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The WRKY6 Transcription Factor Modulates *PHOSPHATE1* Expression in Response to Low Pi Stress in *Arabidopsis* [W] [OA]

Yi-Fang Chen,[1] Li-Qin Li,[1] Qian Xu,[1] You-Han Kong, Hui Wang, and Wei-Hua Wu[2]

State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, National Plant Gene Research Centre (Beijing), Beijing 100193, China

Arabidopsis thaliana **WRKY family comprises 74 members and some of them are involved in plant responses to biotic and abiotic stresses. This study demonstrated that WRKY6 is involved in *Arabidopsis* responses to low-Pi stress through regulating *PHOSPHATE1* (*PHO1*) expression. *WRKY6* overexpression lines, similar to the *pho1* mutant, were more sensitive to low Pi stress and had lower Pi contents in shoots compared with wild-type seedlings and the *wrky6-1* mutant. Immunoprecipitation assays demonstrated that WRKY6 can bind to two W-boxes of the *PHO1* promoter. RNA gel blot and β-glucuronidase activity assays showed that *PHO1* expression was repressed in *WRKY6*-overexpressing lines and enhanced in the *wrky6-1* mutant. Low Pi treatment reduced WRKY6 binding to the *PHO1* promoter, which indicates that *PHO1* regulation by WRKY6 is Pi dependent and that low Pi treatment may release inhibition of *PHO1* expression. Protein gel blot analysis showed that the decrease in WRKY6 protein induced by low Pi treatment was inhibited by a 26S proteosome inhibitor, MG132, suggesting that low Pi–induced release of *PHO1* repression may result from 26S proteosome–mediated proteolysis. In addition, WRKY42 also showed binding to W-boxes of the *PHO1* promoter and repressed *PHO1* expression. Our results demonstrate that WRKY6 and WRKY42 are involved in *Arabidopsis* responses to low Pi stress by regulation of *PHO1* expression.**

INTRODUCTION

Phosphorus (P), as a major essential nutrient for plant growth and development, serves various basic biological functions in the plant life cycle (Raghothama, 1999). Phosphate ($H_2PO_4^-$, or in short, Pi) is the major form that is most readily taken up and transported in the plant cell (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, which is one of major limiting factors for crop production in the cultivated soils. A number of studies have shown that plants have evolved different strategies to overcome limited Pi availability. In response to low Pi stress or Pi starvation, plants may increase the Pi uptake from the soil by alteration of root architecture and function (López-Bucio et al., 2003; Ticconi and Abel, 2004; Osmont et al., 2007). Under Pi-limiting conditions, plants may also increase their Pi acquisition by changing their metabolic and developmental processes (Raghothama and Karthikeyan, 2005), such as increasing phosphatase activity (Lipton et al., 1987) and secretion of organic acids (Marschner, 1995).

PHOSPHATE1 (PHO1) has been shown to play roles in Pi translocation from root to shoot (Hamburger et al., 2002), which is also important for plant adaptation to a low Pi environment. A single nuclear recessive mutation in *PHO1* led to its inability to load Pi into xylem (Poirier et al., 1991; Hamburger et al., 2002). *PHO1* is predominantly expressed in the stellar cells of the root and the lower part of the hypocotyls and is believed have a role in Pi efflux out of root stellar cells for xylem loading (Hamburger et al., 2002). However, PHO1 shares no homology with any previously described Pi transporter proteins in plants and fungi (Hamburger et al., 2002). It is interesting that PHO1 contains a SPX domain, which can be found in several proteins that are involved in phosphate transport and/or Pi signaling pathways in plants and yeast. For example, an SPX protein in yeast named PHO81 is a key regulator in transporting and sensing phosphate, as well as in sorting proteins to endomembranes (Lenburg and O'Shea, 1996; Wykoff and O'Shea, 2001). In *Arabidopsis*, the SPX proteins SPX1-SPX3 are involved in Pi signaling pathways and regulate the expression of the Pi transporter genes *Pht1;4* and *Pht1;5* (Duan et al., 2008). Thus, the possibility cannot be excluded that PHO1 may not be a direct Pi transporter but rather may regulate Pi loading of the xylem either by directly influencing the activity of transporter proteins or via signal transduction.

PHO1 gene expression can be induced by Pi starvation (Stefanovic et al., 2007; Ribot et al., 2008; also see Figure 5B in this study), but the transcription factors that regulate *PHO1* expression remain unknown. Transcriptome analysis has demonstrated that expression of many genes is significantly changed in *Oryza sativa* (Wasaki et al., 2003) and *Arabidopsis thaliana* (Wu et al., 2003; Misson et al., 2005) under Pi-limiting conditions, indicating that transcriptional regulation may play important roles in plant responses to low Pi stress. More recently, a number of regulatory components that may be involved in plant responses to low Pi stress have been reported, such as microRNA miR339 (Bari et al., 2006; Chiou et al., 2006), *Arabidopsis* posttranslation regulators PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (At PHF1) (González et al., 2005) and E3 SUMO Ligase (At SIZ1) (Miura et al., 2005), transcription factors PHOSPHATE

[1] These authors contributed equally to this work.

[2] Address correspondence to wuwh@public3.bta.net.cn.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Wei-Hua Wu (wuwh@public3.bta.net.cn).

