SHORT-ROOT stabilizes PHOSPHATE1 to regulate phosphate allocation in Arabidopsis

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The coordinated distribution of inorganic phosphate (Pi) between roots and shoots is an important process that plants use to maintain Pi homeostasis. SHORT-ROOT (SHR) is well characterized for its function in root radial patterning. Here we demonstrate a role of SHR in controlling Pi allocation from root to shoot by regulating PHOSPHATE1 in the root differentiation zone. We recovered a weak mutant allele of SHR in Arabidopsis that accumulates much less Pi in the shoot and shows a constitutive Pi starvation response under Pi-sufficient conditions. In addition, Pi starvation suppresses SHR protein accumulation and releases its inhibition on the HD-ZIP III transcription factor PHB. PHB accumulates and directly binds the promoter of PHOSPHATE2 to upregulate its transcription, resulting in PHOSPHATE1 degradation in the xylem-pole pericycle cells. Our findings reveal a previously unrecognized mechanism of how plants regulate Pi translocation from roots to shoots.

As sessile organisms, plants have to orchestrate the uptake, usage and redistribution of inorganic phosphate (Pi) to maintain its homeostasis. Under Pi-deficient conditions, shoot growth is inhibited, and more resources are allocated to the root to promote its growth to forage Pi from poor soil. PHOSPHATE1 (PHO1) is specifically expressed in the root pericycle and is critical for Pi loading into the xylem and transport to the shoot. However, previous studies indicate that PHO1 is positively regulated by Pi deficiency at both the transcriptional and post-translational levels to promote Pi translocation from roots to shoots, which contradicts findings that show higher proportions of Pi or biomass being allocated to roots when Pi is limited.\(^{(1)}\) (Supplementary Fig. 1). The underlying molecular mechanism that represses Pi allocation from roots to shoots under Pi starvation was unknown. Here we show that Pi starvation inhibits Pi allocation to the shoot by repressing SHORT-ROOT (SHR) in the root differentiation zone. The repression of SHR releases the transcription factor PHB, which directly activates PHOSPHATE2 (PHO2), resulting in PHO1 degradation in the xylem-pole pericycle cells.

Results

Isolation of the phod1 mutant. To identify new cellular factors required for maintaining Pi homeostasis, we performed a forward genetic screen in Arabidopsis using \(pPHT1;4\)-GUS as the reporter gene.\(^{(2)}\) \(pPHT1;4\)-LUC is specifically induced by Pi starvation, and the seedling emits a luminescence signal (Supplementary Fig. 2a). In this report, we characterized a mutant, phosphate deficiency1 (phod1), in which \(pPHT1;4\)-LUC was constitutively induced on Pi-replete medium (Fig. 1a). To confirm that the induction of \(pPHT1;4\)-LUC in phod1 was caused by the mutation of a regulatory component, we introduced the phod1 mutation into a \(pPHT1;4\)-GUS reporter line by a genetic cross. Consistent with the LUC expression pattern, the \(pPHT1;4\)-GUS activity was also enhanced by the phod1 mutation (Supplementary Fig. 2e). Furthermore, the endogenous \(PHT1;4\) transcript level was increased in the phod1 mutant by fivefold in the shoot and by threefold in the root (Fig. 1b).

To identify the causative mutation for the constitutive activation of \(PHT1;4\), phod1 was backcrossed, and the genomic DNA from the pooled F\(_2\) seedlings with increased LUC signal was deep sequenced (Supplementary Fig. 2b,c). The data were analysed following a mapping-by-sequencing workflow.\(^{(3)}\) In the phod1 mutant, a point mutation (C866T) changes Thr289 to Ile in SHR (Supplementary Fig. 2d). To confirm that the increased expression of \(PHT1;4\) in the mutant is due to the mutation in the SHR gene, we complemented the phod1 mutant by an SHR genomic DNA fragment fused to GFP or FLAG driven by its native promoter (Fig. 1a,b and Supplementary Fig. 2f,g).

