Arabidopsis WRKY45 Transcription Factor Activates *PHOSPHATE TRANSPORTER1;1* Expression in Response to Phosphate Starvation[1][W][OPEN]

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The WRKY transcription factor family has more than 70 members in the Arabidopsis (*Arabidopsis thaliana*) genome, and some of them are involved in plant responses to biotic and abiotic stresses. This study evaluated the role of WRKY45 in regulating phosphate (Pi) uptake in Arabidopsis. WRKY45 was localized in the nucleus and mainly expressed in roots. During Pi starvation, *WRKY45* expression was markedly induced, typically in roots. *WRKY45* overexpression in Arabidopsis increased Pi content and uptake, while RNA interference suppression of *WRKY45* decreased Pi content and uptake. Furthermore, the *WRKY45*-overexpressing lines were more sensitive to arsenate, the analog of Pi, compared with wild-type seedlings. These results indicate that WRKY45 positively regulates Arabidopsis Pi uptake. Quantitative real-time polymerase chain reaction and β-glucuronidase staining assays showed that *PHOSPHATE TRANSPORTER1;1* (*PHT1;1*) expression was enhanced in the *WRKY45*-overexpressing lines and slightly repressed in the *WRKY45* RNA interference line. Chromatin immunoprecipitation and eclectrophoretic mobility shift assay results indicated that WRKY45 can bind to two W-boxes within the *PHT1;1* promoter, confirming the role of WRKY45 in directly up-regulating *PHT1;1* expression. The *pht1;1* mutant showed decreased Pi content and uptake, and overexpression of *PHT1;1* resulted in enhanced Pi content and uptake. Furthermore, the *PHT1;1*-overexpressing line was much more sensitive to arsenate than *WRKY45*-overexpressing and wild-type seedlings, indicating that *PHT1;1* overexpression can enhance Arabidopsis Pi uptake. Moreover, the enhanced Pi uptake and the increased arsenate sensitivity of the *WRKY45*-overexpressing line was impaired by *pht1;1* (*35S:WRKY45-18::pht1;1*), demonstrating an epistatic genetic regulation between WRKY45 and *PHT1;1*. Together, our results demonstrate that WRKY45 is involved in Arabidopsis response to Pi starvation by direct up-regulation of *PHT1;1* expression.

Phosphorus is a major essential nutrient for plant growth and development and serves various basic biological functions in plant life cycle (Raghothama, 1999). Phosphate ($H_2PO_4^-$ or, in short, Pi) is the major form that is absorbed and transported into the plant cells (Ullrich-Eberius et al., 1981; Tu et al., 1990). The Pi concentration in the soil, typically 10 μM or less, results in Pi starvation for plant growth and survival, and plants have evolved different strategies to overcome the limited Pi availability. In response to Pi deficiency, plants increase Pi uptake by altering root architecture (López-Bucio et al., 2003; Ticconi and Abel, 2004; Osmont et al., 2007), by altering the expression of Pi-related genes (Bustos et al., 2010), or by changing their metabolic and developmental processes (Raghothama and Karthikeyan, 2005).

Analysis of the Arabidopsis (*Arabidopsis thaliana*) genome revealed that there are at least nine members of the Pi transporter family (PHOSPHATE TRANSPORTER1 [PHT1] family; Okumura et al., 1998; Mudge et al., 2002). PHT1;1 and PHT1;4, two members of the Arabidopsis PHT1 family, have been demonstrated to be Pi transporters participating in Pi uptake from the soil. Arabidopsis PHT1;1 can complement the yeast (*Saccharomyces cerevisiae*) mutant *pho84* (NS219 [pho3 pho84 ura3]), which lacks the high-affinity Pi transporter Pho84 (Muchhal et al., 1996), and overexpression of the Arabidopsis *PHT1;1* gene in tobacco (*Nicotiana tabacum*) cells increases their Pi uptake capacity (Mitsukawa et al., 1997). *PHT1;1* and *PHT1;4* are highly expressed in the root epidermis and endoderm and root hairs, where they have been proposed to function in Pi uptake from the soil (Karthikeyan et al., 2002; Mudge et al., 2002), and the double mutant *pht1;1Δ4Δ* shows a 75% reduction in Pi uptake capacity compared with the wild-type plant (Shin et al., 2004). PHT1;1 can be regulated at transcriptional and/or posttranscriptional levels. The *PHT1;1* transcript is the most abundant in the Arabidopsis *PHT1* gene family (Mudge et al., 2002), and *PHT1;1* expression is clearly induced during Pi starvation (Muchhal et al., 1996; Karthikeyan et al., 2002; Mudge et al., 2002; Shin et al., 2004). Many regulators have been

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reported to modulate *PHT1;1* expression. The transcription of *PHT1;1* can be positively regulated by phosphate starvation response1 (PHR1), WRKY75, and sugars and negatively regulated by cytokinin, abscisic acid, myb domain protein62 (MYB62), SPX domain protein3, and actin-related protein6/histone H2A variant Htz1 (Martín et al., 2000; Karthikeyan et al., 2002; Shin et al., 2006; Devaiah et al., 2007a, 2009; Duan et al., 2008; Smith et al., 2010). The phosphate transporter traffic facilitator1 (PHF1) protein, a SEC12-related protein, is necessary for PHT1;1 plasma membrane localization (González et al., 2005). Mutation of *PHF1* impairs the localization of PHT1;1 at the plasma membrane, and the *phf1* mutant displays a strong reduction in Pi accumulation compared with the wild-type plant (González et al., 2005). PHT1;1 can be phosphorylated (Nühse et al., 2004), and when the Ser-514 residue of PHT1;1 is changed to Asp, the PHT1;1^{S514D} protein accumulates in the endoplasmic reticulum (Bayle et al., 2011).

Transcriptome analysis demonstrated that the expression of many genes, including *PHT1* family genes, significantly changes in *Oryza sativa* (Wasaki et al., 2003) and Arabidopsis (Wu et al., 2003; Misson et al., 2005) in response to Pi starvation, indicating that transcriptional regulation plays important roles in plants' responses to low-Pi stress. Furthermore, a number of transcription factors have been reported in responses to Pi starvation, such as AtPHR1 (Rubio et al., 2001), rice Pi starvation-induced transcription factor1 (Yi et al., 2005), AtWRKY75 (Devaiah et al., 2007a), zinc finger of Arabidopsis thaliana6 (AtZAT6; Devaiah et al., 2007b), AtMYB62 (Devaiah et al., 2009), and AtWRKY6 (Chen et al., 2009). WRKY proteins are plant-specific transcription factors and are characterized by the presence of one or two highly conserved WRKY domains (Eulgem et al., 2000; Maeo et al., 2001; Zhang and Wang, 2005). The WRKY domain contains the conserved amino acid sequence motif WRKYGQK, followed by a Cys_2His_2 or $Cys_2HisCys$ zinc finger motif, and both conserved motifs of WRKY domain are necessary for the binding affinity of the WRKY protein to the consensus cis-acting element W-box (C/T)TGAC(C/T; Eulgem et al., 2000; Maeo et al., 2001; Zhang and Wang, 2005). Several WRKY proteins, such as AtWRKY75 and AtWRKY6, have been reported to play important roles in plant responses to Pi starvation (Devaiah et al., 2007a; Chen et al., 2009).