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STARVATION RESPONSE1 (At PHR1) (Rubio et al., 2001) and *O. sativa* Pi STARVATION-INDUCED TRANSCRIPTION FACTOR1 (Os PTF1) (Yi et al., 2005), and *Arabidopsis* MYB62 transcription factor (At MYB62) (Devaiah et al., 2009) and *Arabidopsis* WRKY75 transcription factor (At WRKY75) (Devaiah et al., 2007a).

WRKY proteins are plant-specific transcription factors encoded by a multigene family comprising 74 members in *Arabidopsis* (http://www.Arabidopsis.org/browse/genefamily/WRKY-Som.jsp), and many of them have been found to play important roles in plant responses to biotic and abiotic stresses. In addition to a number of *WRKY* genes that have been demonstrated to be involved in plant responses to pathogen infection and other defense-related stimuli (Dong et al., 2003; Kalde et al., 2003; Li et al., 2004; Eulgem and Somssich, 2007), some *WRKY* genes have also been shown to function in plant responses to various abiotic stress, such as drought (Pnueli et al., 2002; Rizhsky et al., 2002; Seki et al., 2002; Mare, et al., 2004), cold (Huang and Duman, 2002; Seki et al., 2002; Mare, et al., 2004), heat (Rizhsky et al., 2002), salinity (Seki et al., 2002), wounding (Hara et al., 2000), and Pi starvation (Devaiah et al., 2007a). However, little is known about the specific interaction of a given WRKY protein with a defined target gene. Recent studies using an oligodeoxynucleotide decoy strategy have revealed that SUSIBA, a WRKY protein, can bind to SURE (sugar responsive) and W-box elements in the *iso1* promoter (Sun et al., 2003). *Petroselinum crispum* WRKY1 has been shown to bind to the W-box of its native promoter as well as to that of Pc *WRKY3* and Pc *PR1-1* based on chromatin immunoprecipitation (ChIP) analysis (Turck et al., 2004). Candidate target genes for At WRKY53 were identified with a pull-down assay (Miao et al., 2004), and electrophoretic mobility shift assays identified candidate targets for *Hordeum vulgare* WRKY transcription factor WRKY38 (Zou et al., 2008), *Arabidopsis* WRKY26 and WRKY11 (Ciolkowski et al., 2008), *Nicotiana tabacum* WRKY1, WRKY2, and WRKY4 (Yamamoto et al., 2004), and *O. sativa* WRKY71 (Zhang et al., 2004).

At WRKY6 was first reported to be associated with senescence- and defense-related processes, and it could activate the expression of its target gene *SIRK*, a receptor-like protein kinase in the process of senescence (Robatzek and Somssich, 2002). Here, we report a previously unknown function of WRKY6 in plant responses to low Pi stress. We demonstrate that plants overexpressing *WRKY6* become more sensitive to low Pi stress and display a similar phenotype as the *pho1* mutant. WRKY6 negatively regulates *PHO1* expression by binding to two W-box consensus motifs within the *PHO1* promoter, and the repression of *PHO1* expression by WRKY6 is released under low Pi conditions.

RESULTS

WRKY6 Overexpression Plants Showed Similar Phenotypes as the *pho1* Mutant under Low Pi Conditions

The growth of the aerial portion of the *Arabidopsis WRKY6*-overexpressing line (*35S:WRKY6-9*) was impaired when the plants were grown in a potting soil mixture (Figure 1A). However, the overall growth of the *WRKY6* null mutant *wrky6-1*, an *En-1* insertion mutant, was obviously better than wild-type plants (Figure 1A). The measured free Pi concentration in the potting soils was ~10 μM in this study. Thus, the plants grown under the described conditions as shown in Figure 1A were actually experienced low Pi stress. Under this low Pi stress condition, the *35S:WRKY6-9* line displayed thinner stalks and smaller leaves compared with wild-type plants (Figure 1A). Figure 1B shows that the *En-1* insertion in *wrky6-1* disrupted the transcription of the *WRKY6* gene.

Besides the *35S:WRKY6-9* line, two more *WRKY6*-overexpressing lines, *Super:WRKY6-13* and *Super:WRKY6-18*, were included in our further experiments. The elevated expression of *WRKY6* mRNA in these transgenic lines is shown in Figure 1C. Transcription of *WRKY6* in either wild-type or the *wrky6-1* plants was not detectable in our RNA gel blot experiments. Among the three *WRKY6*-overexpressing lines, *35S:WRKY6-9*, *Super:WRKY6-18*, and *Super:WRKY6-13* displayed the highest, medium, and the lowest *WRKY6* expression, respectively.

Plants usually accumulate more anthocyanin in their aerial portions in response to low Pi stress (Marschner, 1995), and this can result in brown-colored leaves. When grown on Murashige and Skoog (MS) medium with sufficient Pi supply, all tested plants showed no difference in their phenotypes (top panel in Figure 1D). After the low Pi treatment, the *WRKY6*-overexpressing plants, particularly *Super:WRKY6-18* and *35S:WRKY6-9* lines (both have much higher *WRKY6* expression than does the *Super:WRKY6-13* line), displayed dark-brown leaves similar to the phenotype of the *pho1* mutant (bottom panel in Figure 1D). To confirm further the effects of *WRKY6* overexpression on the low Pi response phenotype, another group of *WRKY6*-overexpressing lines (*35S:WRKY6-3*, *35S:WRKY6-5*, and *35S:WRKY6-9*; Robatzek and Somssich, 2002) were tested. As shown in Supplemental Figure 1 online, overexpression of *WRKY6* indeed increased plant sensitivity to low Pi stress. Furthermore, increase of transgenic plant sensitivity to low Pi stress was closely related to *WRKY6* expression level (see Supplemental Figure 1 online).