The loss of function of SHR severely inhibits root growth\(^{(4)}\). However, the T289I mutation in phod1 only weakly affected root growth. Although the primary root length of phod1 was shorter than that of the wild type (WT), it was much longer than that of the knockout line shr-6 (Supplementary Fig. 3a). Unlike the shr-6 null mutant, which has a disorganized root stem cell niche and lacks the endodermis, the root meristem of phod1 is relatively well organized, and the endodermal layer is present (Supplementary Fig. 3b). This result indicates that phod1 is a weak allele of SHR. To confirm this, we transformed the GFP-fused WT (SHR–GFP) or phod1 allele of SHR (SHR\(^{phod1}\)–GFP) driven by its native promoter into the shr-6 null mutant. The resulting shr-6 SHR\(^{phod1}\) transformant could mimic the phod1 mutant phenotype (Supplementary Fig. 3a,i–k).

Although their mRNA levels were comparable (Supplementary Fig. 3c,d), western blot analysis showed that the protein abundance of SHR\(^{phod1}\)–GFP was much lower than that of SHR–GFP (Supplementary Fig. 3e). Whereas the GFP signal detected in the

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vascular tissue and nucleus of endodermis and quiescent cells in the SHR–GFP lines was strong, it was much weaker and barely detectable in the endodermis and quiescent cells of the SHRphod1–GFP lines (Supplementary Fig. 3f). Furthermore, although SHR accumulation was not affected, SHRphod1 was enhanced by MG132 treatment (a 26S proteasome inhibitor), suggesting that SHRphod1 is prone to degradation by the 26S proteasome (Supplementary Fig. 3g). Using the crystal structure of the SHR GRAS domain (PDB-5B3G) as a template, we mapped the T289I substitution (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) and found that the substitution disrupted the H-bonds formed by the Thr residue with side and/or main chain atoms, possibly affecting the stability of the protein (Supplementary Fig. 3h). These results therefore further affirm that phod1 is a weak allele of SHR and that Thr289 is required for SHR protein stability.

SHR regulates Pi homeostasis. Besides altered PHT1;4 expression, the phod1 mutant also exhibited constitutive Pi starvation response (PSR) phenotypes under Pi-replete conditions. The shoots of the phod1 and shr-6 mutants were smaller than those of the WT and accumulated visible levels of anthocyanin (Supplementary Fig. 4a–c). The roots of the mutants had longer and denser root hairs than the WT (Supplementary Fig. 4d). Under Pi-replete conditions, shoot Pi content of the mutants was significantly reduced, while there was a significant increase in root Pi content (Supplementary Fig. 4e). Under Pi-depleted conditions, however, Pi content in all
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four genotypes was comparable, and root Pi content increased significantly in the shr-6 and phod1 mutants (Supplementary Fig. 4e). All these PSR phenotypes were rescued in phod1 by complementation with native-promoter-driven WT SHR (Supplementary Fig. 4a–e). In addition to PHT1;4, we examined the expression of other Pi-starvation-induced (PSI) genes and found that all genes were induced in the phod1 or shr-6 mutants (Supplementary Figs. 4f and 5a,b). We checked the Pi uptake rate in a time course experiment, along with the Pi accumulation in shoots and roots after Pi resupply for two days. The Pi uptake rate in phod1 roots is the same as that of the WT when Pi is supplied (Supplementary Fig. 4g), but Pi translocation to shoots was inhibited in phod1 even though it had accumulated enough Pi in roots (Supplementary Fig. 4h).

Further analysis of the phod1 transcriptome under Pi-replete conditions revealed the upregulation of 282 PSI genes in the shoot, which accounted for about 30% of all upregulated genes in phod1 (Fig. 1c,d). We found that most of the upregulated PSI genes (230/282) are also targets of PHR1/PHL1 under Pi starvation. These results suggest that even under Pi-replete conditions, the shr mutant experiences Pi starvation, which activates PHR1 (the central regulator of PSRs) to induce PSI gene expression [15,16]. We observed that the cellular Pi content was decreased dramatically in the shoots of phod1 and shr-6 mutants compared with the WT and complementation lines, while Pi content increased in the roots of the shr mutants (Fig. 1e). To elevate Pi absorption in the shoot of phod1, seedlings were submerged in Pi solution, which can gradually restore the repression of the expression levels of PSI genes in the shoot by improving Pi concentrations in solution (Supplementary Fig. 5c). This indicated that Pi deficiency resulted in the induction of PSI genes in the shoot of phod1. SHR is expressed in the stele, and the protein migrates to cortex to form a complex with another GRAS transcription factor, SCR, and activates the expression of SCR and other downstream genes [17]. As expected, SCR transcription was markedly repressed in the phod1 mutant under Pi-replete conditions (Supplementary Fig. 5d). Similar to shr mutants, Pi content in the shoot was decreased associated with a significant induction of the PSI genes in the shoot of the shr mutant under Pi-replete conditions (Supplementary Fig. 5e,f).