In this study, the function of WRKY45 in Arabidopsis responses to Pi starvation was investigated. We demonstrated that *WRKY45* expression was highly induced during Pi deprivation, and overexpression of *WRKY45* enhanced Pi uptake and increased arsenate sensitivity. WRKY45 positively regulated *PHT1;1* expression by binding to the W-boxes within the *PHT1;1* promoter, and overexpression of *PHT1;1* improved plant Pi uptake. The *pht1;1* mutant suppressed the enhanced Pi uptake and the increased arsenate sensitivity caused by *WRKY45* overexpression, demonstrating that *PHT1;1* is epistatic to *WRKY45*. In conclusion, our results demonstrate that WRKY45 is a novel transcription factor that regulates Pi uptake by modulating *PHT1;1* expression in Arabidopsis.

RESULTS

WRKY45 Is a Pi Starvation-Responsive Transcription Factor

WRKY proteins are plant-specific transcription factors, and two of them, AtWRKY75 (Devaiah et al., 2007a) and AtWRKY6 (Chen et al., 2009), have been reported to participate in Arabidopsis Pi homeostasis. It has been hypothesized that other WRKY proteins may also play roles in plant responses to Pi starvation. To test this hypothesis, we analyzed the expression of *WRKY* genes in Arabidopsis during Pi starvation. Quantitative real-time (qRT) PCR analysis showed that *WRKY45* was mainly expressed in the roots (Fig. 1A), and when wild-type Arabidopsis seedlings were challenged with Pi starvation, the *WRKY45* was significantly induced, typically in the roots (Fig. 1A).

To further confirm the expression pattern of *WRKY45*, homozygous single-copy *ProWRKY45*:*GUS* transgenic lines were generated. Seven-day-old *ProWRKY45*:*GUS* seedlings were transferred to Murashige and Skoog (MS) medium (Pi-sufficient condition, MS) or MS medium without Pi (Pi-deficient condition, low phosphate medium [LP]) for 5 d and then stained to detect GUS activity. When *ProWRKY45*:*GUS* seedlings were grown under Pi-sufficient condition, *WRKY45* was mainly expressed in the roots (Fig. 1B, a and b), and when *ProWRKY45*:*GUS* seedlings were transferred to Pi-deficient condition, a strong GUS staining appeared in the roots (Fig. 1B, c and d), typically in the root tips (Fig. 1Bd). The qRT PCR and GUS staining results indicate that *WRKY45* expression is induced during Pi starvation, mainly in the roots.

To detect subcellular localization of WRKY45 protein, the coding region of *WRKY45* was fused with the 3′ end of the *GFP* reporter gene and expressed under the control of the Super promoter. The *GFP* gene alone under the control of the Super promoter served as the control. The subcellular localization of WRKY45 was determined in a transient expression system in *Nicotiana benthamiana* leaves. The WRKY45:GFP fusion protein was exclusively localized in the nucleus (Fig. 1C), indicating that WRKY45 was localized in the nucleus.

WRKY transcription factors typically contain one or more WRKY domains (Eulgem et al., 2000; Maeo et al., 2001; Zhang and Wang, 2005). Amino acid sequence analysis revealed that the deduced amino acid sequence of WRKY45 had a highly conserved WRKYGQK motif and a characteristic Cys_2His_2 zinc finger motif (Fig. 1D).

Together, these data suggest that WRKY45 is a Pi starvation-responsive transcription factor.

Overexpression of *WRKY45* Enhances Arabidopsis Pi Uptake

To characterize the role of WRKY45 during Pi starvation, *WRKY45*-overexpressing (*35S*:*WRKY45*) and *WRKY45* RNA interference (RNAi) transgenic lines were generated. qRT PCR results showed that under

