Running Title:

Pht1;5 mobilizes Pi between source and sink organs

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Focus Issue: Plant Phosphorus Nutrition
Title:

Arabidopsis Pht1;5 mobilizes phosphate between source and sink organs, and influences the interaction between phosphate homeostasis and ethylene signaling

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This work was supported by funding to A.P.S. from Louisiana State University and to K.G.R from the McKnight Foundation.

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ABSTRACT

Phosphorus (P) remobilization in plants is required for continuous growth and development. The Arabidopsis thaliana phosphate (Pi) transporter Pht1;5 has been implicated in mobilizing stored Pi out of older leaves. In this study, we used a reverse-genetics approach to study the role of Pht1;5 in Pi homeostasis. Under low Pi conditions, Pht1;5 loss-of-function (pht1;5-1) resulted in reduced P allocation to shoots and elevated transcript levels for several Pi-starvation-response genes. Under Pi-replete conditions, pht1;5-1 had higher shoot P content compared to wild type (WT), but had reduced P content in roots. Constitutive overexpression of Pht1;5 had the opposite effect on P distribution; namely, lower P levels in shoots compared to WT, but higher P content in roots. Pht1;5-overexpression also resulted in altered Pi remobilization as evidenced by a greater than 2-fold increase in accumulation of Pi in siliques, premature senescence, and an increase in transcript levels of genes involved in Pi scavenging. Furthermore, Pht1;5-overexpressors exhibited increased root hair formation and reduced primary root growth that could be rescued by the application of silver nitrate (ethylene perception inhibitor) or aminoethoxyvinyl-glycine (ethylene biosynthesis inhibitor), respectively. Together the data indicate that Pht1;5 plays a critical role in mobilizing Pi from P source to sink organs in accordance with developmental cues and P status. The study also provides evidence for a link between Pi- and ethylene-signaling pathways.
**INTRODUCTION**

Phosphorus (P) is an essential macronutrient required for many physiological and metabolic processes. It is integral to several molecules such as nucleic acids, phospholipids, and ATP, as well as to intermediates of signal transduction events (Schachtman and Shin, 2007; Rouached et al., 2010). To modulate P homeostasis, plants must balance P uptake, mobilization, and partitioning to various organs. Although P is abundant in nature, the bioavailability of utilizable orthophosphate (Pi) is often suboptimal for crop productivity (Marschner, 1995; Raghothama, 1999; Ticoni and Abel, 2004; Tin et al., 2009). Since Pi concentrations in soil solution rarely exceed 2 µM and cellular Pi concentrations are greater than 10 mM, plants must acquire Pi into roots against a steep concentration gradient (Mimura, 1999; Raghothama, 2000). Pi acquisition appears to be largely mediated by plasma-membrane localized high-affinity Pi transporters belonging to the *PHOSPHATE TRANSPORTER1* (*Pht1*) family (Muchhal et al., 1996; Raghothama, 2000; Chiu et al., 2000; Poirier and Bucher, 2002). These proteins are characterized by 12 membrane-spanning domains that are similar to the yeast Pho84p high-affinity Pi transporter (Muchhal et al., 1996; Rausch and Bucher, 2002). In *Arabidopsis thaliana* there are nine such *Pht1* proteins with 60-95% sequence similarity, and their homologues have been identified in several crop species (rice, wheat, potato, tomato and tobacco) (Rausch and Bucher, 2002). Promoter-reporter fusions of *Pht1* members in Arabidopsis demonstrated the Pi-deficiency-induced expression of eight of the nine members in roots (Karthikeyan et al., 2002; Mudge et al., 2002). Further functional characterization of loss-of-function mutants of *Pht1;1* and *Pht1;4* validated their roles in Pi acquisition (Shin et al., 2004).

After Pi is transported into root epidermal cells, it is loaded into the xylem for distribution to shoot tissues (Poirier et al., 1991). Several studies have characterized mutants that are unable to mobilize Pi from source (older leaves) to sink organs (roots and younger leaves; Delhaize and Randall, 1995; Versaw and Harrison, 2002; Aung et al., 2006; Chiu et al., 2006). Under long-term Pi deprivation, Pi is redistributed from older leaves towards sink organs (young leaves, growing roots, and developing seeds) by a process requiring its transfer to phloem vessels (Raghothama, 2000; Bucher et al., 2001). An important process in plant growth and development is efficient nutrient remobilization from older, senescing leaves in order to scavenge resources that may be limiting in nature or energetically costly to acquire (Leopold, 1961). In this context, it has been demonstrated that up to 78% of stored Pi is remobilized from older leaves in
Arabidopsis (Himelblau and Amasino, 2001). Therefore, the translocation of Pi into sink tissues/cells is important for sustaining growth under low Pi conditions. Chloroplast localized Ph2;1, a low-affinity Pi transporter (Km of ~ 0.4 mM), has been shown to mediate Pi translocation within the aerial parts of Arabidopsis (Daram et al., 1999; Versaw and Harrison, 2002). Low-affinity transporters from barley (HvPht1;6) and rice (OsPht1;2) have also been implicated in Pi remobilization from leaves and Pi movement from root to shoot, respectively (Rae et al., 2003; Ai et al., 2009; Preuss et al., 2010). Despite these developments, little is known about the molecular mechanisms that govern Pi translocation and remobilization in higher plants.

Spatial expression patterns of Pht1 members in different tissue types and organs of Arabidopsis suggest their potential involvement not only in Pi acquisition, but also in internal Pi distribution to metabolically active and growing parts of the plant (Karthikeyan et al., 2002, 2009; Mudge et al., 2002; Miller et al., 2009). Among the Pht1 members, Pht1;5 showed Pi-deficiency-induced expression specifically in the phloem cells of older leaves, cotyledons, and flowers (Mudge et al., 2002). Genome-wide transcriptome analysis further corroborated the expression of Pht1;5 over the course of developmentally-regulated senescence in the leaves of Arabidopsis (van der Graaf et al., 2006). However, the functional characterization of Pht1;5 and its potential role in Pi translocation/remobilization has not been elucidated. Here, we used loss-of-function mutants of Pht1;5 and transgenic lines overexpressing this gene in Arabidopsis to demonstrate its role in Pi mobilization between source and sink under different Pi regimes. We also provide evidence for a tangible link between Pi transporters and ethylene signaling.

RESULTS

Expression profile of Pht1;5, and isolation of pht1;5 T-DNA insertion mutants

Previous studies have demonstrated the Pi-starvation-responsiveness of Pht1;5 expression in Arabidopsis (Mudge et al., 2002; Morcuende et al., 2007; Thibaud et al., 2010). To further determine the spatial expression pattern of Pht1;5, wild-type (WT) Arabidopsis plants were germinated hydroponically on one-half-strength MS medium for 5 d and transferred to low (10 µM Pi, P-) and high Pi (1250 µM Pi, P+) for 7 d. Quantitative reverse-transcription PCR (qRT-PCR) analysis was used to measure Pht1;5 transcript levels in different tissues (roots and rosette leaves) grown under P+ and P- conditions. As shown in Figure 1A, Pi deficiency triggered increases in the abundance of Pht1;5 transcripts in both roots and rosette leaves. Consistent with
earlier studies (Karthikeyan et al., 2002; Mudge et al., 2002; Shin et al., 2004), *Pht1;4* transcript levels increased strongly in response to Pi deficiency in roots and rosette leaves (Fig. 1A). The data thus validated the fidelity of the growth condition used for elucidating the effect of Pi deficiency on the spatial expression profile of *Pht1;5*.