To test the hypothesis that the Pi deficiency of phod1 shoots resulted from a defective shoot or root, we performed a reciprocal micrografting experiment with WT and phod1 mutant seedlings. Although the shr mutation resulted in Pi deficiency symptoms, grafting rescued the mutant phenotype when phod1 shoot was used as a scion grafted onto the WT rootstock, similar to the WT scion control (Supplementary Fig. 6a,b). However, when phod1 was used as the rootstock, both phod1 and WT scions accumulated higher levels of anthocyanin and were much smaller than those with WT rootstock (Supplementary Fig. 6a,b). Consistent with the phenotype, Pi concentration decreased substantially in scions with phod1 rootstock (Supplementary Fig. 6c). These results indicate that the phod1 root genotype is necessary and sufficient for decreased Pi concentration in the shoot. Our findings further suggest that SHR function in the root is required for Pi translocation from root to shoot.

SHR post-translationally regulates PHO1. The defect in translocating Pi from root to shoot in phod1 is reminiscent of the pho1 mutant (Supplementary Fig. 7a) [18]. The pho1 mutant showed severe Pi deficiency in shoots, smaller shoots and upregulated PSI genes in shoots compared with the WT, which is similar to the phod1 mutant (Supplementary Fig. 7b,d,e). However, phod1 exhibits anthocyanin accumulation in shoots and denser root hairs (Supplementary Fig. 7b,c), which is different from pho1 grown on agar plates. The Pi content in the shoot of the phod1 pho1 double mutant is similar to that in the phod1 mutant, indicating that the genes may function in the same pathway to regulate Pi translocation. We found that the PHO1 transcript level was not significantly changed in the root of shr mutants (Fig. 2a). To determine the tissue-specific expression patterns of PHO1, we transformed GFP-fused PHO1 driven by its native promoter (PHO1–GFP) into the pho1 mutant. The resultant transgenic plants have the Pi content in the shoot restored, indicating that the fusion protein is functional (Supplementary Fig. 7a). PHO1–GFP is expressed in the pericycle, predominantly in the xylem-pole pericycle cells. However, the introduction of the phod1 mutation into the PHO1–GFP transgenic line decreased the GFP signal dramatically (Fig. 2c). Consistent with the GFP signal, western blot analysis showed that the protein level of PHO1–GFP was much lower in the phod1 mutant than in the WT (Fig. 2b). To verify that Pi deficiency in the mutant is due to decreased PHO1 protein levels, we attempted to increase its expression by transforming the PHO1::PHO1 construct into phod1. Although most transformants showed the mutant phenotype, we identified two lines with high PHO1 transcript levels in which the PSR phenotypes of phod1 were partially rescued (Fig. 2d,e and Supplementary Fig. 7f). The cellular Pi content in the overexpression lines was also significantly increased compared with the phod1 mutant, although still lower than the WT (Fig. 2e). PHO2-dependent PHO1 degradation can be blocked by endosomal protease inhibitor E64d. We found that E64d treatment significantly enhanced PHO1 protein in phod1 (Fig. 2f), and knocking out the PHO2 gene in the phod1 mutant significantly improved its Pi content, although it was still lower than that of the WT (Fig. 2g). The reason for partial rescue may be that phod1 mutation also affects root development, and even as we emphasize the function of SHR in the maintenance of Pi, we have not been able to exclude its role in root apical meristem maintenance and root patterning (Supplementary Fig. 3b,l). These results indicate that shr mutation repressed PHO1 post-translationally.