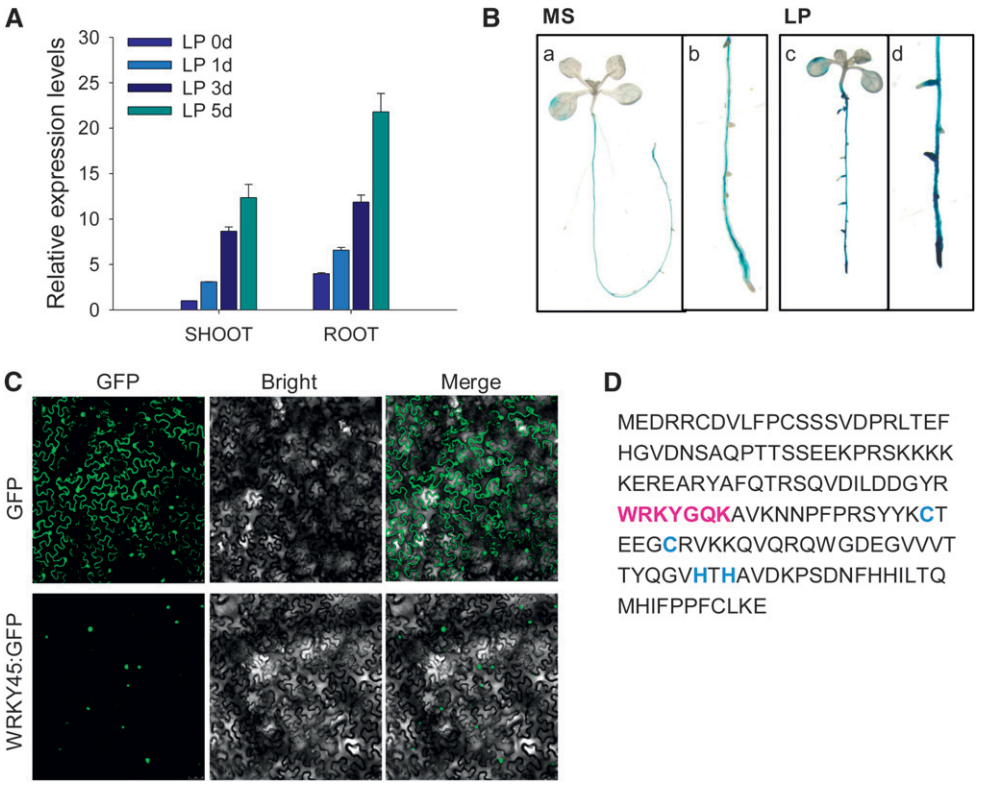

Figure 1. WRKY45 responds to Pi starvation and is localized in the nucleus. A, qRT PCR analysis of *WRKY45* expression. Seven-day-old wild-type seedlings were transferred to LP medium, and then the shoots and roots were harvested separately at the indicated time. The data represent the mean values of three replicates ± SE. B, GUS staining assay of the *ProWRKY45*:*GUS* transgenic line. Seven-day-old *ProWRKY45*:*GUS* seedlings were transferred to MS (a and b) or LP (c and d) medium for 5 d and then stained. Details of the roots of the *ProWRKY45*:*GUS* transgenic seedlings are shown in b and d. C, Subcellular localization of the WRKY45:GFP fusion protein in the *N. benthamiana* leaf. The expression of GFP alone was used as the control. D, Deduced amino acid sequence of WRKY45 showing the highly conserved WRKY domain WRKYGQR and the novel C2H2 zinc finger motif in magenta and blue letters, respectively.

Pi-sufficient (MS) and Pi-deficient (LP) conditions, the *WRKY45* expression was significantly elevated in the *WRKY45*-overexpressing lines and repressed in the *WRKY45* RNAi lines compared with that in wild-type seedlings (Fig. 2A). The Pi content of various plants was measured under Pi-sufficient and Pi-deficient conditions. As shown in Figure 2B, the *WRKY45*-overexpressing lines had higher Pi contents, whereas the *WRKY45* RNAi line had a lower Pi content compared with that of wild-type seedlings (Fig. 2B), suggesting that overexpression of *WRKY45* enhances Arabidopsis Pi accumulation.

To analyze Pi uptake rates, 7-d-old seedlings were transferred into a Pi uptake solution containing 50 μM Pi supplemented with ^{32}P orthophosphate, and Pi

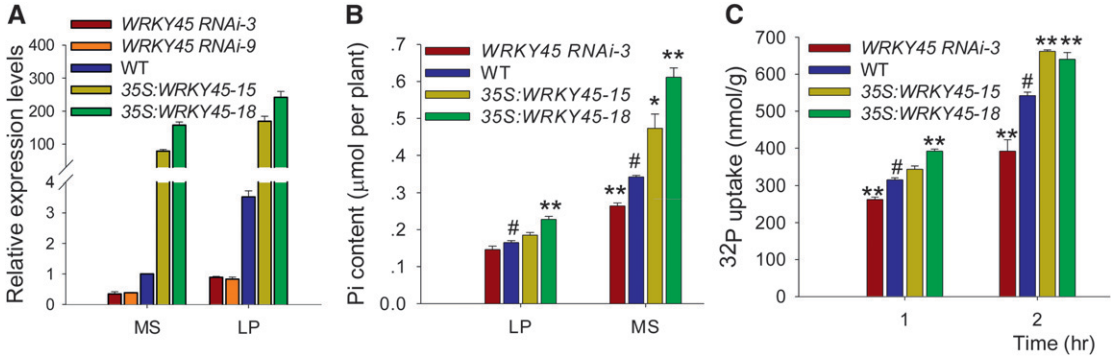

Figure 2. Overexpression of *WRKY45* enhances Arabidopsis Pi accumulation and Pi uptake. A, qRT PCR analysis of *WRKY45* expression in the *WRKY45* RNAi lines, *WRKY45*-overexpressing lines, and wild-type plants. Seven-day-old seedlings were transferred to MS or LP medium and then harvested for RNA extraction. The data are the mean values of three replicates ± SE. B, Pi content analysis. Seven-day-old seedlings were transferred to MS or LP medium for 10 d and then harvested for Pi content analysis. Data are shown as mean ± SE ($n = 3$). Asterisks indicate statistically significant differences compared with the wild type (Student's *t* test, $P < 0.05$). Wild-type plants were used as a control (#). C, Pi uptake was monitored over a 2-h period in 7-d-old seedlings germinated and grown on MS medium. Data are shown as mean ± SE ($n = 3$). Asterisks indicate statistically significant differences compared with the wild type (Student's *t* test, $P < 0.05$). Wild-type plants were used as a control (#). WT, Wild type.

uptake over a 2-h period was measured. As shown in Figure 2C, the *WRKY45*-overexpressing lines displayed a substantial increase, whereas the *WRKY45* RNAi line showed a reduction in Pi uptake capacity compared with that of the wild-type seedlings. Arsenate is an ion structurally analogous to Pi, which is transported into plant roots via Pi transporters (Asher and Reay, 1979; Shin et al., 2004; Catarecha et al., 2007; Castrillo et al., 2013). Thus, arsenate sensitivity was tested among various plant genotypes. No obvious phenotypic differences were observed among the *WRKY45*-overexpressing lines, the *WRKY45* RNAi line, and wild-type seedlings grown under medium without arsenate (Supplemental Fig. S1A). In the presence of arsenate, compared with wild-type seedlings, the *WRKY45*-overexpressing lines displayed an arsenate-sensitive phenotype, and the root length of *WRKY45*-overexpressing lines was significantly shorter than that of wild-type seedlings (Supplemental Fig. S1, A and B), indicating that *WRKY45* overexpression enhanced plant arsenate accumulation. No obvious differences were observed between the *WRKY45* RNAi line and wild-type seedlings grown on medium with arsenate (Supplemental Fig. S1).

WRKY45 Positively Regulates *PHT1;1* Transcription

As the *WRKY45*-overexpressing lines displayed an increase in Pi uptake capacity, we hypothesized that WRKY45 regulates the expression of Pi transporter genes. PHT1;1 and PHT1;4 are the two main Pi transporters for Arabidopsis Pi acquisition from the soil (Shin et al., 2004), which are mainly expressed in the roots (Karthikeyan et al., 2002). Therefore, the expressions of *PHT1;1* and *PHT1;4* were examined in the roots of the *WRKY45*-overexpressing lines, the *WRKY45* RNAi line, and wild-type plants under Pi-sufficient condition. The transcription of *PHT1;1* was elevated in the *WRKY45*-overexpressing lines and slightly repressed in the *WRKY45* RNAi lines compared with that in wild-type roots (Fig. 3A; Supplemental Fig. S2), and there were no obvious differences in *PHT1;4* expression among the various plants (Fig. 3A). Then, the expressions of *PHT1;1* were detected in the roots of *WRKY45* RNAi lines and wild-type seedlings under Pi starvation. The 7-d-old seedlings were transferred to LP medium for 3 d, and then the roots were harvested for RNA extraction. The qRT PCR results showed that the expressions of *PHT1;1* in *WRKY45* RNAi lines (*WRKY45 RNAi-3* and *WRKY45 RNAi-9*) were obviously lower than that in wild-type plants under Pi starvation (Fig. 3B).

