledons during seed germination (Fig. 1B), suggesting that PHO1 might modulate Pi transfer from cotyledons to radicles, which benefitted the growth of radicle through seed coat. Phytin is the K^+, Mg^{2+}, and Ca^{2+} salt of phytic acid (inositol hexaphosphate), and about two-thirds of phosphorus in cereal and legume seeds is stored in the form of phytin (Dwivedi et al., 2017). Phytin is hydrolyzed by enzyme phytase to release phosphate for the growing seedling (Taiz et al., 2015), and in plants, the InsPs were high-abundance phosphate storage compounds in seeds (Raboy, 2003). Previous reports show that the PHO1 protein contains an SPX domain (Wang et al., 2004), which is a sensor domain of InsPs (Wild et al., 2016). In addition, PHO1 is localized in the Golgi/trans-Golgi network and vesicles (Arpat et al., 2012), suggesting that PHO1 may participate in the mobilization of Pi from the phytate store during seed germination.

During seedling stage, *PHO1* is also expressed in guard cells (Zimmerli et al., 2012). *PHO1* expression in guard cells is increased after exogenous ABA treatment, and ABA-mediated stomatal movement is severely impaired in *pho1* mutants (Zimmerli et al., 2012), indicating that PHO1 participates in the ABA signal pathway. Interestingly, the expression of *PHO1* in wild-type seedlings is repressed by exogenous ABA under Pi-sufficient conditions, and ABA addition abolishes the increase of *PHO1* expression in Pi-deficient wild-type seedlings (Ribot et al., 2008), suggesting a cross talk between ABA and Pi-starvation signaling. In this present study, the *pho1* mutants showed ABA-hypersensitive germination phenotypes, whereas the *PHO1*-overexpressing lines were ABA insensitive (Figs. 2 and 3). Previous reports showed that the InsPs played potential roles in hormone signaling, such as auxin (Tan et al., 2007) and jasmonate (Sheard et al., 2010;

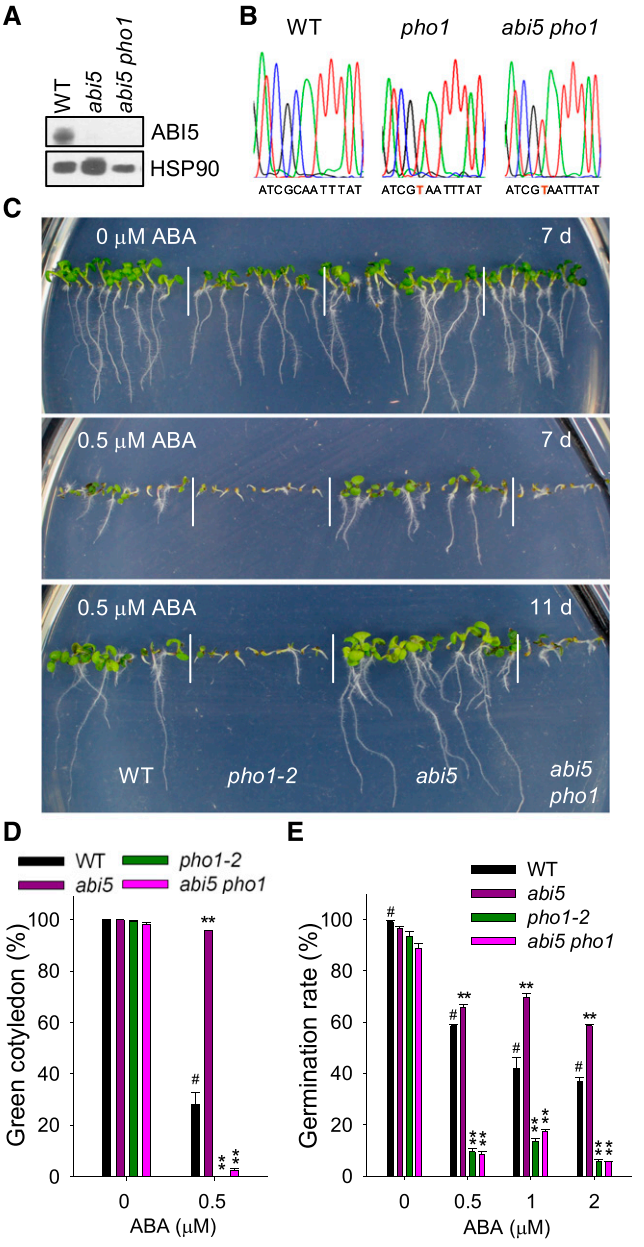

Figure 5. Disruption of *PHO1* abolishes the ABA-insensitive phenotypes of *abi5* mutant. A, Immunoblot analysis of ABI5 protein. The imbibed seeds were grown on MS medium supplemented with 5 μM ABA for 5 d and then harvested for immunoblot analysis. HSP90 was used as the loading control. B, The mutations in *PHO1* gene in the *pho1* mutant and *abi5 pho1* double mutant were evaluated by sequencing. The mutation site in *PHO1* is indicated by red letters. C, Phenotypic comparison. Imbibed seeds were germinated and grown on MS medium with 0 or 0.5 μM ABA for 10 d. D, Cotyledon-greening analysis. Imbibed seeds were germinated and grown on MS medium with 0 or 0.5 μM ABA for 7 d, and then the cotyledon-greening percentages were calculated. Data are shown as mean ± SE ($n = 3$). E, Germination rate measurement. Imbibed seeds were transferred to MS medium with 0, 0.5, 1, or 2 μM ABA for 3 d, and then the seed germination rates were calculated. Data are shown as mean ± SE ($n = 3$). Asterisks indicate significant differences compared with corresponding wild-type plants (#): $^{**}P < 0.01$.