To determine the role of *Pht1;5* in acquisition and mobilization of Pi, a reverse genetics approach was employed. Two homozygous mutants [i.e., SALK_074836 (*pht1;5-1*) and SALK_138009C (*pht1;5-2*)] were identified with T-DNAs inserted at 919 bp (exon) and 1020 bp (intron), respectively, downstream of the translation start site of the *Pht1;5* gene (Fig. 1B). Genetic analysis revealed that the *pht1;5-1* T-DNA segregated as a single insertion locus (data not shown), whereas estimation of the T-DNA copy number of *pht1;5-2* (a confirmed homozygous insertion line, ABRC) via Quantitative PCR revealed the presence of ~5 and ~7 copies of nptII and T-DNA left border compared to *pht1;5-1* (Supplemental Table S2). RT-PCR was performed on seven-day-old Pi-starved WT, *pht1;5-1*, and *pht1;5-2* seedlings for determining the levels of *Pht1;5* expression (Fig. 1C). An amplified product corresponding to *Pht1;5* was detected in WT, whereas no amplification products were detected in *pht1;5-1* or *pht1;5-2*. Further, qRT-PCR analyses of Pi-starved roots and shoots found no detectable amplification for *Pht1;5* in these mutants (data not shown). The results confirmed the identification of two independent loss-of-function mutants for *Pht1;5*.

**Loss-of-function mutation of *Pht1;5* results in altered Pi allocation between shoot and root**

During Pi deficiency, the levels of *Pht1;5* transcripts increased appreciably in both roots and rosette leaves (Fig. 1A). Therefore, a role for Pht1;5 in translocating Pi between root and shoot was hypothesized. To test this, radiolabeled $^{32}$Pi was used to compare the rates of root-to-shoot Pi translocation among WT, *pht1;5-1*, and *pht1;5-2*. A loss-of-function mutant (*pht1;1-2*) of high-affinity transporter *Pht1;1*, which affects both Pi acquisition and allocation (Shin et al., 2004), was included for comparison. Under P+ conditions, the distribution of $^{31}$Pi in the shoots was comparable among WT, *pht1;5-2*, and *pht1;1-2*, and was marginally higher for *pht1;5-1* (Fig. 2A). However, under P- conditions, both *pht1;5* mutants showed 35-40% lower $^{33}$Pi distribution to the shoots relative to WT. Although $^{33}$Pi distribution in *pht1;1-2* was also significantly ($P<0.05$) lower than WT, it was significantly ($P<0.05$) higher than the *pht1;5* mutants (Fig. 2A). These results suggest a more pronounced role for Pht1;5 in allocating Pi to the
shoots during Pi deprivation. To determine whether the attenuated shoot Pi translocation rates observed in the mutants correlated with a change in P accumulation, we compared the total shoot P content of WT, pht1;5-1, and pht1;1-2 (Fig. 2B). Pi deficiency resulted in significant reductions (P<0.05) in the total shoot P accumulation in all genotypes. Although the P content in the P+ shoots of pht1;5-1 was higher than in WT, under P- conditions both mutants (pht1;1-2 and pht1;5-1) accumulated about 20% less P in their shoots compared to WT (Fig 2B). Shoot P measurements in leaves and floral stalks from Pi-starved pht1;5-1 plants show similar decreases compared to WT when grown hydroponically under greenhouse conditions (Supplemental Fig. S1). The decrease in shoot P content in pht1;5-1 was amended by complementing the mutant with Pht1;5 cDNA (Supplemental Fig. S1). The data support a role for Pht1;5 in facilitating mobilization of Pi between root and shoot independently, or in conjunction with, Pht1;1. Whether altered Pi mobilization in the shoots of the pht1;5 mutant has a commensurate effect on Pi-starvation-response (PSR) gene expression was determined via qRT-PCR analysis of several PSR genes (Misson et al., 2005; Shin et al., 2006). Under P+ conditions, other than a marginal increase in At4 transcripts in the pht1;5-1 mutant, the relative transcript levels for the PSR genes tested (At4, Pht1;4, DGD1, and SQD1) were similar between pht1;5-1 and WT (Fig. 2C). In contrast, the transcript levels for all the genes tested were significantly higher in the Pi-deprived shoots of pht1;5-1 compared to WT. These data corroborate that a more aggravated Pi starvation response is being experienced by the shoots of pht1;5-1 compared to WT due to the disruption of Pi mobilization from root to the shoot during Pi deficiency.

To gain further insight into a possible role for Pht1;5 in the maintenance of Pi homeostasis, $^{33}$Pi uptake rate in the roots was evaluated for WT, pht1;5-1, pht1;5-2, and pht1;1-2 seedlings grown under P+ and P- conditions (Fig. 3A). Independent of the Pi regime, there were significant (P<0.05) increases (~10-20%) in the $^{33}$Pi uptake rates (i.e. net accumulation of $^{33}$Pi) in the roots of pht1;5-1 and pht1;5-2 compared to that of WT. On the contrary, there was a significant (P<0.05) decline (~30%) in root Pi uptake for pht1;1-2 compared to WT when grown under Pi-replete conditions, which is consistent with an earlier study (Shin et al., 2004). Despite differential $^{33}$Pi uptake rates observed for the pht1;1 and pht1;5 mutants under variable Pi conditions, the total P content in the roots of these mutants under both P+ and P- conditions were very similar and significantly (P<0.05) lower compared to WT (Fig. 3B). The total P content data for roots (Fig. 3B) and shoots (Fig. 2B) of WT, pht1;5-1, and pht1;1-2 were used to
calculate root:shoot distribution ratios of P (Fig. 3C). Although Pi deficiency exerted no significant ($P<0.05$) differences on the ratio among WT and the mutants, $pht1;5-1$ showed a significant ($P<0.05$) decline in the ratio compared to WT and $pht1;1-2$ when grown under Pi-replete conditions. Considering that $Pht1;5$ is expressed strongly in shoot tissues during Pi-replete conditions (Fig 1A), these data suggest that $Pht1;5$ is involved in mobilization of Pi from shoots to roots during high Pi conditions.

Arsenate ($\text{AsO}_4^{3-}$) is an oxyanion structurally analogous to Pi and is taken up by roots via high-affinity Pi transporters (Asher and Reay, 1979; Shin et al., 2004; Catarecha et al., 2007). To gain further insight into the role of $Pht1;5$ in Pi acquisition, we compared the phenotypic responses of the $pht1;1-2$ and $pht1;5-1$ mutants to arsenate (Fig. 4). Although the toxic effect of arsenate was evident on the growth of both the mutants and WT, the degree of tolerance varied. The shoot fresh weight of WT grown on arsenate was 42% compared to that of untreated WT seedlings (Fig. 4A). The corresponding values for the $pht1;1-2$ and $pht1;5-1$ mutants were 77% and 65%, respectively, which indicated greater tolerance to arsenate by the mutants relative to WT. A similar trend of higher tolerance in the mutants was evident with respect to lateral root development (Fig. 4B). Together these data indicate that loss of $Pht1;5$ confers weak tolerance to arsenate, suggesting that $Pht1;5$ could influence acquisition of both Pi and arsenate.