The GUS staining results showed that *PHT1;1* was mainly expressed in the roots of the single-copy *ProPHT1;1:GUS* transgenic lines (Fig. 3C). Under Pi starvation, *PHT1;1* expression was significantly induced, typically in the root tips (Fig. 3C, c and d), indicating that *WRKY45* and *PHT1;1* had similar expression patterns. In addition, we crossed the *ProPHT1;1:GUS* line with the *WRKY45*-overexpressing lines (*35S:WRKY45-15* and *35S:WRKY45-18*), the *WRKY45* RNAi line (*WRKY45 RNAi-9*),

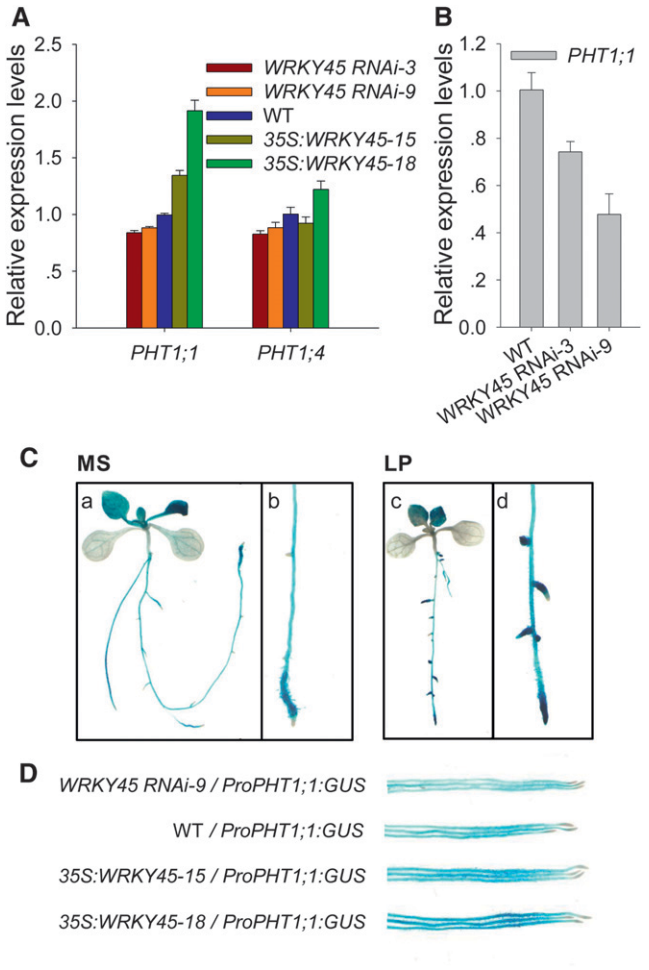

Figure 3. WRKY45 promotes *PHT1;1* expression. A, qRT PCR analysis of *PHT1;1* and *PHT1;4* expressions in the roots of the *WRKY45*-overexpressing lines, *WRKY45* RNAi lines, and wild-type plants. The plants were germinated and grown on MS medium for 7 d, and then the roots were harvested for RNA extraction. The data are the mean values of three replicates ± SE. B, qRT PCR analysis of *PHT1;1* expression in the roots of the *WRKY45* RNAi lines and wild-type plants. The 7-d-old seedlings were transferred to LP medium for 3 d and then harvested for RNA extraction. The data are the mean values of three replicates ± SE. C, GUS staining of the *ProPHT1;1:GUS* transgenic line. Seven-day-old *ProPHT1;1:GUS* seedlings were transferred to MS (a and b) or LP (c and d) medium for 5 d and then stained. Details of the roots of the *ProPHT1;1:GUS* transgenic line are shown in b and d. D, GUS staining showing the expression patterns of *PHT1;1* in the *WRKY45* RNAi line, *WRKY45*-overexpression lines, and wild-type plants. The plants were germinated and grown on MS medium for 7 d and then harvested for GUS staining. WT, Wild type.

and wild-type plants and obtained the *35S:WRKY45-15/ProPHT1;1:GUS*, *35S:WRKY45-18/ProPHT1;1:GUS*, *WRKY45 RNAi-9/ProPHT1;1:GUS*, and wild-type/*ProPHT1;1:GUS* plants, respectively. Under Pi-sufficient condition, the root GUS staining results showed that the *PHT1;1* expression was promoted in the *WRKY45*-overexpressing lines (*35S:WRKY45-15/ProPHT1;1:GUS* and *35S:WRKY45-18/ProPHT1;1:GUS*) and

slightly repressed in the *WRKY45* RNAi line (*WRKY45 RNAi-9*/*ProPHT1;1*:*GUS*) compared with that in the wild type (wild type/*ProPHT1;1*:*GUS*; Fig. 3D). These data demonstrate that WRKY45 positively regulates *PHT1;1* expression.

WRKY45 Binds to the *PHT1;1* Promoter

WRKY proteins regulate their target genes' expression by binding to the W-box(es) in their target gene promoters (Rubio et al., 2001; Wu et al., 2003; Misson et al., 2005). There are several W-box motifs within the *PHT1;1* promoter (Fig. 4A; Martín et al., 2000). Thus, we further hypothesized that WRKY45 may directly regulate *PHT1;1* expression by binding to the W-box (es) of the *PHT1;1* promoter. To test this hypothesis, we generated the WRKY45-Myc transgenic lines. The coding region of *WRKY45* was fused with the 3′ end of the Myc tag and expressed under the control of the *Cauliflower mosaic virus* 35S promoter, generating single-copy *WRKY45*-Myc transgenic lines. Western-blot analysis with anti-Myc showed that the WRKY45-Myc protein was expressed in the *WRKY45*-Myc line (Fig. 4B). The in vivo interaction between WRKY45 and the W-box motifs within the *PHT1;1* promoter was investigated using the chromatin immunoprecipitation (ChIP) method with WRKY45-Myc roots. As shown in Figure 4C, WRKY45 interacted with the *PHT1;1* promoter when the primer combinations containing either site 1 or site 4 were applied, while no interaction was observed between WRKY45 and the *PHT1;1* promoter containing site 2 or site 3 (Fig. 4C). The eclectrophoretic mobility shift assay (EMSA) was also performed. WRKY45 was expressed in *Escherichia coli* as a fusion protein with CTP:CMP-3-deoxy-D-mannooctulosonate cytidylyltransferase (CMP-KDO) synthetase (CKS) and affinity purified from the soluble fraction. The WRKY45-CKS fusion protein can bind to the site 1 and site 4 within the *PHT1;1* promoter, and the binding was abolished by the addition of increasing amounts of unlabeled competitors with the same sequence (Fig. 4D). By contrast, the CKS protein alone did not show any detectable binding to the *PHT1;1* promoter (Fig. 4D). These data demonstrate that WRKY45 can directly bind to two W-boxes within the *PHT1;1* promoter.