A defect in Pi transfer from root to shoot has been reported in the *pho1* mutant (Poirier et al., 1991; Hamburger et al., 2002), resulting in reduced Pi content in the shoot and smaller plant size. Under either Pi-sufficient or Pi-deficient conditions, the *WRKY6*-overexpressing lines showed similar reduced Pi contents in shoots as the *pho1* mutant (Figures 2A and 2B). As a result, the ratios of Pi content in shoot to that in root (Pi_{shoot}/Pi_{root}) for both *WRKY6*-overexpressing lines and the *pho1* mutant were significantly lower than the ratio determined in wild-type plants, particularly under low Pi condition (Figures 2C and 2D). In addition, four *Super:PHO1* lines (*Super:PHO1-1*, *-7*, *-9*, and *-13*) with differential *PHO1* expression were selected for the Pi content assay. As shown in Supplemental Figure 2 online, all *Super:PHO1* lines and the *wrky6-1* mutant displayed higher Pi contents in shoots, whereas the *pho1* mutant showed the lowest Pi content in shoots under both MS and low Pi (LP) conditions. The results demonstrate that Pi content in shoots indeed correlates with *PHO1* expression. These data suggest that WRKY6 may play a role in plant responses to Pi starvation at least partially through regulating PHO1-dependent Pi transfer.

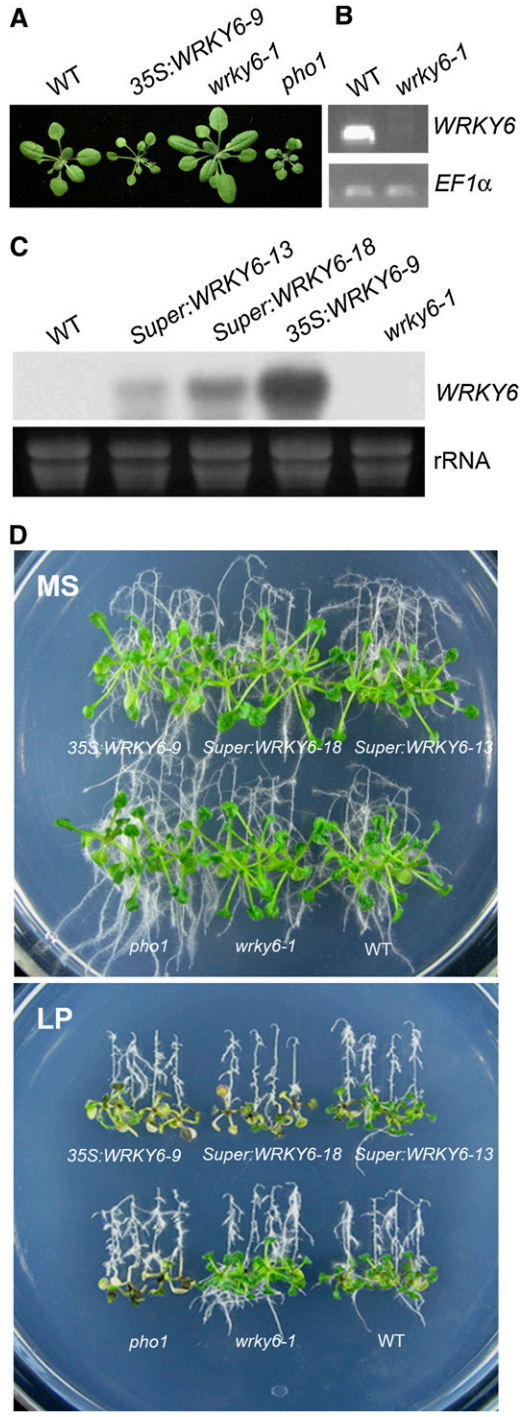

Figure 1. Phenotype Tests of Various Plant Materials.

(A) Phenotype comparison of the *WRKY6*-overexpressing line (*35S:WRKY6-9*), the *WRKY6 En-1* insertion mutant (*wrky6-1*), the *pho1* mutant, and wild-type (Columbia-0 [Col-0]) plants. All plants were grown in a potting soil mixture (rich soil:vermiculite = 2:1, v/v) and kept in growth chambers at 22°C with illumination at 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for an 18-h daily light period for 30 d.

(B) RT-PCR test of *WRKY6* expression in the *wrky6*-1 mutant and wild-type seedlings. Seven-day-old seedlings were used for RNA extraction. *EF1α* was amplified for the control.

(C) RNA gel blot analysis of *WRKY6* expression in the *WRKY6*-overexpressing lines (*Super:WRKY6-13*, *Super:WRKY6-18*, and *35S:WRKY6-9*) and the *wrky6*-1 mutant. Seven-day-old seedlings were used for RNAs extracted. The ethidium bromide–stained rRNA band was shown for the loading controls.

(D) Phenotype comparison of the various plant lines as indicated. The 7-d-old seedlings germinated on MS medium were transferred to MS (top panel) or LP (bottom panel) medium for another 7 d.

It should be noted that the *wrky6-1* mutants were obviously growing better than wild-type plants under the low Pi condition (Figures 1A and 1D, bottom panel) in our experiments, although they showed no difference under Pi-sufficient conditions (Figure 1D, top panel).