PHB directly regulates PHO2 gene expression. We found enhanced PHO2 transcript levels in both shr-6 and scr-1 mutants (Fig. 3a). Analysis of the stele-specific microarray data [19] also revealed higher expression levels of PHO2 in the shr-2 mutant (Supplementary Fig. 8a). Increased PHO2 expression could lead to a concomitant increase in its protein levels and consequently a decrease in PHO1 protein levels. SHR/SCR-activated microRNA miR165/166 can migrate from the endodermis to the stele, where they target the PHB mRNA and other transcripts of HD–ZIP III transcription factors. We found that PHB transcript levels increased in shr and scr mutants (Fig. 3a), consistent with a previous report [20]. Interestingly, the mutation of PHB in the shr-2 phb-6 double mutant repressed PHO2 expression (Supplementary Fig. 8a). The mutation of PHB also rescued the PSR phenotypes and partially restored cellular Pi content (Fig. 3b,c). As PHB mRNA is targeted by miR165/166 for degradation, we fused the FLAG tag to a miRNA-insensitive version of PHB [21] and transformed the native-promoter-driven fusion gene (PHB-FLAG) into the WT to enhance PHB (PHB*) expression. With the increase in PHB expression, the PHO2 transcript level was also increased in the transgenic lines (Supplementary Fig. 8b,c). It is therefore likely that SHR regulates PHO2 gene expression through PHB. Since the PHO2 promoter contains several putative PHB binding sites (Supplementary Fig. 8d–f), we tested whether PHB could bind to these motifs. Both GST-fused full PHB and its HD–ZIP domain could bind to the P1 motif, while the HD domain only or the carboxy-terminal end could not (Fig. 3d). The mutation of the critical amino acid residue (N76I) in the HD domain abolished the binding, which suggested that both the HD and ZIP domains are necessary and sufficient for binding to the P1 region on the promoter (Fig. 3d). The binding of PHB to the P1 region was competitively blocked by an unlabelled P1 probe (Supplementary Fig. 8g). Furthermore, the HD–ZIP domain could also bind to the P2, P3, P4 and P5 motifs in the PHO2 promoter with different affinities (Fig. 3e). The class III HD–ZIP family has five members (PHB, PHV, REV, CNA and ATBBH), all of which can be
targeted by miR165/166; hence, their expressions were all increased in the shr mutant. In vitro electrophoretic mobility shift assay (EMSA) experiments showed that all five proteins could bind to the PHO2 promoter (Supplementary Fig. 8h–k). Both PHB\textsubscript{n} and PHV\textsubscript{n} could significantly activate PHO2 expression in dual-LUC transient transcriptional assays (Fig. 3g). Furthermore, the chromatin immunoprecipitation (ChIP) assay using the PHB\textsubscript{n}-FLAG transgenic line showed that PHB\textsubscript{n}-FLAG was enriched on the PHO2 promoter in vivo (Fig. 3f). These data indicate that the SHR-mediated PHB directly regulates PHO2 gene expression.