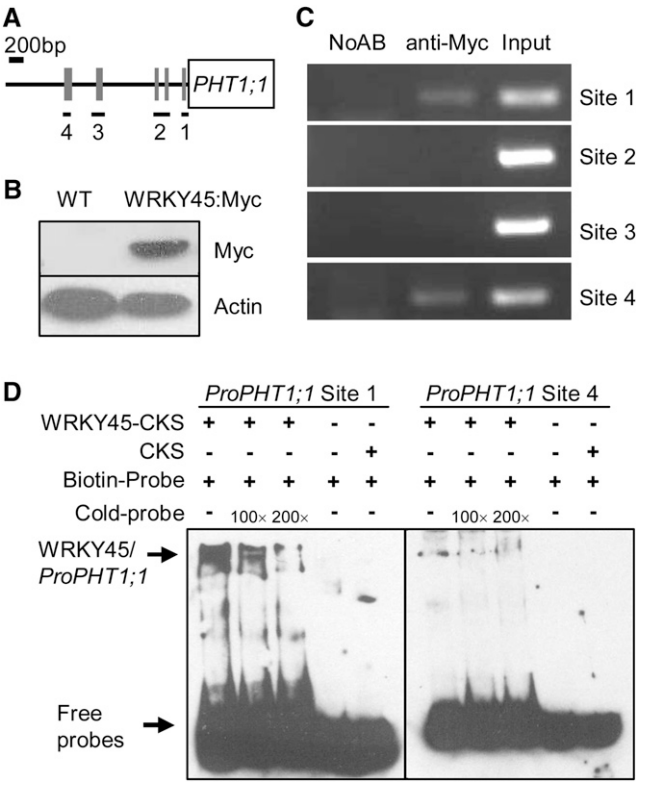

Figure 4. WRKY45 binds to the *PHT1;1* promoter. A, Diagram of the *PHT1;1* promoter region showing the relative positions of the W-boxes. The W-boxes are marked by gray rectangles, and relative positions and sizes of the different PCR-amplified fragments are indicated by black lines under the W-box(es). B, Western-blot analysis was performed with anti-Myc to detect the WRKY45-Myc protein in the WRKY45-Myc transgenic line and wild-type seedlings. C, ChIP analysis to detect the association between WRKY45 and the W-boxes within the *PHT1;1* promoter in the WRKY45-Myc transgenic line. The ChIP signals with (anti-Myc) and without (NoAB) addition of anti-Myc are indicated. D, EMSA assay to analyze the binding of WRKY45 to *PHT1;1* promoter. Each biotin-labeled DNA probe was incubated with WRKY45-CKS protein. An excess of unlabeled probes was added to compete with labeled promoter sequences. Biotin-labeled probes incubated with CKS protein served as the negative control. WT, Wild type.

Overexpression of *PHT1;1* Enhances Arabidopsis Pi Uptake

The *PHT1;1*-overexpressing lines (*Super*:*PHT1;1*) were also generated, and the *pht1;1* mutant was obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc). qRT PCR analysis showed that the *PHT1;1* expression was abolished in the *pht1;1* mutant and overexpressed in the *PHT1;1*-overexpressing lines (Fig. 5A). The Pi uptake capacity of the *PHT1;1*-overexpressing lines was much higher than that of wild-type plants and even much higher than that of the *WRKY45*-overexpressing line (Fig. 5B). By contrast, the *pht1;1* mutant showed a reduction in Pi uptake compared with that of wild-type seedlings (Fig. 5B). In the presence of arsenate, the *PHT1;1*-overexpressing line was much more sensitive to arsenate [As(V)] than were wild-type seedlings and the *WRKY45*-overexpressing line (Supplemental Fig. S3). These data indicate that overexpression of *PHT1;1* enhances Arabidopsis Pi uptake.

Epistatic Relationship between WRKY45 and *PHT1;1*

Epistatic relationship between WRKY45 and *PHT1;1* was assessed by crossing *35S*:*WRKY45* and *pht1;1* mutant to produce *35S*:*WRKY45*::*pht1;1*. F2 progeny were genotyped for the presence of both *35S*:*WRKY45* and

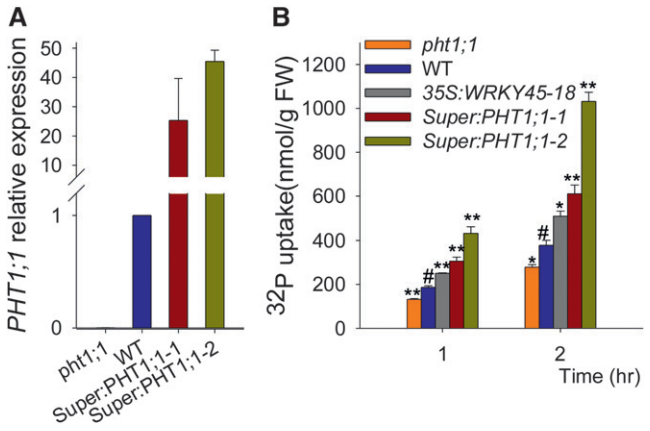

Figure 5. Overexpression of *PHT1;1* enhances plant Pi uptake. A, qRT PCR analysis of *PHT1;1* expression in the *pht1;1* mutant, *PHT1;1*-overexpressing lines, and wild-type seedlings. Seven-day-old seedlings were used for RNA extraction. The data are the mean values of three replicates ± SE. B, Pi uptake was monitored over a 2-h period in 7-d-old seedlings germinated and grown on MS medium. Data are shown as mean ± SE ($n = 3$). Asterisks indicate statistically significant differences compared with the wild type (Student's *t* test, $P < 0.05$). Wild-type plants were used as a control (#). WT, Wild type.

pht1;1 mutantation, and the homozygous at both loci was selected for evaluation. In the *35S:WRKY45-18::pht1;1*, the *WRKY45* was overexpressed and *PHT1;1* expression was repressed (Fig. 6A). The Pi uptake of *35S:WRKY45-18::pht1;1* was lower compared with *35S:WRKY45-18* (Fig. 6B), and in the presence of arsenate, *pht1;1* suppressed the As(V) sensitivity of *35S:WRKY45* (Supplemental Fig. S4). These results indicate that *PHT1;1* is genetically epistatic to *WRKY45*.