However, Robatzek and Somssich (2001, 2002) had not observed phenotype difference between the *wrky6-1* mutants and wild-type plants. After we have grown the plants under different environmental conditions, we believe that this difference mainly resulted from growth conditions, particularly light period. In the studies by Robatzek and Somssich (2001, 2002), plants were grown first under short-day conditions followed by long-day periods, while plants were grown under a constant long-day (18 h light) condition in our experiments. When *Arabidopsis* plants were grown under a short-day condition (10-h light), almost no phenotype difference between wild-type and *wrky6-1* plants was observed (as shown in Supplemental Figure 3 online).

WRKY6 Interacts with Two W-Box Motifs of the *PHO1* Promoter

To test the hypothesis that WRKY6 regulates *PHO1* expression, we first tested whether WRKY6 could bind the *PHO1* promoter. It is known that WRKY proteins usually bind to the W-box motifs of their target gene promoters (Eulgem et al., 2000). Analysis of the primary sequence of the *PHO1* promoter revealed six W-box consensus motifs within the *PHO1* promoter and four of them (named W_Q, W_X, W_Y, and W_Z, respectively) are located at the very end of promoter nearing the coding region (Figure 3A). The in vivo interaction between WRKY6 and the W-box motifs of the *PHO1* promoter was investigated using the ChIP-qPCR (chromatin immunoprecipitation quantitative PCR) method. As shown in Figure 3B, WRKY6 strongly interacted with the *PHO1* promoter when the primer combinations encompassing either W_Y or W_Z were applied, while no interaction was observed between WRKY6 and *PHO1* promoter containing only W_Q or W_X box. These results demonstrated that WRKY6, as a transcription factor, can bind to two (W_Y and W_Z) W-box motifs within the *PHO1* promoter nearing the coding region, suggesting regulation of *PHO1* transcription by WRKY6.

WRKY6 Negatively Regulates *PHO1* Transcription

Based on the results of the phenotype tests (Figure 1), Pi content measurements (Figure 2), and ChIP analysis (Figure 3B), we further hypothesized that WRKY6 may negatively regulate *PHO1*

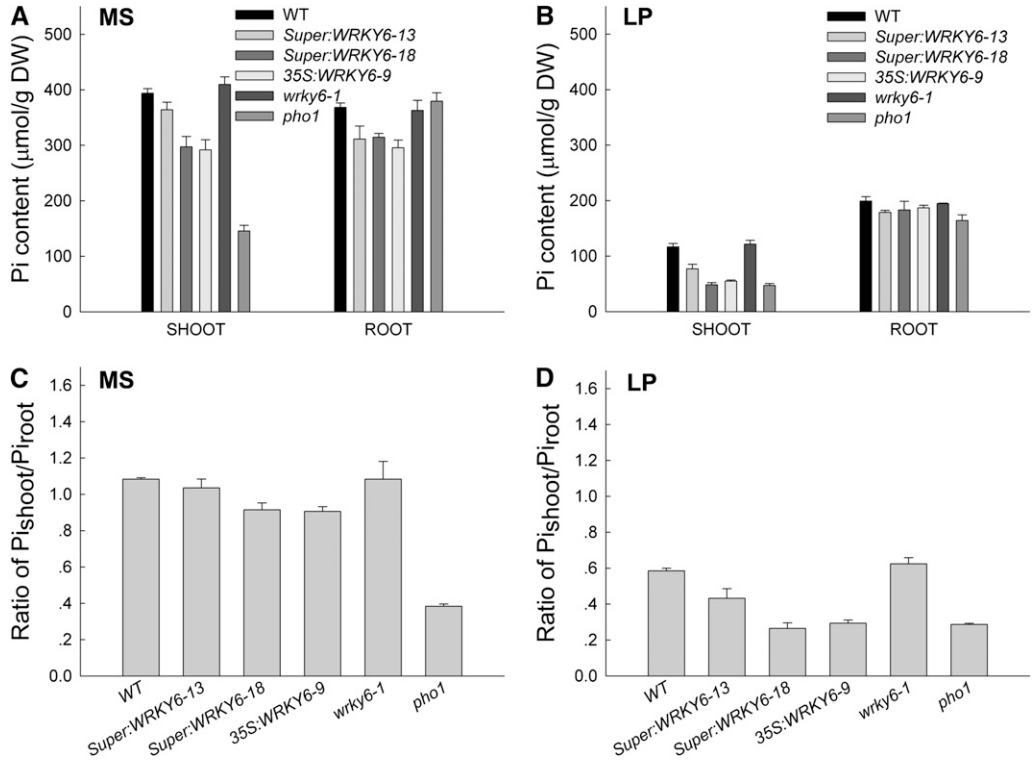

Figure 2. Pi Content Measurements in Various Plant Materials.

The 7-d-old seedlings of *WRKY6*-overexpressing lines (*Super:WRKY6-13*, *Super:WRKY6-18*, and *35S:WRKY6-9*), the *wrky6*-1 mutant, the *pho1* mutant, and wild-type plants germinated on MS medium were transferred to MS (**[A]** and **[C]**) or LP (**[B]** and **[D]**) medium for another 7 d, and then the shoots and roots of the seedlings were harvested separately for Pi content measurements.