Fig. 2 | PHO1 localization pattern and protein levels are altered in phod1. a, Quantitative analysis of PHO1 gene expression in the shoot and root of the WT, shr-6, phod1 and SHR–GFP/pho1. The data are means ± s.d. n = 2 (biologically independent replicates). The data were analysed with a two-tailed t-test. b, Western blot analysis of PHO1–GFP protein levels in the roots of the WT and phod1. NC, negative control. The experiments in b were repeated independently two times, with similar results. c, Accumulation of PHO1–GFP in the WT and phod1. The white arrowheads mark the endodermal transversal wall. Ep, epidermis; Co, cortex; En, endodermis; Xy, xylem. Scale bars, 20 μm. The experiments in c were repeated independently three times, with similar results. d, Morphological appearance (d) and Pi content (e) of two-week-old WT, phod1 and transgenic lines with different expression levels of PHO1. Scale bars, 0.5 cm (d). The data are means ± s.d. n = 3 (biologically independent replicates). The sets of data were analysed by one-way ANOVA followed by Duncan’s post hoc test, and the letters represent statistically significant differences at P < 0.05 for multiple comparisons (e). f, Western blot analysis of PHO1–GFP in roots of the WT and phod1 after E64d treatment. The experiments in f were repeated independently two times, with similar results. g, Pi content in the shoots of nine-day-old seedlings of the WT, phod1 and the phod1 pho2 double mutant. The data are means ± s.d. n = 3 (biologically independent replicates). The sets of data were analysed by one-way ANOVA followed by Duncan’s post hoc test, and the letters represent statistically significant differences at P < 0.05 for multiple comparisons.
Pi starvation represses SHR to regulate PHO1 stability. To assess SHR responsiveness to Pi starvation, we evaluated the relative abundance of SHR transcripts and the encoding protein in plants grown on media containing different concentrations of Pi (Fig. 4a and Supplementary Fig. 9a). SHR transcription was induced in the roots under Pi starvation (Supplementary Fig. 9a). However, SHR–FLAG protein levels decreased in roots with decreasing availability of Pi (Fig. 4a,b), but not of nitrogen, to the plant (Supplementary Fig. 9b,c). To avoid the side effect of root growth inhibition mediated by the photo-Fenton reaction\(^{39}\), we covered the roots of the SHR–FLAG line with aluminium foil and found that the SHR–FLAG protein still decreased markedly (Fig. 4c and Supplementary Videos 1 and 2). The suppression of SHR–FLAG by Pi deficiency could not be blocked by the addition of MG132 (a 26S proteasome inhibitor) or a mixed protease inhibitor cocktail (Fig. 4c). In addition, SHR–FLAG protein immunoprecipitated from transgenic plants was incubated with total proteins extracted from Col-0 plants grown in Pi-replete or Pi-depleted conditions, and the degradation rate of SHR–FLAG was similar (Fig. 4c). Furthermore, we examined SHR protein stability by applying cycloheximide (CHX), a protein translation inhibitor, to SHR–FLAG transgenic plants. Blocking of translation by CHX resulted in a similar decline of SHR–FLAG protein under Pi-replete to Pi-depleted conditions (Fig. 4g). These results suggest that Pi starvation represses SHR by inhibiting its translation.

Consistent with the repression of SHR, PHB–GFP signal is stronger under Pi-depleted conditions than under Pi-replete conditions, especially at the xylem pole (Fig. 4h and Supplementary Videos 3 and 4). As PHB activates PHO2, which directs PHO1 degradation, PHO1–GFP abundance was rescued by PHB mutation in the phd1 mutant (Fig. 4i). Although PHO1–GFP signal increased within two days on Pi-depleted medium, it decreased after long-term Pi starvation (Fig. 4i). The change in PHO1–GFP was confirmed by western blot analysis (Fig. 4k), which was coincident with the decline of SHR protein under long-term Pi starvation (Fig. 4i).

When seedlings were transferred to Pi-deficient conditions, Pi content decreases in both the shoot and the root, but a higher proportion of Pi is allocated to the shoot during the early stage. However, a reversal in the allocation strategy was observed under long-term Pi starvation. Accordingly, the Pi content root/shoot ratio was significantly increased under long-term Pi-depleted conditions (Fig. 4m). The mutation of SHR in the phd1 mutant could mimic the Pi allocation strategy under Pi-deficient conditions (Fig. 4n), and a
similar result was also observed for total P content (Supplementary Fig. 10). These results indicate that Pi starvation represses SHR to regulate PHO1 abundance in xylem-pole pericycle cells to allocate a higher proportion of Pi to the root, which ensures root vitality to forage more Pi from the soil.

**Discussion**

To respond to Pi deficiency, plants have evolved sophisticated strategies to orchestrate Pi uptake, usage and redistribution between shoots and roots. During the onset of Pi deficiency, Pi content decreases before miR399 is induced in the shoot and transduced as a systemic signal to the root, where it targets PHO2 mRNA, thus stabilizing PHO1. Pi starvation also activates the ubiquitin E3 ligase PRU1 to direct the degradation of WRKY6, releasing its repression of PHO1 gene expression. Enhanced PHO1 levels elevate the ability of Pi allocation from roots to shoots to alleviate shoot Pi deficiency. However, after long-term Pi deficiency, SHR is repressed to reduce PHO1, which limits Pi translocation from roots to shoots, and relatively more Pi is retained in the root to support its vitality and expansion to forage more Pi from the soil (Fig. 4o). To support this, the root/shoot ratio increases in terms of both biomass and Pi content (Fig. 4m,n and Supplementary Fig. 1). Here we provide biochemical and molecular evidence that Pi starvation represses SHR to release the downstream transcription factor PHB, which directly regulates PHO2 to promote PHO1 degradation, inhibiting Pi translocation from roots to shoots.