**Overexpression of $Pht1;5$ affects distribution and remobilization of Pi between source and sink**

To further examine the *in planta* role of $Pht1;5$ in Pi allocation we overexpressed the $Pht1;5$ coding region under the control of the $ACTIN 2$ ($ACT2$) promoter in WT Arabidopsis. The $ACT2$ promoter imparts strong constitutive expression in vegetative tissues (An et al., 1996; Kandasamy et al., 2002). Two independently generated transgenic lines (5A and 11C) with relatively high levels of $Pht1;5$ expression compared to WT (Supplemental Fig. S2) were selected for phenotypic characterization. After four weeks of growth the $Pht1;5$-overexpressors showed substantial increases in shoot biomass and leaf area compared to WT and $pht1;5-1$ (Fig. 5A). A more detailed phenotypic characterization was carried out on WT, $pht1;5-1$, and overexpressors that were grown for an additional two weeks. The $Pht1;5$-overexpressors exhibited significant ($P<0.05$) increases in total leaf area (Fig. 5B), floral stalk thickness (Fig. 5C), and total leaf dry weight (Fig. 5D) compared to WT and $pht1;5-1$. On the other hand,
*pht1;5-1* initially displayed slower growth at the four week-stage (Fig. 5A) but morphological parameters after two more weeks were only marginally affected compared to the WT (Fig. 5, B and D). These phenotypic differences were observed under both short- (Fig. 5) and long-day (not shown) growth conditions.

To examine the effect of *Pht1;5*-overexpression on P distribution and acquisition, the total shoot and root P content and $^{33}$Pi root uptake rates of WT and *Pht1;5*-overexpressors were determined. Seedlings were grown on agar-solidified Pi-replete medium. Relative to WT, there was a significant ($P<0.05$) decline in the total P content in the shoots of both overexpressors, but an increase in total root P content (Fig. 6A). Interestingly, while the WT showed higher ($P<0.05$) shoot P content than root, there was no difference in P content between the two organs in the *Pht1;5*-overexpression lines. This suggests altered P distribution between source and sink in the overexpressors (Fig. 6B). It is plausible that *Pht1;5*-overexpression results in recycling of Pi from the shoots back to the roots. Elevated (~60%) root $^{33}$Pi uptake rates in the *Pht1;5*-overexpressors compared to WT (Fig. 6C) further supported this notion. Although the $^{33}$Pi uptake rates in roots of the Pi-deprived *Pht1;5*-overexpressors were attenuated compared to WT, the P contents were comparable (data not shown). This indicates a differential effect of *Pht1;5*-overexpression on Pi homeostasis under different Pi regimes.

*Pht1;5*-overexpressors that were grown to maturity under greenhouse conditions consistently displayed chlorosis in older leaves that subsequently senesced earlier than those of WT (Fig. 7A). To gain insight into this premature senescence, $^{33}$Pi tracer studies were carried out to compare the Pi accumulation among a *Pht1;5*-overexpressor (11C), the *pht1;5-1* mutant, and WT (Fig. 7B). Compared to WT, $^{33}$Pi accumulation in the *Pht1;5*-overexpressor was significantly ($P<0.05$) lower in rosette leaves, comparable in cauline leaves, marginally higher in floral stalks, and ~2-fold higher in siliques. The $^{33}$Pi distribution in *pht1;5-1* tissues was consistently and significantly ($P<0.05$) lower relative to WT. The data suggest increased mobilization of Pi in the *Pht1;5*-overexpressor from source (i.e. rosette leaves) to sink (i.e. siliques), which may be attributed to enhanced Pi scavenging mechanisms that facilitate release of Pi from bound sources such as nucleic acids (Green, 1994; Raghorthama, 1999; Misson et al., 2005). qRT-PCR analysis was used to quantify the transcript levels of ribonuclease (*RNS1*; Bariola et al., 1994) and phosphatase (*ACP5*; del Pozo et al., 1999) genes involved in Pi remobilization in leaves of four-week-old *Pht1;5*-overexpressors grown under Pi-replete
conditions. As anticipated, there were increases in the relative transcript levels of RNS1 (14 to 28 fold) and ACP5 (1.5 to 2 fold) in the Phl1;5-overexpressors compared to WT (Fig. 7C). These molecular data support a role for Phl1;5 in facilitating remobilization of Pi from senescing to metabolically active parts of the plant.

*Phl1;5-overexpression alters root hair development and primary root growth in association with ethylene signaling*

To study whether differential nutrient partitioning between root and shoot affects root development in the Phl1;5-overexpression lines, different root traits of the overexpressors were compared with those of WT and the phl1;5-1 mutant. Among the root traits examined, increased root-hair proliferation is an early response of plants to Pi deficiency (Bates and Lynch, 1996; Ma et al., 2001; Jain et al., 2007b). Irrespective of the Pi regime, the Phl1;5-overexpressors showed significant (*P*<0.05) increases in both the number and length of root hairs compared to WT and phl1;5-1 (Fig. 8). A second Pi-deficiency-induced root response is a reduction in primary root growth due to premature cell differentiation (López-Bucio et al., 2002, 2003; Sánchez-Calderón et al., 2005; Jain et al., 2007a). Under P+ and P- conditions, there were significant (*P*<0.05) reductions in the primary root length of both Phl1;5-overexpressors compared to WT and phl1;5-1 (Fig. 9, A and B). Pi-deficiency-mediated root proliferation aimed at enhancing Pi mining capacity results in an increased root/shoot biomass ratio (Hermans et al., 2006; Hammond and White, 2008). Analysis of this trait revealed a significant (*P*<0.05) reduction in the Phl1;5-overexpressors relative to WT and phl1;5-1 under P- conditions, whereas a reverse trend was observed under P+ conditions (Fig. 9C). Together these data indicate accentuated Pi-deficiency responses of Phl1;5-overexpressors under variable Pi conditions.

Modulation of root traits has been shown to be a manifestation of complex interactions among signaling pathways of Pi deficiency and phytohormones, such as auxin and ethylene (Williamson et al., 2001; López-Bucio et al., 2002; Nacry et al., 2005; Jain et al., 2007a; Lei et al., 2011). Several lines of evidence indicate that ethylene stimulates auxin biosynthesis, and synergistic interactions between them modulate root growth and root hair development (Osmont et al., 2007; Stepanova and Alonso, 2009). Since overexpression of Phl1;5 caused a dramatic inhibition of primary root growth and alteration in root hair development under both P+ and P- conditions, its root responses towards ethylene precursor/inhibitors were investigated. WT and
Phl1;5-overexpressors (5A and 11C) were grown on one-half-strength MS medium for 5 d and then transferred to P+ supplemented with ethylene precursor (1 μM ACC), biosynthesis inhibitor (0.2 μM AVG), perception inhibitor (20 μM Ag⁺), or simultaneous application of ACC + AVG, and ACC + Ag⁺ (Fig. 10). The effects of these treatments on root hair development and primary root length were documented after 1 and 7 d, respectively. Treatment with AVG resulted in significant (P<0.05) reductions in total root hair length of WT and the Phl1;5-overexpressors (Fig. 10B). However, Ag⁺ treatment resulted in a substantial decrease in total root hair length for the Phl1;5-overexpressors, but only a marginal decrease for WT (Fig. 10B). A similar phenomenon was observed with the impact of AVG on primary root growth. Treatment with AVG had no effect on WT primary root length, but it dramatically increased (P<0.001) primary root growth in the overexpressors, resulting in lengths comparable to WT (Fig. 10, A and C). The Ag+ treatment significantly (P<0.05) reduced the primary root length of the WT, but did not impose any detrimental effect on the root length of the Phl1;5-overexpression lines (Fig. 10C). Although medium supplemented with ACC or ACC + AVG exerted strong (P<0.001) inhibitory effects on the primary root growth of all the genotypes tested, the Phl1;5-overexpressors displayed augmented sensitivity towards ACC compared to WT (Fig. 10C). Together these results implicate ethylene signaling in modulating the primary root and root hair phenotypes of Phl1;5-overexpression lines.