(A) and **(B)** Pi contents in roots and shoots of tested plant materials. Three replicates were included for each treatment, and experiments were repeated three times. Data are shown as means ± SE (n = 3).

(C) and **(D)** Comparison of the ratio of Pi_{shoot} to Pi_{root}. The ratio was calculated from the data presented in **(A)** and **(B)**. Data are shown as means ± SE (n = 3).

transcription. To test this hypothesis, we first compared the transcription of *PHO1* in the roots of *WRKY6*-overexpressing lines, *wrky6-1*, and wild-type plants, since both *WRKY6* and *PHO1* are highly expressed in roots (Robatzek and Somssich, 2001; Hamburger et al., 2002). As shown in Figure 4A, the transcription of *PHO1* in roots was repressed in the *WRKY6*-overexpressing lines. Repression of *PHO1* expression was also closely related to the *WRKY6* expression levels in *WRKY6*-overexpressing lines, with the strongest repression in *35S:WRKY6-9* plants and the weakest repression in *Super:WRKY6-13* plants.

We further tested if WRKY6 binding to *PHO1* W-box motifs was required for its function in regulation of *PHO1* transcription. Different truncated *PHO1* promoter fragments (indicated above each panel of Figures 4B to 4D) driving the β-glucuronidase (GUS) reporter gene were transformed into *35S:WRKY6-9*, *wrky6-1*, and wild-type plants. In wild-type plants, the GUS reporter gene was expressed when driven by *PHO1* promoter fragment containing all four W-box motifs (W_Q, W_X, W_Y, and W_Z), two W-box motifs (W_Y and W_Z), or no W-box motifs, respectively (Figures 4B to 4D). When all four W-box motifs were deleted from the *PHO1* promoter, the expression of the reporter gene in *35S:WRKY6-9* and wild-type roots was dramatically increased, indicating the removal of negative regulation (Figures 4B to 4E). On the other hand, strong GUS expression was detected in *wrky6-1* roots regardless of which promoter fragment was used (Figures 4B to 4E). The GUS expression level was much higher in *wrky6-1* roots than in wild-type roots when the reporter gene was driven by promoters containing W_Y and W_Z (Figures 4B, 4C, and 4E). However, there was almost no difference in the GUS staining among the roots of all three different types of plants when no W-box motif existed (Figures 4D and 4E). More importantly, in the roots of *35:WRKY6-9* plants expressing the GUS reporter gene driven by W_Y- and W_Z-containing promoter fragments, only weak GUS staining can be detected (Figures 4B, 4C, and 4E). The results demonstrate that binding to *PHO1* W-box motifs was required for WRKY6 regulation of *PHO1* transcription. All these data support the notion that WRKY6 is the negative regulator of *PHO1* transcription.

Repression of *PHO1* Transcription by WRKY6 Is Removed under Low Pi Stress

Consistent with previous reports (Stefanovic et al., 2007; Ribot et al., 2008), we observed that *PHO1* transcription was induced in response to low Pi stress. The *PHO1* transcription level in

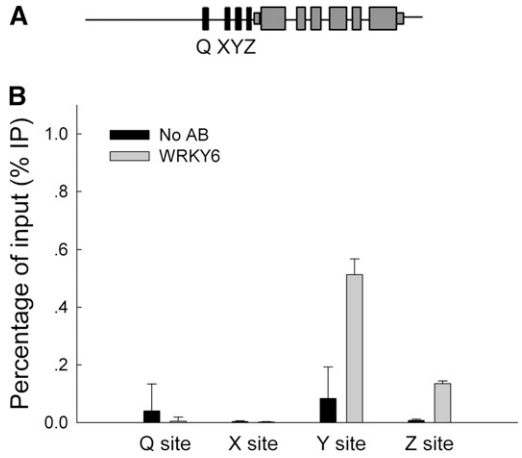

Figure 3. ChIP Assays for At WRKY6 Binding to the W-Box of the *PHO1* Promoter in Vivo.

(A) Diagram of the *PHO1* promoter region showing the relative positions of four of six W-boxes (Q, −1718 to −1625; X, −1269 to −1181; Y, −966 to −936; and Z, −775 to −618). W-boxes are marked by black rectangles, and the untranslated region and exons of *PHO1* are marked by gray boxes.

(B) ChIP-qPCR analysis of the *PHO1* promoter sequence. ChIP assays were performed with chromatin prepared from wild-type *Arabidopsis* roots. The gray and black bars represent the ChIP signals with (WRKY6) and without (NoAB) addition of anti-WRKY6 serum, respectively. The experiments were repeated three times, and three replicates were included for each sample in each experiment. The data are presented as means ± SE (n = 3).

wild-type roots was increased after the plants had been transferred to the low Pi medium for 3 d (Figure 5B; Ribot et al., 2008). It was further proposed that the low Pi stress might trigger the plant responses through suppression of *WRKY6* expression. However, as shown in Figure 5A, after wild-type plants were challenged with low Pi stress, *WRKY6* expression level was increased during the first 3 h and then decreased, but stayed above its zero time expression level for ~48 h. Another hypothesis we proposed was that the Pi starvation inhibits WRKY6 functioning in suppression of *PHO1* expression, such as through a possible blockage of WRKY6 binding to W-box motifs of the *PHO1* promoter or a low Pi–induced WRKY6 protein degradation. ChIP-qPCR experiments were conducted to test whether WRKY6 protein still can bind to W_Y and W_Z boxes of the *PHO1* promoter under the low Pi condition. As shown in Figure 5C, the interaction between the WRKY6 protein and W_Y or W_Z box of the *PHO1* promoter was severely impaired under the low Pi condition. To confirm further the interaction of WRKY6 with the W_Y and W_Z boxes of the *PHO1* promoter, ChIP-qPCR experiments were performed using the wild type, *wrky6-1* mutant, and three *WRKY6*-overexpressing lines. As shown in Figure 6, the strong interaction of WRKY6 with W_Y or W_Z boxes of *PHO1* promoter was displayed again in all three *WRKY6*-overexpressing lines under the normal conditions (on MS medium), while this interaction was reduced under the low Pi condition (on LP medium).