**Methods**

**Plant materials and growth conditions.** The following transgenic lines have been following transgenic lines have been following transgenic lines have been constructed using binary vector pPHT1::GFP:pPH2, pPHT1::LUC and pPHT1::GUS. For the following Arabidopsis mutants, seeds were ordered from the Arabidopsis Biological...
Resource Center: shr-6 (SALK_002744), pho1-2 (CS8507), pho2 (SAIL_47_E01) and phb (SALK_008924 and SALK_021684) the shr-6 pho1 double mutant was generated by a genetic cross between shr-6 and pho1. The plants were grown in 1/2 MS agar medium with 1.0% (w/v) sucrose and 1.1% (w/v) agar under a 16 h/8 h light/dark cycle at 23°C.

Mutant isolation and mapping by sequencing analysis. EMS-mutagenized M2 seedlings were sowed directly on 1/2 MS medium plates. Then, 100 mM luciferin was sprayed on the cotyledon old seedlings, and the fluorescence images were taken with a CCD camera (PyLoNi300B, Princeton Instrument). Seedlings with enhanced LUC signals were selected as candidates, and the phenotype was confirmed in the next generation. To map the mutated genes, the mutants were back-crossed to the WT, a total of 250 F1 seedlings were employed to enhance LUC signals were pooled and the genomic DNA was isolated to perform next-generation sequencing. The sequencing reads were mapped to the Arabidopsis genome (v.TAIR10) with SHORE (v.0.9.0) and GenomeMapper (v.0.4.4)⁴⁷, and candidate causative mutations were identified with algorithms developed in SHOREmap v.3.0.

Plasmid construction and complementation analysis. For complementation, SHR genomic DNA with about 2 kb of promoter region was amplified from Col-0 genomic DNA and cloned into pCAMBIA1300 vector harbouring a C-terminal GFP or FLAG tag by the homologous recombination method. The mutation site was introduced into SHR through site-directed mutagenesis with the primers listed in Supplementary Table 1. PHO1 genomic DNA with about 2 kb of native promoter region was amplified with pCAMBIA1300 vector harbouring a C-terminal GFP tag. PHB genomic DNA with about 2 kb of promoter was cloned into pCAMBIA1300, and the mutation in the mir165/166 target site was introduced into PHB by the primers listed in Supplementary Table 1. The construct was transformed into WT plants. Agrobacterium strain GV3101 was used to transform the constructs into different genotypes by the standard floral dip method⁴⁷.

Pi, total P and anthocyanin estimation. To determine Pi content, the tissues of eight-day-old seedlings were collected in 1.5 ml Eppendorf tubes and ground into fine powder by metal beads after snap-freezing in liquid nitrogen. Pi content was then measured using the phosphomolybdate colorimetric assay as previously described⁴⁸. Elemental analysis of total P through inductively coupled plasma mass spectrometry was performed as previously described⁴⁹. Shoots and roots of 12-day-old seedlings under P⁺ or P⁻ conditions were separately collected and subsequently rinsed with deionised water four times. After being dried at 65 °C for three days and ground to a fine powder under a room temperature, each sample was weighed in triplicate or in quadruplicate on an analytical balance. All samples were digested by concentrated nitric acid, and further estimation of total P was performed on a NexION 350d inductively coupled plasma mass spectrometer (PerkinElmer) coupled with an Apex desolvation system and an SC-4 DX autosampler (Elemental Scientific). As previously described, all the samples were normalized with a heuristical algorithm⁴⁹. For anthocyanin estimation, anthocyanin was extracted with methanol containing 1% HCl (v/v). After the removal of chlorophyll with an equal volume of chloroform, the anthocyanin content was measured as previously described⁵⁰. Relative anthocyanin contents were calculated and shown as A₅₃₀–₆₅₇ g⁻¹ fresh weight. Data analysis was carried out with SPSS v16.0.

Grafting of Arabidopsis plants. Five-day-old seedlings grown on 1/2 MS medium were used to do the reciprocal grafting under a dissecting microscope in a clean hood as previously described⁵¹. Two-week-old grafted plants without adventitious roots were used for the subsequent analyses.