DISCUSSION

Plants possess multiple Pi transport systems, regulated at both high- and low-affinities, that facilitate Pi uptake from the rhizosphere and subsequently distribute it to cells and subcellular compartments. The movement of Pi inside the plant is an intricate and complex process since source and sink relationships are constantly changing depending on the rate of growth, stage of development, and Pi availability (Raghothama, 1999; Bucher et al., 2001; Miller et al., 2009). The data presented here demonstrate that Phl1;5 is required for normal mobilization of Pi between source and sink tissues.

Phl1;5 influences shoot Pi status by regulating Pi distribution between root and shoot

Among the members of the Phl1 family, Phl1;5 was shown to be expressed in cotyledons and hypocotyls of germinating seedlings, floral buds, older leaves at the onset of senescence, and
in Pi-starved roots (Mudge et al., 2002). Our experiments corroborated these results, and also showed that \textit{Pht1;5} is expressed in leaves under both P+ and P- conditions (Fig. 1A). Functional inactivation of this gene in \textit{pht1;5} mutant seedlings (\textit{pht1;5-1} and \textit{pht1;5-2}) grown under Pi-deficient conditions led to low shoot P content, despite also resulting in an increased root Pi uptake rate (Figs 2 and 3). Shoots of mature Pi-starved \textit{pht1;5-1} plants showed a similar reduction in P content compared to WT (Supplemental Fig. S1). Decreased shoot P content of \textit{pht1;5} mutants correlated with the up-regulation of several Pi-starvation-response (PSR) genes (\textit{At4}, \textit{Pht1;4}, \textit{DGD1}, and \textit{SQD1}; Fig. 2C). Together these results indicate that the loss of \textit{Pht1;5} affects mobilization of Pi between root and shoot during Pi deficiency. \textit{Pht1;5} may play a role similar to \textit{Pht1} transporters from rice (\textit{OsPht1;2} and \textit{OsPht1;6}) and barley (\textit{HvPht1;6}), which have been implicated in Pi mobilization (Rae et al., 2003; Ai et al., 2009). Interestingly, \textit{Pht1;5}, \textit{OsPht1;2}, and \textit{HvPht1;6} are all expressed in stele cells in the root during Pi deficiency, consistent with them playing a role in root-to-shoot mobilization of Pi (Mudge et al., 2002; Rae et al., 2003; Ai et al., 2009).

Since \textit{Pht1;5} is predominately expressed in shoot tissues during Pi-replete conditions (Mudge et al. 2002; Fig. 1A), \textit{Pht1;5} may play a role in Pi transfer from shoot to root. Our data demonstrate that the \textit{pht1;5-1} mutant accumulates more P in shoots and less in roots compared to WT (Figs 2 and 3). Also under Pi-replete conditions, the root Pi uptake rates for the \textit{pht1;5} mutants were 10 - 20\% higher than WT (Fig. 3A). This likely results from enhanced uptake capacity by other Pi-starvation-induced \textit{Pht1} transporters. In a previous study, loss of \textit{Pht1;4} showed similar increases in shoot P accumulation and root Pi uptake under Pi-replete conditions (Shin et al., 2004). This suggests that \textit{Pht1;4} and \textit{Pht1;5} may have partially overlapping functions during high Pi conditions. Notably, \textit{Pht1;4} expression has been detected in the root stele, and in shoot tissues under Pi-replete conditions (Karthikeyan et al., 2002; Mudge et al., 2002; Misson et al., 2004; Fig. 1A), consistent with a role for \textit{Pht1;4} in Pi mobilization. A key difference between the loss of \textit{Pht1;4} and \textit{Pht1;5} is the impact on root Pi uptake during Pi deficiency. \textit{pht1;4} mutants experience a drop in root Pi uptake during Pi deficiency (Shin et al., 2004), whereas \textit{pht1;5} mutants exhibit an increase (Fig. 3A). This supports the notion that \textit{Pht1;4}, but not \textit{Pht1;5}, contributes significantly to root Pi acquisition. Nevertheless, loss of \textit{Pht1;5} results in moderate tolerance to arsenate (Fig. 4), suggesting that \textit{Pht1;5} has a role in influencing root acquisition of arsenate, and by inference, of Pi.
For the most part, the two independent T-DNA insertion lines, *pht1;5-1* and *pht1;5-2*, showed similar physiological traits (Figs. 2 and 3). However, distribution of $^{33}$P in the shoots of the mutants differed (Fig. 2A). This may be due to the presence of multiple T-DNA copies in *pht1;5-2* as estimated via qPCR (Supplemental Table S2). Thus, *pht1;5-1* was characterized more extensively in this study.

Additional evidence supporting the role of Pht1;5 in Pi mobilization came from analysis of plants overexpressing *Pht1;5*. Under Pi-replete conditions, *Pht1;5*-overexpressors accumulated more P in roots, but less in shoots, relative to WT (Fig. 6B), which is the opposite trend as that observed for the *pht1;5-1* mutant (Fig. 3C). Interestingly though, despite reduced shoot P concentrations the *Pht1;5*-overexpression lines showed increased shoot biomass (Fig. 5). Our results suggest that overexpression of *Pht1;5* results in an imbalance of Pi supply and demand thereby affecting root/shoot biomass allocation (Fig. 9) and overall plant growth (Figs 5 and 6). In an earlier study, Lai et al. (2007) reported that increased meristematic activity results in Pi consumption and the magnitude of organ growth activity specifies Pi demand. Therefore it is plausible that an increase in growth and Pi remobilization in *Pht1;5*-overexpression lines depletes Pi reserves in the shoot at a faster rate. Together the results suggest that Pht1;5 plays a role in modulating Pi distribution between shoot and root in accordance with Pi status. Differences in physiological responses between WT and the *Pht1;5*-overexpressors were less evident under Pi-deficiency conditions (data not shown), which could be due to strong induction of *Pht1;5* during Pi deprivation in WT (Fig. 1A and Supplemental Fig. S2).

**Pht1;5 facilitates Pi remobilization from source to sink in adult plants**

*Pht1;5* is the only Arabidopsis Pht1 member known to be induced in leaves by developmentally-regulated senescence (Mudge et al., 2002; van der Graaf et al., 2006; Arabidopsis eFP Browser http://bbc.botany.utoronto.ca/efp/). Interestingly, *Pht1;5*-overexpressors displayed premature leaf senescence (Fig. 7A). Radiotracer experiments of feeding $^{33}$Pi to mature plants revealed that the *Pht1;5*-overexpressors accumulated 2-fold higher levels of $^{33}$Pi in siliques relative to WT, while the accumulation in rosette leaves was lower (Fig. 7B). The *Pht1;5*-overexpressors also had increased transcript levels for *RNS1* and *ACP5* in rosette leaves (Fig. 7C). During Pi limitation and senescence, scavenging processes involving the activation of these genes facilitate the release of Pi from organic forms of P (phosphate-
esters/nucleic acids) thereby making it available for recycling (Bariola et al., 1994; Green, 1994; del Pozo et al., 1999). The localization of Phl1;5 in the phloem of older leaves substantiates its role in Pi remobilization (Mudge et al., 2002). Together the data support a role for Phl1;5 in facilitating remobilization of Pi released by scavenging processes from senescing, source (rosette leaves) to metabolically active sink (siliques) organs. A QTL mapping study for seed nutrient content performed using two recombinant inbred populations, Columbia (Col) × Landsberg erecta (Ler) and Cape Verde Islands (Cvi) × Ler, identified Phl1;5 as a putative locus that governs seed P content (Waters and Grusak, 2008). The role of Phl1;5 in loading Pi into seeds warrants further investigation.