WRKY45 Negatively Regulates *WRKY75* Expression

The Pi uptake of *pht1;1* mutant was lower than that of the wild-type seedlings, even lower than that of *WRKY45 RNAi-9* (Fig. 7A), indicating that in addition to WRKY45, there are other transcription factor(s) regulating the expression of *PHT1;1*. Previous reports demonstrated that several transcription factors, such as PHR1 (Rubio et al., 2001), WRKY75 (Devaiah et al., 2007a), and ZAT6 (Devaiah et al., 2007b), regulate plant Pi uptake or *PHT1* family genes' expression. PHR1 can bind to P1BS sequence within its target gene promoters to regulate target gene expression (Bustos et al., 2010). Promoter sequence analysis showed that there was no P1BS motif within the *WRKY45* promoter (data not shown), and the expression of *WRKY45* was not regulated in the *phr1* glucocorticoid receptor:PHR1-overexpressing plant (Bustos et al., 2010), indicating that PHR1 cannot regulate the expression of *WRKY45*. There was no W-box within the *PHR1* promoter (Fig. 7B), and the WRKY45 cannot regulate the expression of *PHR1* (Fig. 7C). These data indicate that the function of WRKY45 in up-regulating *PHT1;1* expression is independent of PHR1.

Promoter sequence analysis showed that there was a W-box within the promoters of *ZAT6* and *WRKY75* separately (Fig. 7B). It is proposed that WRKY45 may regulate the expressions of *ZAT6* and/or *WRKY75*. The qRT PCR assays showed that WRKY45 did not regulate the *ZAT6* expression (Fig. 7D), and the expression of *WRKY75* was repressed in the *WRKY45*-overexpressing lines (Fig. 7E). To further test the function of WRKY45 on regulation of *WRKY75* expression, transient expression experiments in tobacco leaves were performed. As shown in Figure 7F, WRKY45 inhibited *WRKY75* promoter activity. These data demonstrate that WRKY45 negatively regulates the expression of *WRKY75*.

Promoter sequence analysis results also showed that there were two W-boxes within the *WRKY45* promoter (Fig. 7B), and the transient expression assays showed that WRKY75 can repress the activity of the *WRKY45*

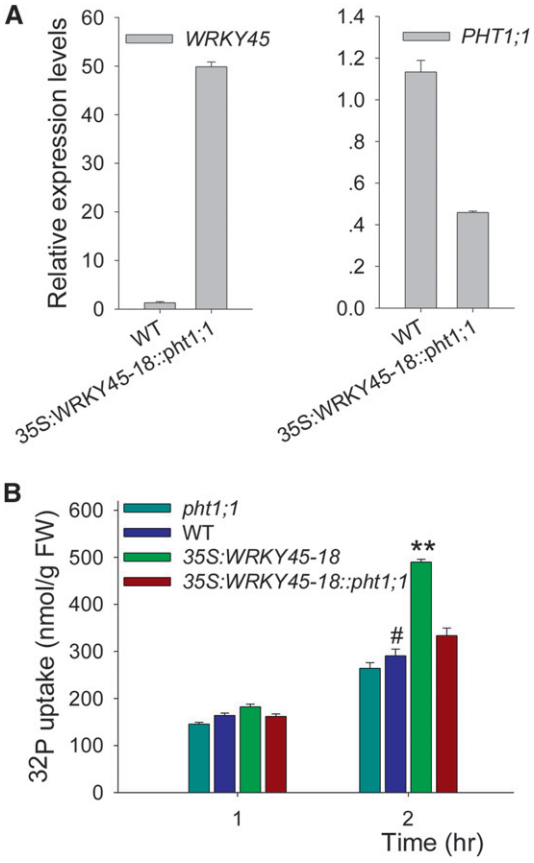

Figure 6. The Pi uptake enhancement caused by *WRKY45* overexpression is suppressed in the *pht1;1* mutant. A, qRT PCR analysis of *PHT1;1* or *WRKY45* expression in the *35S:WRKY45-18::pht1;1* lines and wild-type seedlings. Seven-day-old seedlings were used for RNA extraction. The data are the mean values of three replicates ± SE. B, Pi uptake was monitored over a 2-h period in 7-d-old seedlings germinated and grown on MS medium. Data are shown as mean ± SE ($n = 3$). Asterisks indicate statistically significant differences compared with the wild type (Student's *t* test, $P < 0.05$). Wild-type plants were used as a control (#). WT, Wild type.