Histochemical GUS staining was assayed by the normal procedure. Histochemical GUS staining was assayed as previously described⁵². The GUS-stained seedlings were photographed using a Zeiss Imager M2.

Real-time quantitative PCR. Total RNA was extracted from shoots and roots using the RNeasy Plant Kit (Qiagen), and 1 µg of RNA was reverse-transcribed following the manufacturer’s instructions (YESEN, 11121E650). The complementary DNA was used as a template, and real-time PCR was performed on a CFX96 real-time PCR detection system (Bio-RAD). ACT2 was used as the reference gene. The primers used in real-time quantitative PCR are listed in Supplementary Table 1.

Transcriptome analysis. The shoots of eight-day-old WT and pho2 seedlings under P⁺ conditions were collected in triplicate. Total RNA was extracted via the RNeasy Plant Kit (Qiagen) and by adding DNase to digest genomic DNA contamination during the isolation of RNA. RNA-seq was carried out with the Illumina HiSeq 2000 platform, and the clean reads were mapped to Arabidopsis genome build TAIR10 using the aligner STAR v.2.5.3a⁵³. The quantification was carried out with StringTie v.1.3.3 (ref. ⁵⁴). Differentially expressed genes (DEGs) were analysed using DESeq2 (v.1.20.0)⁵⁵. The genes that were either up- or downregualted with a twofold or higher change with an adjusted P value of ≤0.05 were regarded as DEGs. Gene Ontology enrichment analysis of the DEGs was performed using clusterProfiler (v.4.0.5)⁵⁶. The list of the DEGs is available in Supplementary Data 1.

Western blotting. To determine the protein levels of SHR–GFP, SHR–PHO1–GFP, SHR–FLAG and PHO1–GFP, seedling tissues were ground into fine powder in liquid nitrogen and dissolved in 5% SDS. The solution was then boiled at 95°C for 5 min and centrifuged for 10 min at 15,000 g. The supernatant was mixed with 5x SDS loading buffer and separated on a 10% SDS–PAGE gel. Anti-GFP (Rock, 118146001.1, 1:1,000 dilution), anti-FLAG (Sigma, F1804, 1:4,000 dilution) and anti-ACTIN (CWBIO, CW0265, 1:5,000 dilution) antibodies were used to detect the proteins.

Transient expression assays in Arabidopsis protoplasts. Briefly, five-day-old Arabidopsis seedlings were harvested from 1/2 MS solid medium. The roots were shredded in 10 ml of enzyme digestion solution (1.5% cellulose, 1.5% pectolyse, 0.4 M mannitol, 20 mM MES, 20 mM KC1, 10 mM CaCl₂, 0.1% BSA). The digestion solution was gently shaken at 80 rpm for 2 h. Protoplasts were harvested for plasmid transformation, reporters and internal controls. The plasmid transformation in protoplasts and the LUC and GUS activity assays was performed as previously described⁵⁷.

Protein degradation assays in vitro. SHR–FLAG protein of eight-day-old seedlings was immunoprecipitated and purified using beads fused with FLAG antibody, and the SHR–FLAG protein was equally divided into PCR tubes. Total protein of WT seedlings under P⁺ or P⁻ conditions was crudely extracted by solution (20 mM Tris–HCl, 150 mM NaCl, pH 7.4). After protein purification, equal amounts of crude proteins from P⁺ and P⁻ conditions were added into the lysis buffer and the degradation of SHR–FLAG was performed. The supernatant was regarded as DEGs. Gene Ontology enrichment analysis of the DEGs was performed as previously described⁵⁷.

ChIP assays. The roots of seedlings were collected and immediately crosslinked in 1% formaldehyde as previously described⁵⁸. Protein A/G Magnetic Beads (MCE, HY-K0202) were used for pre-clearing samples and antibody binding. Anti-FLAG (Sigma, F1804) was used for the immunoprecipitation of protein with FLAG. ChIP products were eluted with 50 µl of ddH₂O, and 1µl of product was used as the template for each quantitative PCR reaction. The primers for quantitative PCR with ChIP are listed in Supplementary Table 1.