The regulation of Phl1;5 and its interactions with ethylene- and senescence-related pathways

Modulation of root system architecture (RSA) to increase root surface area for enhancing Pi adsorption is a characteristic adaptive response of Pi-starved plants (Jain et al., 2007b; Schachtman and Shin, 2007; Rouached et al., 2010). Constitutive Phl1;5-overexpression caused increases in the number and length of root hairs, as well as a reduction of primary root length under both P+ and P- conditions (Figs 8 and 9). Several lines of evidence suggest that the modulation of Pi-deficiency-induced changes in RSA result from cross-talk among sugar, nutrient, and phytohormone signaling (López-Bucio et al., 2003; Jain et al., 2007b; Rubio et al., 2009). Thus, the alteration of root growth in the Phl1;5-overexpressors could be regulated independently of the Pi regime. Previous studies have implicated ethylene in Pi-deficiency-induced root hair formation and primary root elongation (Ma et al., 2001, 2003; Zhang et al., 2003; Lei et al., 2011). Ethylene has also been implicated in tolerance to potassium deprivation and iron signaling responses (Zaid et al., 2003; Shin and Schachtman, 2004; Jung et al., 2009). We found that treatment with AVG, an inhibitor of ACC synthase and ethylene biosynthesis, rescued the primary root phenotype of Phl1;5-overexpressors (Fig. 10, A and C), while Ag+ treatment decreased the total length of root hairs of Phl1;5-overexpressors to near WT-levels (Fig. 10B). Expression of PhPT1, a high-affinity Pi transporter in petunia, was shown to be induced during senescence and in response to ethylene treatment (Chaplin and Jones, 2009), highlighting a potential link among ethylene, senescence, and activation of Pi transporters. In a recent study, Lei et al. (2011) showed that the Pi-deficiency-induced expression of a Phl1;4...
promoter-luciferase reporter transgene was strongly enhanced by ACC, but attenuated by Ag+ treatment. Further, the expression of several PSR genes (including Phl1;1 and Phl1;4) were induced and suppressed in null mutant alleles of CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) and ein2-5, (ethylene insensitive 2), respectively. These studies along with our results provide evidence for a tangible connection between Phl1 transporters and ethylene signaling. Another possible link between ethylene and Phl1;5 activity could be ethylene-dependent modulation of reactive-oxygen-species (ROS), which may regulate Phl1;5. A similar phenomenon was recently identified for the regulation of HAK5, which encodes a high-affinity potassium transporter (Jung et al., 2009). Since ethylene and auxin function synergistically in regulating several root traits (Schmidt and Schikora, 2001; López-Bucio et al., 2002; Ma et al., 2003), the potential role of auxin in influencing the exaggerated root hair development and reduced primary root growth of Phl1;5-overexpressors can not be ruled out. However, the cross talk with ethylene signaling appears to be conditional as evidenced by comparable apical hook formation and hypocotyl lengths of etiolated seedlings of WT, phl1;5 mutants, and Phl1;5-overexpressors (data not shown).

Loss-of-function/RNAi mutants of PHO2, PHR1 and WRKY75 impact pathways that regulate Pi acquisition and distribution, thereby playing critical roles in systemic Pi responses (Delhaize and Randall, 1995; Rubio et al., 2001; Bari et al., 2006; Devaiyah et al., 2007; Thibaud et al., 2010). Using qRT-PCR, we found that relative Phl1;5 transcript levels in loss-of-function pho2 and phr1 mutants were comparable to WT, but those in a WRKY75-RNAi line were reduced under both P+ and P- conditions (Supplemental Fig. S3). This supports a role for WRKY75 in the regulation of Phl1;5. Previously, WRKY75 was shown to positively regulate Pi acquisition and negatively regulate lateral root and root hair development in Arabidopsis (Devaiah et al., 2007). The presence of two WRKY-box (W-box) cis-elements (TTGACC/T) in the Phl1;5 promoter further suggest regulatory control by WRKY transcription factors. WRKY proteins have also been implicated in defense responses and senescence-regulated processes (Eulgem and Somssich, 2007). For instance, WRKY6 is known to be a positive regulator of genes involved in senescence signaling and pathogen defense responses (Robatzek and Somssich, 2002). Overexpression or loss-of-function of WRKY6 resulted in alterations in the transcript abundance of Phl1;5, suggesting a role for WRKY6 in Phl1;5 regulation (Chen et al., 2009). Interestingly, WRKY6 has also been shown to modulate Pi distribution between root and
shoot by negatively regulating the expression of \textit{PHO1}, which encodes a protein involved in loading Pi into root xylem (Poirier et al., 1991; Chen et al., 2009). One characteristic feature of WRKY proteins is their ability to autoregulate their own promoter as well as cross-regulating other WRKYs (Rushton et al., 2010). It is possible that feed-forward or feed-back regulation of these determinants could occur concomitantly with the alteration in shoot Pi levels due to the activity of \textit{Pht1;5}. More experiments are required to dissect the molecular mechanisms that regulate \textit{Pht1;5} and its responses that trigger senescence-regulated processes.

In conclusion, our data indicate that \textit{Pht1;5} plays a complex role in differential partitioning of Pi to plant organs, thereby influencing overall plant growth. Under Pi-replete conditions, \textit{Pht1;5} expression is limited to shoots and is modulated by developmental cues to promote Pi distribution to sink tissues, including roots of young seedlings and reproductive tissues in mature plants. Upon perception of low Pi, \textit{Pht1;5} expression is induced in both shoots and roots. Under these conditions, \textit{Pht1;5} is required for proper Pi translocation from root to shoot, likely via a role in loading Pi into root xylem. \textit{Pht1;5} also indirectly influences Pi acquisition by regulating shoot P status.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

\textit{Arabidopsis (Arabidopsis thaliana)} ecotype Columbia (Col-0), \textit{pht1;1-2} (Shin et al., 2004), \textit{pho2} (Delhaize and Randall, 1995), \textit{WRKY75 RNAi} line (Devaiah et al., 2007), and \textit{phr1} (Bari et al., 2006) were used in this study. For studying growth at the seedling stage, seeds were sterilized in 70% (v/v) ethanol for 1 min, followed by 50% (v/v) commercial bleach and 0.1% (v/v) Tween 20 for 10-15 min. After 3-5 washes with sterile water, seeds were stratified at 4°C for 2-3 d and suspended in 0.2% (w/v) agar. Seeds were germinated on one-half-strength Murashige and Skoog (MS) medium, 1.5% (w/v) sucrose (Suc), buffered to pH 5.7 with 0.5 mM MES (2-[N-morpholino]ethanesulfonic acid). Depending on the experiment, seedlings were grown on meshes in standard Magenta (GA-7) boxes under asceptic hydroponic conditions as described (Jain et al., 2009) or vertically grown on Petri plates with medium solidified with 1.2% (w/v) agar (Sigma A1296, lot no. 096K01581 with a P content of ~ 30 µM). Seedlings were then transferred to a growth room set to these conditions: 22°C, 16-h photoperiod and average photosynthetically active radiation (PAR) between 60-70 µmol m⁻² s⁻¹. After 5 d, uniformly
grown seedlings with primary root lengths ranging between 15–25 mm were transferred to P+ (1.25 mM KH2PO4) or P- (P-, 0 mM KH2PO4) medium as described (López-Bucio et al., 2002; Jain et al., 2009). Although hydroponic conditions are best suited for studying nutrient starvation stress conditions, we observed that lack of added Pi was detrimental to seedling growth and led to eventual death before the completion of the experiment. Therefore the P- treatments were supplemented with 10 μM KH2PO4 for the seedlings to deplete during the course of the experiment. In the Pi-deprived medium, KH2PO4 was replaced by equimolar amount of K2SO4.

Ethylene responsive root growth assays on seedlings were performed using ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) with or without the addition of aminoethoxyvinyl-glycine (AVG) and silver nitrate (AgNO3) as described in Ruzicka et al., (2007).

