Arabidopsis WRKY45 Transcription Factor Activates PHOSPHATE TRANSPORTER1;1 Expression in Response to Phosphate Starvation

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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 electrophoretic 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 phi1;1 (35S:WRKY45:18:phi1;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 (H2PO4− 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;1ΔAΔ 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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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; Mao et al., 2001; Zhang and Wang, 2005). The WRKY domain contains the conserved amino acid sequence motif WRKYGQK, followed by a Cys2His2 or Cys2HisCys 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; Mao 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.

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
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 32P orthophosphate, and Pi accumulation 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 (#).
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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 plants grown under medium without arsenate (Supplemental Fig. S1). 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), 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 electrophoretic mobility shift assay (EMSA) was also performed. WRKY45 was expressed in Escherichia coli as a fusion protein with CTP-CMP-3-deoxy-D-manno-octulosonate 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.

Overexpression of PHT1;1 Enhances Arabidopsis Pi Uptake

The PHT1;1-overexpressing lines (SuperPHT1;1) were also generated, and the plt1;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 plt1;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 plt1;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 plt1;1 mutant to produce 35S:WRKY45::plt1;1. F2 progeny were genotyped for the presence of both 35S:WRKY45 and...
pht1;1 mutation, 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 (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 promoter.
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 (Richter 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 pht1phl1 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 phl1-1 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 KH2PO4), and the agar was replaced by agarose (Fomera) to avoid phosphorous contamination.

For arsenate treatment, the sterilized seeds were plated on MS medium with 10 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, phr1-1 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 SuperPHT1;1, WRKY45 and PHT1;1 complementary DNA fragments were ampliﬁed by PCR using gene-speciﬁc primers (Supplemental Table S1) and cloned into pH121 or pCAMBIA1300 vector. To construct the WRKY45 RNAi line, the WRKY45 fragment was

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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 (Crocker et al., 2009). 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-C175 protein was purified from Escherichia coli. The fragments of the PHT1:1 promoter that 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 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 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.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AtWRKY45 (At3g04340), HPT1:1 (At5g43350), PHT1:4 (At2g39840), PHT1:5 (At5g31380), and ZAT6 (At5g04340).

Supplemental Data

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

Supplemental Figure S1. Arsenite tolerance phenotype of WRKY45-overexpressing lines, WRKY45 RNAl 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 RNAl line, and wild-type plants.
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