Mosblech et al., 2011). PHO1 interacted with InsPs (Wild et al., 2016) and was involved in the ABA signaling pathway in seed germination (Figs. 2 and 3) as well as in guard cells (Zimmerli et al., 2012). The implication of InsP in ABA signaling has been shown by FIERY1/SAL1 (Xiong et al., 2001; Wilson et al., 2009). Consequently, it is possible that PHO1 participates in the ABA signal transduction during seed germination via its interaction with InsP.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type plant used in this study was Arabidopsis (*Arabidopsis thaliana*) Col-0. The *pho1-2* (Hamburger et al., 2002), *pho1-4* (Hamburger et al., 2002), *pho1-5* (Liu et al., 2012), and *abi5* (Feng et al., 2014) mutants and *PHO1*-overexpressing (*PHO1pro:PHO1-12* and *PHO1pro:PHO1-15*; Liu et al., 2012), *ABI5*-overexpressing (*35S:ABI5*) (Bu et al., 2009), and *ProPHO1:GUS* (Chen et al., 2009) lines were described previously.

The phenotype comparison assays were conducted as previously described (Feng et al., 2014; Huang et al., 2016). For seed harvest, Arabidopsis plants were grown at 22°C for a 16-h daily light period in a potting soil mixture (rich soil:vermiculite = 2:1, v/v). Each plant was foliar sprayed every 4 d with 600 μL of 1/8 MS solution containing 3 mM Pi. All genotypes were foliar sprayed during growth and grown in the same green house. The germination experiments were performed with the seeds of different genotypes that were grown together at the same time and under the same environmental conditions. The relative humidity was approximately 70% (±5%).

Determination of Germination Rate and Green Cotyledon

The imbibed seeds were germinated and grown on MS medium supplemented with 0, 0.3, or 0.5 μM ABA, and then the germination rate and green cotyledon were measured on the third day and seventh day, separately. Germination rate was measured based on the emergence of the radicle through seed coat, as observed under a microscope. Green cotyledon was determined based on the appearance of green cotyledons in a seedling. More than 100 seeds were measured in each replicate. Each experiment was conducted in three biological replicates, and similar results were obtained.

qRT-PCR Assay and GUS Staining

The total RNA of seedlings and seeds was extracted with RNeasy Plant Mini kit (Bioteke), and treated with DNase I (Takara) to eliminate genomic DNA contamination. The qRT-PCR assay was conducted as described previously (Feng et al., 2014; Huang et al., 2016). *Actin2/8* expression was used as an internal control. The primers used are listed in Supplemental Table S1. Each experiment was done in three biological replicates.

GUS staining experiment was performed as previously described (Chen et al., 2009).

Transient Expression Assay in *Nicotiana benthamiana*

The coding sequence of *ABI5* was cloned into vector *Super1300* (Chen et al., 2009) to form a *Super:ABI5* construct. The primers used are listed in Supplemental Table S1. The construct *ProPHO1:GUS* was described previously (Chen et al., 2009). The *ProPHO1:GUS* and *Super:ABI5* were singly or cotransformed into *N. benthamiana* leaves. For each infiltration sample, *Super:LUC* was added as an internal control. The GUS and LUC activities of infiltrated leaves were quantitatively determined, and the GUS/LUC ratio was used to quantify promoter activity.

Protein Expression and EMSA Experiment

The coding sequence of *ABI5* was cloned into *pET28a-SUMO* vector (Novagen). The primers used are listed in Supplemental Table S1. The recombinant plasmid and *pET28a-SUMO* were introduced into *Escherichia coli* strain BL21 separately, and the SUMO-His-ABI5 and SUMO-His proteins were purified using Ni-Sepharose 6 Fast Flow (GE Healthcare).

The EMSA was conducted as described previously (Huang et al., 2016). The fragments of *PHO1* promoter (P1 and P2) were obtained by PCR using biotin-labeled or -unlabeled primers (see Supplemental Table S1). Biotin-unlabeled fragments (cold-probe) of the same sequences were used as competitors.

ChIP-qPCR Assay

The *abi5* mutant and wild-type Arabidopsis were germinated and grown on MS medium with 0.5 μM ABA for 1.5 d and then harvested for ChIP experiment. The ChIP experiment was performed as described previously (Chen et al., 2009; Huang et al., 2016), and the primers used are listed in Supplemental Table S1.

Accession Numbers

Sequence data from this article can be found in GenBank (http://www.ncbi.nlm.nih.gov) under the following accession numbers: PHO1 (At3g23430), ABI5 (At2g36270), ACT2 (At3g18780), and ACT8 (At1g49240).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The characterization of Arabidopsis plants sprayed with external Pi.

Supplemental Figure S2. Germination phenotype of *PHO1*-underexpressing line.

Supplemental Figure S3. Sequences of normal and mutated *PHO1* promoters.

Supplemental Table S1. Primer sequences used in this study.

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