For phenotypic characterization of older developmental stages, greenhouse studies were conducted on plants that were germinated and grown on Premier Pro-mix TA (Premier Horticulture Inc., PA). Once every week plants were irrigated with modified one-half-strength Hoagland’s solution comprising 2.5 mM Ca(NO3)2.4H2O, 1.25 mM K2SO4, 1 mM MgSO4.7H2O, 250 μM KH2PO4, 0.13 μM KCl, 2.25 μM MnSO4.H2O, 12.5 μM H3BO3, 2.0 μM ZnSO4.7H2O, 0.25 μM CuSO4.5H2O, 0.1 μM Na2MoO4.2H2O, and 27.5 mg L-1 Fe-sequestrene, and buffered to pH 5.7 with 0.5 mM MES.

**Isolation of mutant lines**

Putative T-DNA insertion lines in the Pht1;5 gene were identified by searching the SIGNAL-SALK database (http://signal.salk.edu/cgi-bin/tdnaexpress). Homozygous plants were isolated by PCR screening seed populations (obtained from ABRC stock center) using T-DNA left-border (LBa1, 5’-TGGTTCACGTA GTGGCCCATCG-3’), gene-specific right (RP, 5’-GTTTGAGCTTTTGAGACGGTG-3’) and gene-specific left (LP, 5’-ACCTTTGCTAATT CTGGCCG-3’) primer combinations to confirm insertions in the Pht1;5 locus. The T-DNA insertion site was PCR-amplified and sequenced at the Purdue University Genomics Core Facility. The homozygous mutants were designated pht1;5-1 (SALK_074836) and pht1;5-2 (SALK_138009C).

**Construction of Pht1;5-overexpression lines**
For overexpression of Pht1;5, the 1.6 kb Pht1;5 cDNA was reverse-transcribed with SuperScript III (Invitrogen) from total RNA extracted from Pi-starved Arabidopsis seedlings, and the primers PHT5_S1 (5’-TACGTCGGGCCCCCATGGCGGAGAAAAAAGGAAAAGAAGT-3’) and PHT5_A1629 (5’-TAGCTGAAGCTTGGATCTCAAACC GGACTTTTCTACCGGA-3’) (restriction sites are underlined) were used for amplification. The flanking NcoI and BamHI restriction sites were used for cloning the Pht1;5 coding sequence into a constitutive, vegetative ACT2 promoter/terminator cassette (pACT2; Kandasamy et al., 2002), within the binary vector pCambia-1300. Wild-type Arabidopsis plants (ecotype Col-0) or the pht1;5-1 mutant were transformed using Agrobacterium-mediated transformation (Clough and Bent, 1998). Hygromycin resistance was used for identifying homozygous T3 plant lines.

RNA Expression Analyses

Total RNA was isolated from ground plant tissues using the RNeasy Plant Mini Kit (Qiagen Inc., www.qiagen.com). One microgram of total RNA was treated with RQ1 RNase-free DNase I (Promega Inc., www.promega.com) and was reverse transcribed using Superscript III™ Reverse Transcriptase (Invitrogen Inc., www.invitrogen.com). Reverse-transcription PCR (RT-PCR) was performed on 2 µL of the cDNA using gene-specific primers. Thermal cycling consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles (30 s at 94°C, 30 s at 60°C, 90 s at 72°C) and final 7 min extension at 72°C. Quantitative RT-PCR analysis was performed on an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems Inc., www.appliedbiosystems.com). UBC and At4g26410 (Czechowski et al., 2005) were used as reference genes, and relative expression levels of the genes were computed by the 2^−ΔΔCt method of relative quantification (Livak and Schmittgen, 2001). In Fig. 1A, transcript abundances of Pht1;4 and Pht1;5 across different tissues were calculated as 2^−ΔCt. The primers used are listed in Supplemental Table S1.

Estimation of T-DNA copy number

T-DNA copy number in the genomes of the pht1;5 mutants was estimated via quantitative PCR as described previously by Mason et al., (2002). QuickExtract Plant DNA Extraction Solution (Epicentre, Madison, WI) was used to obtain genomic DNA. Reactions for two biological replicates per genotype were performed in triplicate on an Applied Biosystems 7500
real-time PCR system using SYBR Green detection chemistry (Applied Biosystems Inc.,
www.appliedbiosystems.com). Starting quantities for two regions of the SALK transgene, nptII
and a portion near the left border, and two endogenous single-copy genes, Actin 2 and Pht1;4,
were determined by LinRegPCR software (Ramakers et al., 2003). For each transgene a virtual
calibrator (Masson et al., 2002) was created to use for normalization of the data to represent T-
DNA copy number.

Total P Estimation

Total P content was estimated using a modification of the U.S. Environmental Protection
Agency Method 365.2. Approximately 50 mg of plant material was taken in preweighed glass
vials (Kimble Chase Life Sciences, NJ), dried overnight at 70°C, weighed, and flamed to ash in a
furnace. The samples were dissolved in 100 μL concentrated HCl, and 10 μL of the digested
sample was diluted with 790 μL of deionized water. To this, 200 μL of molybdenum-blue
reagent (5N H₂SO₄, 4% (w/v) ammonium molybdate and 10% (w/v) ascorbic acid) was added
and samples were incubated at 45°C for 20 min prior to spectrophotometric determination of P.

Pi Uptake Assay

Pi uptake was performed as described previously (Shin et al. 2004) with minor modifications.
Seedlings (~50) were raised under aseptic conditions hydroponically on one-half-strength MS
medium for 5d and transferred to P+ (1.25 mM Pi) and P- (0.01 mM Pi) media for 7d. The P+
and P- seedlings along with the meshes were transferred to Petri plates (100 mm x 15 mm)
containing P+ or P- nutrient solution supplemented with 0.15 μCi ml⁻¹ [³³P] orthophosphate.
After 2h, samples were incubated in ice-cold desorption medium (0.1 mM CaCl₂, 5 mM MES,
and 2 mM KH₂PO₄, pH 5.7) for 30 min. The samples were rinsed with desorption solution
followed by distilled water and blotted dry before separating roots and shoots. After their fresh
weights were recorded, tissues were dried overnight at 65°C, and ³³Pi activity was measured by
using a liquid scintillation counter (Tri-Carb 2810, PerkinElmer). The root ³³Pi uptake rate was
calculated as pmol Pi g fresh weight⁻¹ hour⁻¹ and distribution was calculated as as pmol Pi g fresh
weight⁻¹.

For ³³Pi feeding and tracing experiments, WT, the pht1;5-1 mutant, and Pht1;5-
overexpressor (11C) were grown on agar-solidified one-half-strength MS medium for 10 d and
transferred to well-aerated tubes containing one-fifth-strength Hoagland’s nutrient solution containing 100 μM Pi. After 10 d, roots were immersed in the same nutrient solution supplemented with 0.15 μCi ml⁻¹ [³³P] for 2 h. Roots were desorbed with unlabelled Hoagland’s solution for 30 min and rinsed with tap water thoroughly before transferring back to the nutrient solution. Plants were allowed to grow for 7 d before harvesting different parts of the plant. Tissue samples were dried overnight at 65°C, weighed, and ³³Pi activity was measured as mentioned above.