EMSA. The expression and purification of GST-fused proteins in E. coli BL21 were performed as previously described⁵⁹. The binding sites of HD–ZIP III proteins on the PH2 promoter were identified using the online resource AthaMap⁶⁰. Biotin-labelled probes (30 bp of DNA) and non-biotin-labelled competitor probes were chemically synthesized (Sangon Biotech). For the DNA–protein binding assay, 1 µg of recombinant protein and 50 fmol probe or additional dose of competitors was mixed in a reaction volume of 20 µl. EMSA was performed using a Pierce LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer’s instructions. The migration of biotin-labelled probes was detected using ECL and the ChemiDoc imaging system (Bio-Rad). All of the probe and competitor sequences used in EMSA are listed in Supplementary Table 1.

Accession numbers. The gene sequences reported in this article can be found in the Arabidopsis Genome Initiative Information Resource (http://www.arabidopsis.org/), and the locus numbers are as follows: SHR (AT1G37350), SGR (AT3G54220), MIR165a (AT1G01183), MIR166b (AT4G08885), PHB (AT2G34710), PHV (AT3G0490), REV (AT5G06090), CNA (AT1G52150), ATHBA (AT3G38880), PHO1 (AT3G23430), PHO2 (AT3G37770), PHT1;1 (AT2G32630), IPS1 (AT3G20992), AT4 (AT5G03545), SPX1 (AT2G0150), RNS1 (AT2G02990), PAP17 (AT3G17770), MIR399a (AT5G62162), MDG3 (AT2G18110) and PAP10 (AT2G16430).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq data in this study have been deposited in the NCBI Gene Expression Omnibus with the accession code GSE199834. Source data are provided with this paper.

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Author contributions

M.L. and X.X. conceived and designed the study. X.X. and J.Z. performed most of the experiments and analysed the data with M.L. The other authors assisted in the experiments and discussed the results. X.X., V.S. and M.L. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection | The Cq value for qPCR were collected by CFX96 real-time PCR detection system (Bio-RAD). The OD values for Pi and anthocyanin estimation were collected by Thermo Scientific Varioskan LUX detection system. Total P estimation was performed on a NexION 350d ICP-MS spectrometer (PerkinElmer) coupled with an Apex desolation system and an SC-4 DX autosampler (Elemental Scientific). Transcriptome data and mapping-by-sequencing data was collected as shown in manuscripts.

Data analysis | For mapping-by-sequencing analysis, reads were mapped to the Arabidopsis genome (version TAIR10) with SHORE (version 0.9.0) and GenomeMapper (version 0.4.4), and candidate causative mutations were identified with algorithms developed in SHOREmap (v.3.0). For transcriptome analysis, STAR (version 2.5.3a), StringTie (version 1.3.3), DESeq2 (version 1.32.0) and clusterProfiler (version 4.0.5) were used as shown in manuscripts. Statistical analyses were performed with SPSS v16.0.

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We have provided a data availability statement in the manuscript. The RNA-Seq data in this study has been deposited in the NCBI Gene Expression Omnibus with the accession code GSE199834.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  No sample-size calculation was performed. Samples size for Pi content assay was determined according to the typical number in similar studies. (Dong, et al, 2019. Inositol Pyrophosphate InsP8 Acts as an Intracellular Phosphate Signal in Arabidopsis).

Data exclusions  No data were excluded from the analyses.

Replication  Each experiment has biological repeats. All experimental findings have been repeated two or three times to confirm their reproducibility.

Randomization  The plant materials were randomly allocated into different groups, and collected randomly.

Blinding  Sample groups were labeled as letters without the sample information, another person was blinded to the group for data analysis.

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| ☐ | Animals and other organisms | | |
| ☐ | Human research participants | | |
| ☑ | Clinical data | | |
| ☑ | Dual use research of concern | | |

Antibodies

| Antibodies used | The commercial antibodies Anti-GFP (Roch, 11814460001), anti-FLAG (Sigma, F1804) and anti-ACTIN (CWBIO, CW0264). |
|-----------------|---------------------------------------------------------------|
| Validation      | These antibodies in our studies are all commercial antibodies, which have been validated by many previous studies, such as the studies of Boltryk, et al, Nat Commun (2021), Yunhao, et al, Nature (2011). In our study, Col-0 plants were used as a negative control to validate the specificity of antibody to the target proteins. |