It was further proposed that low Pi stress may induce degradation of WRKY6 protein so that repression of *PHO1* transcription could be weakened. Protein gel blot analysis was performed using anti-WRKY6 serum in the total proteins extracted from the roots of seedlings grown on the low Pi medium. As shown in Figure 7A, the low Pi treatment induced a time-dependent decrease of WRKY6 protein content. Yeast two-hybrid assays (see Supplemental Table 1 online) showed that WRKY6 interacted with a RING-type finger E3 ligase (At1g74410), indicating that WRKY6 protein degradation may be mediated by the 26S proteosome. Addition of 10 μM MG132, a 26S proteosome inhibitor (Lee et al., 2009), blocked the low Pi–induced decrease of WRKY6 protein (Figures 7B and 7C), suggesting that a 26S proteasome–mediated WRKY6 proteolysis is involved in WRKY6-regulated *PHO1* expression in response to low Pi stress.

WRKY42 Interacts with the *PHO1* Promoter and Negatively Regulates *PHO1* Transcription

To identify other proteins that interact with WRKY6, we performed yeast two-hybrid experiments using WRKY6 as bait in fusion with the Gal4 DNA binding domain. As listed in Supplemental Table 1 online, there are at least a dozen proteins that interact with WRKY6. Among these WRKY6 interacting proteins, WRKY42, as the closest homolog of WRKY6 (Eulgem et al., 2000), may have similar function to WRKY6. To test this hypothesis, we tested possible binding of WRKY42 with the *PHO1* promoter. The ChIP-qPCR experiments showed that, similar to WRKY6, WRKY42 can bind to both the Y and Z W-box motifs within the *PHO1* promoter but not to the Q and X W-box motifs (Figure 8A). To further test possible function of WRKY42 on regulation of *PHO1* expression, transient expression experiments in tobacco leaves were performed. The results showed that, similar to WRKY6, WRKY42 inhibited *PHO1* promoter activity (Figure 8B). The coinjection of *Super:WRKY6* and *Super:WRKY42* showed much stronger repression on ProPHO1:GUS expression than did injection of either *Super:WRKY6* or *Super:WRKY42* alone (Figure 8B). However, WRKY75 had no effect on *PHO1* expression and did not influence the inhibition of *PHO1* expression by WRKY6 (Figure 8B).

Taking all these results together, we concluded that WRKY6 functions in plant responses to low Pi stress by negatively regulating *PHO1* expression. Under normal conditions with sufficient Pi supply, WRKY6 (and probably also WRKY42) can bind to the W-box motifs W_Y and W_Z within the *PHO1* promoter and represses the transcription of *PHO1*. Under Pi-deficient conditions, WRKY6 protein content is decreased via a 26S proteosome–mediated proteolysis, and the interaction of WRKY6 and the *PHO1* is limited. As a result, repression of *PHO1* transcription by WRKY6 is relieved, which might be important for plant adaptation to a Pi-deficient environment.

DISCUSSION

Plant-specific WRKY transcription factor family proteins have been implicated in the regulation of genes involved in plant responses to biotic as well as abiotic stresses, such as pathogen-induced stress (Dong et al., 2003; Eulgem and Somssich, 2007), drought, cold, and salinity stresses (Seki et al., 2002; Dong