**Analysis of RSA**

Root traits were documented as described (Jain et al., 2007a). Briefly, the seedlings grown on agar-solidified medium were scanned at 600 dpi using a desktop scanner (UMAX PowerLook III) and transferred to 70% (v/v) ethanol facilitating preservation for subsequent detailed analyses. The scanned images were used for measuring the elongation of the primary root after transfer from one-half-strength MS to P+ and P- media. For documenting the number of first-and higher-order lateral roots and their lengths, seedlings stored in ethanol were gently spread on agar (1%, w/v) plates with a camel hair brush and scanned at 600 dpi. 10-15 scanned images of the seedlings per genotype for every treatment were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

**Documentation of Root Hairs**

Root hair numbers and lengths were recorded as described (Jain et al., 2007a). Briefly, seedlings were initially grown on one-half-strength MS medium with agar (1.2% w/v) for 5d and transferred to agar-solidified P+ and P- media. After 2 d or as mentioned images of root hairs growing in the 5-mm region from the tip of the primary root were captured using a stereomicroscope (Nikon SMZ-U). Root hairs were measured for ~10 seedlings per genotype for every treatment using ImageJ software.

**Statistical Analyses**

Data were analyzed by one-way (Figs 2A, 3A, 5B-D, 6B-C) or two-way ANOVA (Figs 2B, 3B-C, 4B, 6A, 7B, 8B-C, 9B-C, 10B-C) and Tukey’s Honestly Significant Difference (HSD) test
was carried out for multiple comparisons using the SPSS 10 program (www.spss.com). Different letters represent means that were statistically different at $P < 0.05$ or as mentioned.

**Accessions**

*Pht1;5* corresponds to Gen Bank No. NM_128843 and Arabidopsis Genome Initiative locus At2g32830.

**Supplemental Data**

**Supplemental Figure S1.** Total P content in *pht1;5-1* mutant complemented with *Pht1;5* cDNA.

**Supplemental Figure S2.** Relative expression of *Pht1;5* in two independent *Pht1;5*-overexpression lines.

**Supplemental Figure S3.** Relative expression of *Pht1;5* in Pi-signaling mutants.

**Supplemental Table S1.** Primers used for PCR analyses.

**Supplemental Table S2.** Quantitative PCR estimation of T-DNA copy number in the *pht1;5* mutants.

**ACKNOWLEDGEMENTS**

We thank Debra M. Sherman and Chia-Ping Huang of the Purdue Life Sciences Microscopy Facility for technical assistance with the microscopy work, Prof. Richard Meagher (University of Georgia, Athens) for gifting the *Pht1;5*-overexpression lines, and the Ministry of Science and Technology, Department of Biotechnology, Government of India, for awarding a Ramalingaswamy Fellowship (2009) to A.J.

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Figure 1. Spatial expression pattern of *Pht1;5* and isolation of *pht1;5* loss-of-function mutants. A, Quantitative RT-PCR analysis of the expression of *Pht1;4* and *Pht1;5* in different organs under different Pi regimes. WT and the mutants were raised hydroponically under aseptic conditions on one-half-strength MS medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 7 d. B, Schematic representation of the *Pht1;5* gene and the location of T-DNA inserts (filled triangles) in the different *pht1;5* alleles. Open and filled boxes represent untranslated regions and exons, respectively. C, Reverse-transcription PCR analysis of *Pht1;5* expression in the WT and *pht1;5* mutants. Seedlings were grown hydroponically under aseptic conditions on one-half-strength MS medium for 5 d and transferred to P- medium for 7 d. Whole seedlings were harvested. Transcript abundance was determined using primers specific to *Pht1;5* and *UBC* genes.

Figure 2. *Pht1;5* loss-of-function affects Pi distribution in shoots. WT and the mutants were raised hydroponically under aseptic conditions on one-half-strength MS medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 7 d. A, Distribution of Pi in the shoots (n = four replicates of ~50 seedlings each). B, Total P content in the shoots (n = four replicates of 25 seedlings each). A and B, Values are the means, and different letters indicate means that differ significantly (*P*<0.05). Error bars indicate standard error of the mean. C, Quantitative RT-PCR analyses of relative expression of Pi starvation response genes in the shoots of WT and *pht1;5-1*. *At4g26410* was used as an internal control and the values, normalized to WT P+ levels, are the RQ ± maximum/minimum values of two independent biological replicates run in triplicate. Asterisks (*) and (**) indicate ≥1.5-fold and ≥2.0-fold changes, respectively, compared to WT.
Figure 3. Pht1;5 loss-of-function disrupts distribution and assimilation of Pi in the roots. WT and mutants were grown hydroponically on one-half-strength MS medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 7 d. A, Rate of Pi uptake in the roots ($n = 4$ replicates of ~50 seedlings each). Seedlings grown under P+ and P- were transferred to respective medium supplemented with Pi for 2 h and roots were analyzed for Pi uptake. B, Total P content in the roots ($n = 4$ replicates of 25 seedlings each). C, Distribution ratio of P between root and shoot ($n = 4$ replicates of 25 seedlings each). Histograms show the means, and different letters indicate means that differ significantly ($P<0.05$). Error bars represent standard error of the mean.

Figure 4. Loss of Pht1;5 results in moderate arsenate tolerance. WT and the mutants were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high (P+) Pi medium with and without 100 μM arsenate for 7 d. A, Images of representative seedlings are shown ($n = 20$). Shoot fresh weights are presented relative to that of the respective seedlings grown in the absence of arsenate ($n = 4$ replicates of 20 seedlings each). B, Total number of lateral roots ($n = 20$). Histograms are means, and different letters indicate means that differ significantly ($P<0.05$). Error bars represent standard error of the mean.

Figure 5. Pht1;5-overexpression increases shoot biomass. Seeds were germinated in potting mix in the greenhouse. A, Four-week-old WT, pht1;5-1 and Pht1;5-overexpressors (5A and 11C) fertilized with one-half-strength Hoagland’s solution. B and C, Rosette leaves and floral stalks from six-week-old plants were dissected and scanned at 200 dpi for documenting total leaf area (B) and stalk thickness (C). D, Rosette leaves were dried at 65°C for 24 h to determine the leaf biomass ($n = 6$ plants for each genotype). Histograms (B-D) are means, and different letters indicate means that differ significantly ($P<0.05$). Error bars represent standard error of the mean.

Figure 6. Overexpression of Pht1;5 results in altered Pi homeostasis. A and B, WT and Pht1;5-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high Pi medium (P+) for 7 d. A, Total P content in the tissues indicated ($n = 5$ replicates of 20 seedlings each). B, P distribution between root and shoot ($n = 5$ replicates of 20 seedlings each). C, Rate of $^{33}$Pi uptake in the roots. Seedlings grown initially under P+ medium were transferred to the same media supplemented with $^{33}$Pi for 2 h and roots were analyzed for $^{33}$Pi uptake ($n = 4$ replicates of ~50 seedlings each). Histograms are means, and different letters indicate means that differ significantly ($P<0.05$). Error bars represent standard error of the mean.

Figure 7. Pht1;5-overexpressors exhibit premature senescence and increased Pi remobilization. A, Eight-week-old WT and Pht1;5-overexpressors (5A and 11C) showing senescent rosette leaves. B, WT, pht1;5-1, and 11C were grown on agar-solidified one-half-strength MS medium for 10 d and transferred to well-aerated tubes with one-fifth-strength Hoagland’s nutrient solution supplemented with 100 μM Pi. After 10 d, roots were immersed in nutrient solution supplemented with $^{33}$Pi for 2 h. Roots were rinsed with unlabelled nutrient solution and plants were transferred back to the nutrient solution for 7 d before scintillation counting ($n = 5$). Histogram bars represent means, and different letters indicate means that differ significantly ($P<0.05$). Error bars indicate standard error of the mean. C, Quantitative RT-PCR analyses showing increased expression of genes involved in Pi remobilization in Pht1;5-overexpressors. Three-week-old plants were grown hydroponically in modified one-half-strength Hoagland’s
nutrient solution for 7 d and transferred to high Pi media for 7 d. *Atg26410* was used as an
internal control and the values, normalized to WT levels, are the RQ ± maximum/minimum
values of two independent biological replicates run in triplicate. Asterisks (*) and (**) indicate
≥1.5-fold and ≥2.0-fold changes, respectively, compared to WT.