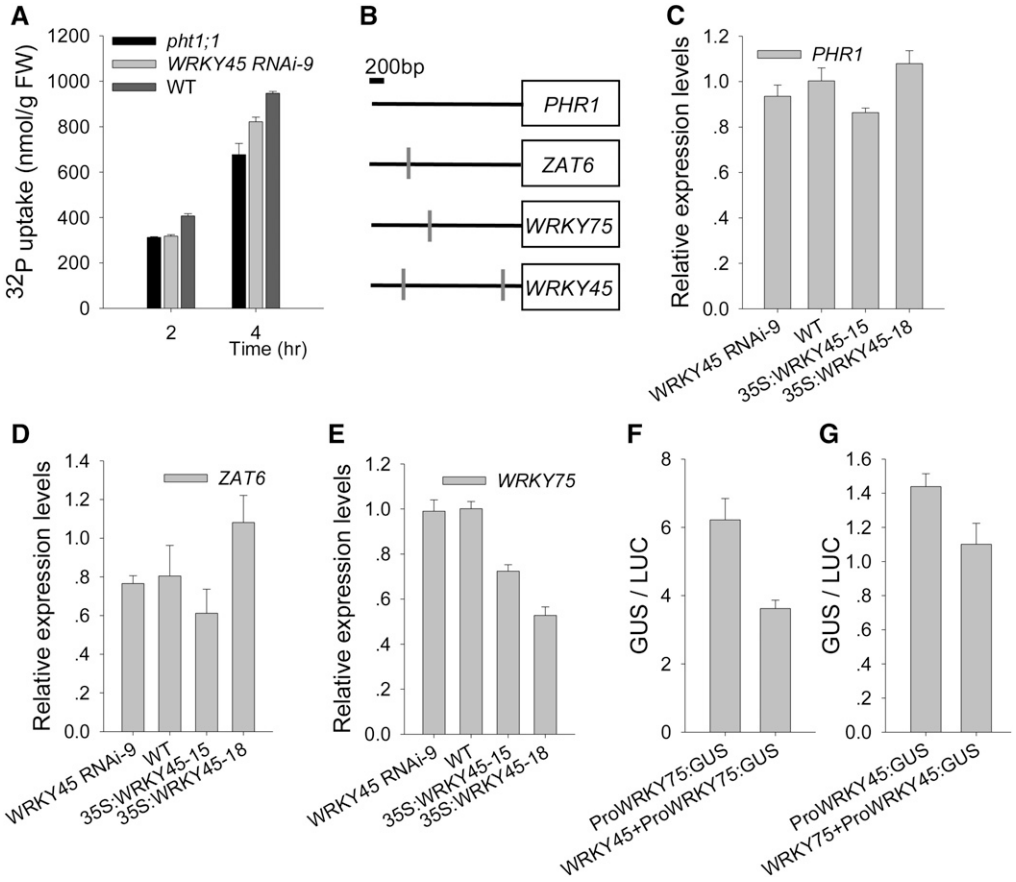

Figure 7. Expressions of Pi-related transcription factor genes in *WRKY45*-overexpressing lines. A, Pi uptake was monitored over a 4-h period in 7-d-old seedlings germinated and grown on MS medium. Data are shown as mean ± SE ($n = 3$). B, Diagram of the promoter regions of *PHR1*, *ZAT6*, *WRKY75*, and *WRKY45* showing the relative positions of the W-boxes. The W-boxes are marked by gray rectangles. C to E, qRT PCR analysis of *PHR1* (C), *ZAT6* (D), and *WRKY75* (E) expressions in the *WRKY45* RNAi line, *WRKY45*-overexpressing lines, and wild-type plants. Seven-day-old seedlings were harvested for RNA extraction. The data are the mean values of three replicates ± SE. F and G, Transient overexpression of *WRKY45* fused to *ProWRKY75:GUS* (F) and transient overexpression of *WRKY75* fused to *ProWRKY45:GUS* (G) in *N. benthamiana* leaves. Each data bar represents the means ± SE ($n = 3$). WT, Wild type.

promoter (Fig. 7G), indicating that WRKY75 negatively regulated the *WRKY45* expression.

DISCUSSION

WRKY45 Is a Positive Regulator of Plant Pi Uptake

Previous reports have revealed the importance of transcriptional control in plant responses to Pi starvation (Wu et al., 2003; Misson et al., 2005), suggesting that transcription factors play important roles in plant responses to Pi starvation. The molecular mechanisms of gene expression regulation during Pi starvation are poorly understood. Some transcription factors, such as PHR1 (Rubio et al., 2001), WRKY75 (Devaiah et al., 2007a), ZAT6 (Devaiah et al., 2007b), basic helix-loop-helix32 (Chen et al., 2007), MYB62 (Devaiah et al., 2009), and WRKY6 (Chen et al., 2009), have been identified to be involved in the response to Pi starvation. This study demonstrates that the Arabidopsis transcription factor WRKY45 is a positive regulator in plant response to Pi starvation. *WRKY45* expression was induced by Pi starvation (Fig. 1), and overexpression of *WRKY45* enhanced Arabidopsis Pi uptake and increased arsenate sensitivity (Fig. 2). The genetic and biochemical data support the notion that WRKY45 positively regulates the *PHT1;1* expression by binding to the *PHT1;1* promoter (Figs. 3 and 4). Overexpression of *PHT1;1* enhanced Arabidopsis Pi uptake (Fig. 5) and increased arsenate sensitivity (Supplemental Fig. S3), and genetic data further confirmed that *PHT1;1* is genetically epistatic to *WRKY45* (Fig. 6; Supplemental Fig. S4). We demonstrated that WRKY45, as a positive regulator of plant responses to Pi starvation, directly regulates *PHT1;1* expression to enhance plant Pi uptake.

Several transcription factors, such as PHR1 (Rubio et al., 2001), WRKY75 (Devaiah et al., 2007a), and ZAT6 (Devaiah et al., 2007b), have been reported to

regulate plant Pi uptake or *PHT1* family gene expression. The expression of *PHR1* is not induced during Pi deprivation, and PHR1 is a small ubiquitin-like modifier (SUMO)ylation target of SAP and Miz protein (SIZ1; SUMO E3 ligase; Rubio et al., 2001; Miura et al., 2005). PHR1 cannot regulate the expression of *WRKY45* (Bustos et al., 2010), and the expression of *PHR1* was not regulated by WRKY45 (Fig. 7C), indicating that the regulating of WRKY45 on *PHT1;1* is independent of PHR1.

Previous reports demonstrated that some WRKY proteins have redundant function, as Arabidopsis WRKY40, WRKY18, and WRKY60 play partial redundant functions in plant responses to pathogens (Xu et al., 2006) and abscisic acid signal (Shang et al., 2010), and Arabidopsis WRKY6 and WRKY42 both activate the expression of senescence-induced receptor-like kinase (Robatzek and Somssich, 2002). Robatzek and Somssich (2002) also found that Arabidopsis WRKY6 negatively regulated the expression of *WRKY42*. Similar to previous reports, WRKY75 (Devaiah et al., 2007a) and WRKY45 (Fig. 3) both positively regulate *PHT1;1* expression, and WRKY45 and WRKY75 can repress each other's expression (Fig. 7, E–G).

PHR1 (Rubio et al., 2001), WRKY75 (Devaiah et al., 2007a), ZAT6 (Devaiah et al., 2007b), and MYB62 (Devaiah et al., 2009) can regulate *PHT1;1* and *PHT1;4* expression. By contrast, WRKY45 directly up-regulated *PHT1;1* expression (Figs. 3 and 4), while *PHT1;4* expression was not regulated in *WRKY45*-overexpressing lines (Fig. 3A), suggesting that WRKY45 specifically regulates *PHT1;1* expression.

The Transcription of *PHT1;1* Is Specifically Regulated

Pi is an essential nutrient for plants, and the low bioavailability of Pi in the soil is a major factor limiting growth, development, and productivity of plants. The Pi transporters transport Pi from external media containing very low levels of Pi into the cytoplasm (Nilsson et al., 2007). Two Arabidopsis Pi transporters, PHT1;1 and PHT1;4, have been demonstrated to play a significant role in Pi acquisition from both poor- and rich-Pi environments (Shin et al., 2004). The expressions of *PHT1;1* and *PHT1;4* are induced preferentially in the roots during Pi starvation (Shin et al., 2004), consistent with their role in Pi acquisition. In addition to these two members of the PHT1 family, the expressions of other family members are also induced during Pi starvation (Muchhal et al., 1996; Karthikeyan et al., 2002). Thus, it has been concluded that the transcription of Pi transporter genes is activated upon Pi starvation, although the mechanisms are still not fully understood.

The *PHT1;1* gene is the Pi transporter gene with the most abundant expression level in the Arabidopsis *PHT1* gene family (Mudge et al., 2002). Several transcription factors have been reported to influence *PHT1;1* expression. In the *WRKY75* RNAi line, the expression of *PHT1;1* was repressed, and the Pi uptake capacity was reduced during Pi deprivation (Devaiah et al., 2007a), indicating that WRKY75 may positively regulate *PHT1;1* expression directly or indirectly. PHR1 and PHR1-Like1 (PHL1) are two important MYB-related transcription factors. In the *phr1* mutant or the *phr1phl1* double mutant, the transcript level of *PHT1;1* was low (Rubio et al., 2001), and overexpression of *PHR1* in the wild-type plant resulted in increased Pi content (Nilsson et al., 2007), suggesting that PHR1 and PHL1 can regulate *PHT1;1* expression. In this study, we demonstrated that WRKY45 can bind to the two W-box motifs within the *PHT1;1* promoter to directly up-regulate *PHT1;1* expression. These data indicate that the transcription of *PHT1;1* is positively regulated by several transcription factors.