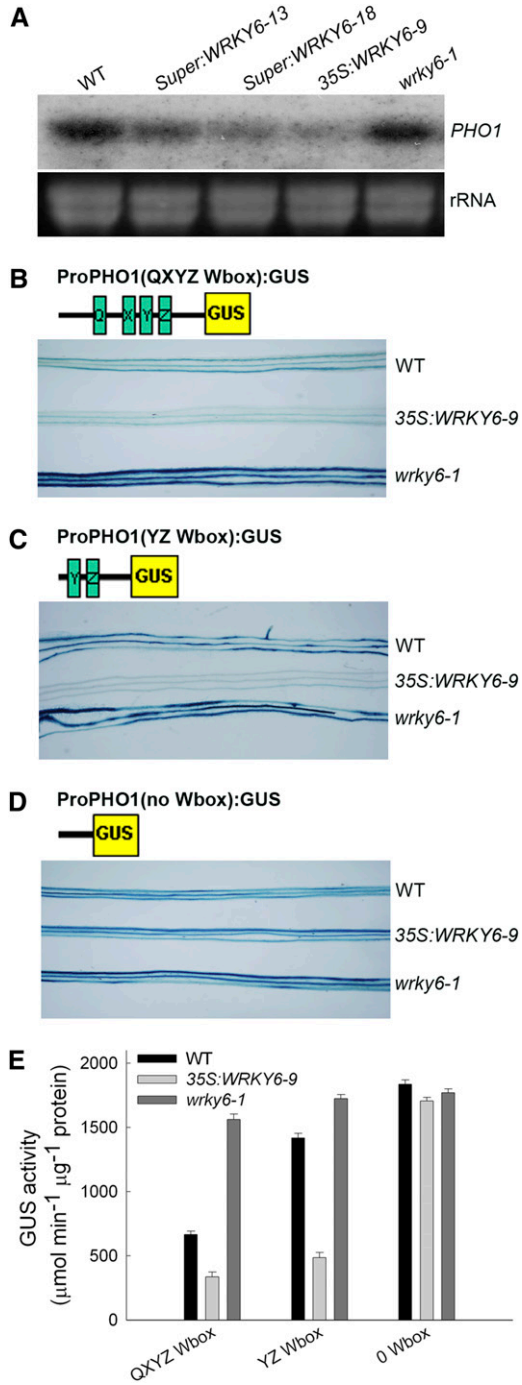

Figure 4. Suppression of *PHO1* Expression by WRKY6.

(A) RNA gel blot analysis of *PHO1* expression in the roots of the *WRKY6*-overexpressing lines (*Super:WRKY6-13*, *Super:WRKY6-18*, and *35S:WRKY6-9*), the *wrky6-1* mutants, and wild-type plants. rRNA is shown as a loading control.

(B) to **(D)** GUS staining showing expression patterns of *PHO1* in transgenic plants carrying distinct *PHO1* promoter constructs (indicated above each panel; green boxes show W boxes, and yellow box represents the *GUS* gene) in *35S:WRKY6-9*, *wrky6-1* mutant, or wild-type backgrounds. The three roots in each group are representatives from three independent transgenic lines for each background. All *PHO1* promoter–driven GUS transgenic lines are homozygous lines, and each line contains a single copy of insertion.

(E) Relative GUS activities in different transgenic plants.

et al., 2003). WRKY factors act primarily by binding to conserved W-box elements in the promoters of specific targets to direct temporal and spatial expression of these genes (Ulker and Somssich, 2004). Among 74 members in the *Arabidopsis* WRKY family, only WRKY75 has been reported to be involved in modulation of Pi acquisition and root development (Devaiah et al., 2007a). This study demonstrated that WRKY6 (and probably also WRKY42) plays an important role in modulation of plant responses to low Pi stress via regulation of *PHO1* expression.

WRKY6 Is a Negative Regulator for *PHO1* Transcription

Plant responses to Pi starvation involve the transcriptional regulation of numerous genes to establish an adaptive mechanism (Franco-Zorilla et al., 2004). Several *Arabidopsis* transcription factors were identified functioning in the Pi starvation response, such as PHR1 (Rubio et al., 2001), ZAT6 (Devaiah et al., 2007b), BHLH32 (Chen et al., 2007), MYB62 (Devaiah et al., 2009), and a WRKY family protein WRKY75 (Devaiah et al., 2007a). *WRKY75* can be induced by low Pi stress and is believed act as a positive regulator of Pi acquisition under Pi-deficient conditions (Devaiah et al., 2007a).

In this study, we first observed that the *WRKY6* overexpression lines displayed similar phenotypes as the *pho1* mutant under low Pi stress, including growth inhibition and anthocyanin accumulation. We further demonstrated that WRKY6 protein can bind to two W-boxes of the *PHO1* promoter and that *PHO1* transcription was repressed by overexpression of *WRKY6* under normal Pi supply conditions. This repression of *PHO1* transcription by WRKY6 was relieved under low Pi conditions, indicating that the regulation of *PHO1* transcription by WRKY6 is Pi dependent. In addition, protein blot analysis showed that the low Pi treatment–induced WRKY6 decrease was inhibited by a 26S proteosome inhibitor MG132. This suggests that the low Pi–induced release of *PHO1* repression may result from 26S proteosome–mediated WRKY6 proteolysis. Such a Pi-dependent mechanism may make WRKY6 a key regulator for plant responses to low Pi stress.

Mechanism of *PHO1* Regulation by WRKY6 and WRKY42

Under normal growth conditions, WRKY6 represses *PHO1* expression to balance Pi homeostasis through its binding to two W-boxes at the end of the coding region of the *PHO1* promoter. When a low Pi stress signal is sensed by an unknown signaling mechanism and relayed to the E3 ubiquitin ligase, a 26S proteosome–mediated WRKY6 protein degradation is activated and WRKY6 binding to the *PHO1* promoter W box motifs is weakened so that *PHO1* transcription is induced to cope with the Pi-deficient environment. As a result, PHO1-facilitated Pi loading from root to xylem occurs and translocation of Pi from root to shoot could be promoted so that plants can adapt to a Pi-deficient environment. The plant may use WRKY6 (and probably