**Figure 8.** *Pht1;5*-overexpression leads to increases in root hair number and length. WT, *pht1;5-1*,
and *Pht1;5*-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS
medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 2 d. A, Primary root tips
of the representative seedlings showing root hairs (*n* = 10). Data are presented for numbers (B)
and total lengths of the root hairs in a 5-mm section from the primary root tip (C). Histograms
are means, and different letters indicate means that differ significantly (*P*<0.05). Error bars
represent standard error of the mean.

**Figure 9.** *Pht1;5* loss-of-function and overexpression affects root system architecture. WT, *pht1;5-1*,
and *Pht1;5*-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS
medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 7 d. A, Root system architecture
recorded on a dry weight basis (*n* = 5 replicates of 20 seedlings each). B and C, Histograms are means, and different letters indicate means that differ
significantly(*P*<0.05). Error bars represent standard error of the mean.

**Figure 10.** Ethylene signaling and biosynthesis inhibitors rescue the root phenotype of *Pht1;5-
overexpressors. WT and the *Pht1;5*-overexpressors (5A and 11C) were grown on agar-solidified
one-half-strength MS medium for 5 d and then transferred to P+ medium (control), or the same
medium supplemented with the inhibitors shown, for 7 d. A, Phenotype of WT and 11C
seedlings grown in the presence of indicated treatments. B, Total root hair length in a 5-mm
section from the primary root tip (*n* = 10). C, Primary root length under the indicated treatments
(*n* = 16). Histograms are means, and different letters indicate means that differ significantly
(*P*<0.05). Asterisks (**) represent means that differ significantly at (*P*<0.001) compared with the
untreated control sets.
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A, Quantitative RT-PCR analysis of the expression of *Pht1;4* and *Pht1;5* in different organs under different Pi regimes. WT and the mutants were raised hydroponically under aseptic conditions on one-half-strength MS medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 7 d. B, Schematic representation of the *Pht1;5* gene and the location of T-DNA inserts (filled triangles) in the different *pht1;5* alleles. Open and filled boxes represent untranslated regions and exons, respectively. C, Reverse-transcription PCR analysis of *Pht1;5* expression in the WT and *pht1;5* mutants. Seedlings were grown hydroponically under aseptic conditions on one-half-strength MS medium for 5 d and transferred to P- medium for 7 d. Whole seedlings were harvested. Transcript abundance was determined using primers specific to *Pht1;5* and *UBC* genes.
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Figure 4. Loss of Phl1:5 results in moderate arsenate tolerance. WT and the mutants were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high (P+) Pi medium with and without 100 µM arsenate for 7 d. A, Images of representative seedlings are shown (n = 20). Shoot fresh weights are presented relative to that of the respective seedlings grown in the absence of arsenate (n = four replicates of 20 seedlings each). B, Total number of lateral roots (n = 20). Histograms are means, and different letters indicate means that differ significantly (P<0.05). Error bars represent standard error of the mean.
**Figure 5.** *Pht1;5*-overexpression increases shoot biomass. Seeds were germinated in potting mix in the greenhouse. A, Four-week-old WT, *pht1;5-1* and *Pht1;5*-overexpressors (5A and 11C) fertilized with one-half-strength Hoagland’s solution. B and C, Rosette leaves and floral stalks from six-week-old plants were dissected and scanned at 200 dpi for documenting total leaf area (B) and stalk thickness (C). D, Rosette leaves were dried at 65°C for 24 h to determine the leaf biomass (*n* = 6 plants for each genotype). Histograms (B-D) are means, and different letters indicate means that differ significantly (*P*<0.05). Error bars represent standard error of the mean.
Figure 6. Overexpression of Pht1;5 results in altered Pi homeostasis. A and B, WT and Pht1;5-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high Pi medium (P+) for 7 d. A, Total P content in the tissues indicated (n = 5 replicates of 20 seedlings each). B, P distribution between root and shoot (n = 5 replicates of 20 seedlings each). C, Rate of $^{33}$Pi uptake in the roots. Seedlings grown initially under P+ medium were transferred to the same media supplemented with $^{33}$Pi for 2 h and roots were analyzed for $^{33}$Pi uptake (n = 4 replicates of ~50 seedlings each). Histograms are means, and different letters indicate means that differ significantly (P<0.05). Error bars represent standard error of the mean.
Figure 7. Pht1;5-overexpressors exhibit premature senescence and increased Pi remobilization. 
A, Eight-week-old WT and Pht1;5-overexpressors (5A and 11C) showing senescent rosette leaves. B, WT, pht1;5-1, and 11C were grown on agar-solidified one-half-strength MS medium for 10 d and transferred to well-aerated tubes with one-fifth-strength Hoagland’s nutrient solution supplemented with 100 µM Pi. After 10 d, roots were immersed in nutrient solution supplemented with $^{33}$Pi for 2 h. Roots were rinsed with unlabelled nutrient solution and plants were transferred back to the nutrient solution for 7 d before scintillation counting ($n = 5$). Histogram bars represent means, and different letters indicate means that differ significantly ($P<0.05$). Error bars indicate standard error of the mean. C, Quantitative RT-PCR analyses showing increased expression of genes involved in Pi remobilization in Pht1;5-overexpressors. Three-week-old plants were grown hydroponically in modified one-half-strength Hoagland’s...
nutrient solution for 7 d and transferred to high Pi media for 7 d. *At4g26410* was used as an internal control and the values, normalized to WT levels, are the RQ ± maximum/minimum values of two independent biological replicates run in triplicate. Asterisks (*) and (**) indicate ≥1.5-fold and ≥2.0-fold changes, respectively, compared to WT.
Figure 8. Pht1;5-overexpression leads to increases in root hair number and length. WT, pht1;5-1, and Pht1;5-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high (P+) or low (P-) Pi medium for 2 d. A, Primary root tips of the representative seedlings showing root hairs (n = 10). Data are presented for numbers (B) and total lengths of the root hairs in a 5-mm section from the primary root tip (C). Histograms are means, and different letters indicate means that differ significantly (P<0.05). Error bars represent standard error of the mean.
Figure 9. *Pht1;5* loss-of-function and overexpression affects root system architecture. WT, *pht1;5-1*, and *Pht1;5*-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS medium for 5 d and transferred to high (P+) or low (P−) Pi medium for 7 d. A, Root system architecture of the representative seedlings (*n* = 12). B, Primary root length (*n* = 12). C, Root-shoot biomass ratio recorded on a dry weight basis (*n* = 5 replicates of 20 seedlings each). B and C, Histograms are means, and different letters indicate means that differ significantly (*P*<0.05). Error bars represent standard error of the mean.
Figure 10. Ethylene signaling and biosynthesis inhibitors rescue the root phenotype of Pht1;5-overexpressors. WT and the Pht1;5-overexpressors (5A and 11C) were grown on agar-solidified one-half-strength MS medium for 5 d and then transferred to P+ medium (control), or the same medium supplemented with the inhibitors shown, for 7 d. A, Phenotype of WT and 11C seedlings grown in the presence of indicated treatments. B, Total root hair length in a 5-mm section from the primary root tip (n = 10). C, Primary root length under the indicated treatments (n = 16). Histograms are means, and different letters indicate means that differ significantly (P<0.05). Asterisks (**) represent means that differ significantly at (P<0.001) compared with the untreated control sets.