In addition to positive regulation, *PHT1;1* expression is also negatively regulated. Upon Pi starvation, the expression of *PHT1;1* in *ZAT6*-overexpressing lines is lower than that in wild-type plants (Devaiah et al., 2007b), indicating that *PHT1;1* expression can be negatively regulated even during Pi starvation. The *PHT1;1* expression is also down-regulated in the *MYB62*-overexpressing lines (Devaiah et al., 2009).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used in this study. The Arabidopsis transfer DNA insertion line (Salk_088586c) of *PHT1;1* was obtained from the Arabidopsis Biological Resource Center (Ohio State University) and named *pht1;1* mutant.

The Arabidopsis seeds were surface sterilized and incubated at 4°C in darkness for 3 d. Then, the seeds were plated on MS medium containing 1.25 mM Pi, 3% (w/v) Suc, and 0.8% (w/v) agar and grown at 22°C with constant illumination at 80 μmol m^{-2} s^{-1}, unless otherwise indicated.

For Pi starvation treatment, 7-d-old seedlings were transferred to LP medium. The LP medium was made by modifying the MS medium to contain 10 μM Pi (supplied with KH_2PO_4), and the agar was replaced by agarose (Promega) to avoid phosphorous contamination.

For arsenate treatment, the sterilized seeds were plated on MS medium with 500 μM Pi and 150 μM As(V).

Quantification of Total Pi

Seven-day-old Arabidopsis seedlings were transferred to MS medium for 7 to 10 d, and then the seedlings were harvested for Pi content measurements. The total Pi content in the samples was quantified as described previously (Clough and Bent, 1998; Wasaki et al., 2003).

Pi Uptake Assay

The *WRKY45*-overexpressing lines, *WRKY45* RNAi lines, *PHT1;1*-overexpressing lines, *pht1;1* mutant, and wild-type plants were germinated and grown on MS medium for 7 d, and then the seedlings were used for Pi uptake assays. A group of 15 seedlings was used as one biological sample. Pi uptake assay was modified from Devaiah et al. (2007a), and radioactivity was measured with a scintillation counter (Beckman Coulter).

Plasmid Construction and Plant Transformation

To construct *35S:WRKY45* and *Super:PHT1;1*, *WRKY45* and *PHT1;1* complementary DNA fragments were amplified by PCR using gene-specific primers (Supplemental Table S1) and cloned into pBI121 or pCAMBIA1300 vector. To construct the *WRKY45 RNAi* line, the *WRKY45* fragment was

amplified by PCR using the primers listed in Supplemental Table S1 and cloned into pBI121 vector. To construct *ProWRKY45*:*GUS* and *ProPHT1;1*:*GUS*, the *WRKY45* and *PHT1;1* promoter fragments were amplified by PCR using the primers listed in Supplemental Table S1 and cloned into pCAMBIA1381 vector. All constructs were introduced into an *Agrobacterium tumefaciens* strain (GV3101) and transformed into plants via floral dip transformation (Clough and Bent, 1998).

qRT PCR

qRT PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) following the manufacturer's protocol. Three real-time PCR reactions were repeated independently using *Actin2/8* gene as an internal control. The primers used are listed in Supplemental Table S1.

Subcellular Localization Assay

For the subcellular localization assay, *WRKY45* fused to *GFP* and *GFP* alone were cloned into pSuper1300:GFP vector, resulting in *WRKY45*:*GFP* and *GFP* constructs. The plasmids were then transformed into *A. tumefaciens* (GV3101). The transient expression assays were conducted as described previously (Chen et al., 2009). GFP fluorescence in the transformed leaves was imaged using a confocal laser scanning microscope (LSM510, Carl Zeiss).

ChIP Assay

The WRKY45-Myc transgenic line was generated first by cloning the coding sequence of WRKY45-Myc into pBI121 vector, resulting in the *35S*:*WRKY45-Myc* construct. Then, the *35S*:*WRKY45-Myc* plasmid was transformed into wild-type plants by the floral dip method (Clough and Bent, 1998). The WRKY45-Myc transgenic line used in the ChIP assay was a single-copy homozygous line.

The 7-d-old WRKY45-Myc seedlings were transferred to LP medium for 10 d, and then the roots were harvested for ChIP experiments with anti-Myc, which were conducted as described previously (Saleh et al., 2008; Chen et al., 2009). The primer combinations amplifying fragments within the *PHT1;1* promoter are listed in Supplemental Table S1.

EMSA Assay

The EMSA was conducted using LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's protocol. The recombinant WRKY45-CKS protein was purified from *Escherichia coli*. The fragments of the *PHT1;1* promoter were obtained by PCR using biotin-labeled or -unlabeled primers (Supplemental Table S1). Biotin-unlabeled fragments of the same sequences were used as competitors.

Transient Expression Assays in *Nicotiana benthamiana*

The transient GUS expression assays were performed as described elsewhere (Chen et al., 2009). The constructs (*ProWRKY45*:*GUS* and *ProWRKY75*:*GUS*) alone and in various combinations were transformed into *A. tumefaciens* strain GV3101. The infiltrated plants were kept under a 14-h-light/10-h-dark photoperiod at 23°C for 72 h to express GUS and Luciferase (LUC) proteins. The GUS and LUC activities of the infiltrated leaves were quantitatively determined, and the GUS/LUC ratio was used to quantify the promoter activity.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AtWRKY45 (At3g01970), PHT1;1 (At5g43350), PHT1;4 (At2g38940), PHR1 (At4g28610), WRKY75 (At5g13080), and ZAT6 (At5g04340).

Supplemental Data

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

Supplemental Figure S1. Arsenate tolerance phenotype of *WRKY45*-overexpressing lines, *WRKY45* RNAi lines, and wild-type seedlings.

Supplemental Figure S2. RNA gel-blot analysis of *PHT1;1* expression in the roots of the *WRKY45*-overexpressing lines, *WRKY45* RNAi line, and wild-type plants.

Supplemental Figure S3. Arsenate tolerance phenotype of *PHT1;1*-overexpressing line and wild-type seedlings.

Supplemental Figure S4. Arsenate tolerance phenotype of *35S*:*WRKY45-18*, *pht1;1* mutant, *35S*:*WRKY45-18*::*pht1;1*, and wild-type seedlings.

Supplemental Table S1. Primer sequences used in this study.

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