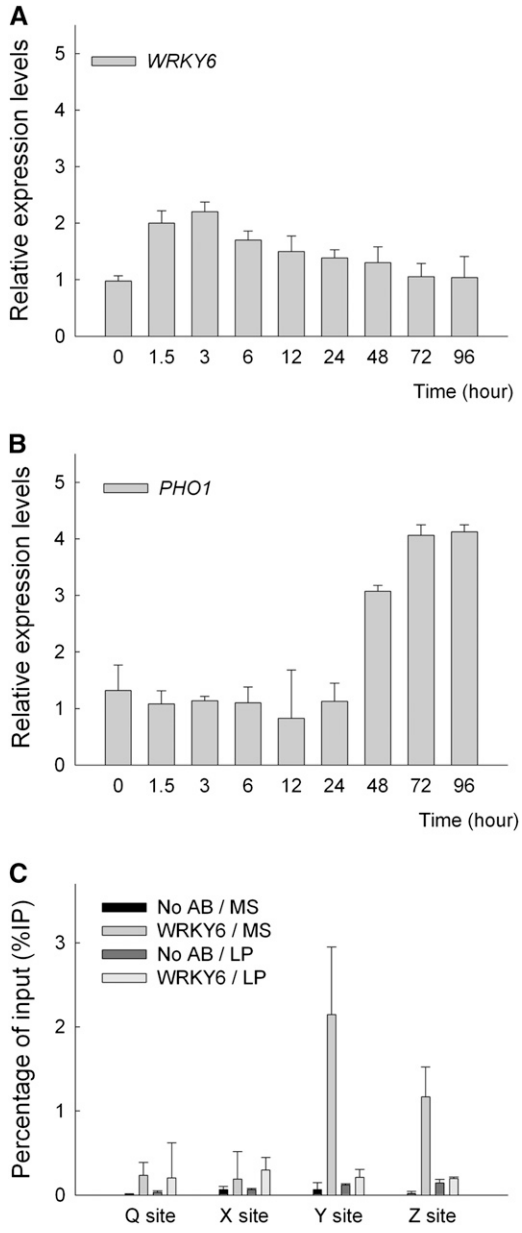

Figure 5. Repression of *PHO1* Expression by WRKY6 Was Released in Response to Low Pi Stress.

(A) qPCR analysis of *WRKY6* expression induced by Pi starvation.
(B) qPCR analysis of *PHO1* expression induced by Pi starvation.
(C) ChIP-qPCR assays to detect the association between WRKY6 and W-boxes within the *PHO1* promoter in wild-type plants under the normal (MS) and LP conditions. The ChIP signals with (WRKY6) and without (NoAB) addition of anti-WRKY6 serum are indicated. The data are presented as means ± SE ($n = 3$). The experiments were repeated three times, and three replicates were included for each sample in each experiment.

also WRKY42) as a key regulator that responds to varied Pi supply conditions and regulates Pi distribution in different organs via regulation of *PHO1* as well as other unknown components.

Our results also showed that, similar to WRKY6, WRKY42 can bind to both the Y and Z W-box motifs of the *PHO1* promoter but not to the Q and X W-box motifs (Figure 8A). In addition, WRKY42 alone can also inhibit *PHO1* expression (Figure 8B). Alternatively, considering that WRKY6 can interact with WRKY42 (see Supplemental Table 1 online), one may wonder if these two factors can form heterocomplexes to regulate *PHO1* expression. Xu et al. (2006b) reported that three different kinds of WRKY proteins (WRKY18, WRKY40, and WRKY60) can interact with each other and form heterocomplexes, and the interactions between these WRKY factors influence their DNA binding activities. Robatzek and Somssich (2002) showed that WRKY6 can act as a negative regulator of its own and *WRKY42* expression even though the mechanism and function remains unknown.

To test possible synergic effects of WRKY6 and WRKY42 on *PHO1* expression, we coinjected *Super:WRKY6* and *Super:WRKY42* in tobacco leaves to test their effects on ProPHO1:GUS expression. The results (Figure 8B) indicate that WRKY6 and WRKY42 together had stronger repression on *PHO1* expression. However, still we cannot conclude that they work together (by forming heterodimers) or work independently at this point. The ChIP-qPCR data showed that WRKY6 and WRKY42 had differential interactions with Y and Z W-boxes within the *PHO1* promoter. WRKY6 displayed a stronger interaction with the Y box than with the Z-box (Figures 3B and 5C), while WRKY42 displayed a stronger interaction with the Z-box than with the Y-box (Figure 8A). These results indicate that WRKY6 and WRKY42 may regulate *PHO1* expression in different ways. To clarify further if WRKY6 and WRKY42 work independently or together as a complex in regulation of *PHO1* expression is an important issue for comprehensively understanding complex mechanisms of *PHO1* regulation by WRKY factors.

Regulation of *PHO1* by Other Possible Regulatory Factors

Although the Q and X W-boxes within the *PHO1* promoter do not bind to WRKY6, we have noticed that the deletion of the sequences containing the Q- and X-boxes reduced the inhibitory effect of WRKY6 on *PHO1* expression (Figures 4B, 4C, and 4E), particularly for wild-type plants. One of possible explanation for this phenomenon is that, within the deleted sequences, some other regulatory elements related to plant responses to low Pi stress may exist. The analysis of the deleted sequences using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) shows that, in addition to Q and X W-boxes, there are a number of regulatory elements for the following transcription factors: GATA factors, CCAAT-box transcription factor, multiprotein bridging factor 1 (MBF1), homeodomain-leucine zipper (HD-Zip) transcription factor, MYB transcription factor, Dof (DNA binding with one finger) factor, heat shock transcriptional factor (HSF), etc. Although there is no report so far regarding *PHO1* regulation by these TFs, at least two of them have been reported involving plant responses to low Pi stress. The HD-Zip factor has been reported to bind to the phosphate response domain of the soybean (*Glycine max*) *VspB* tripartite promoter (Tang et al.,

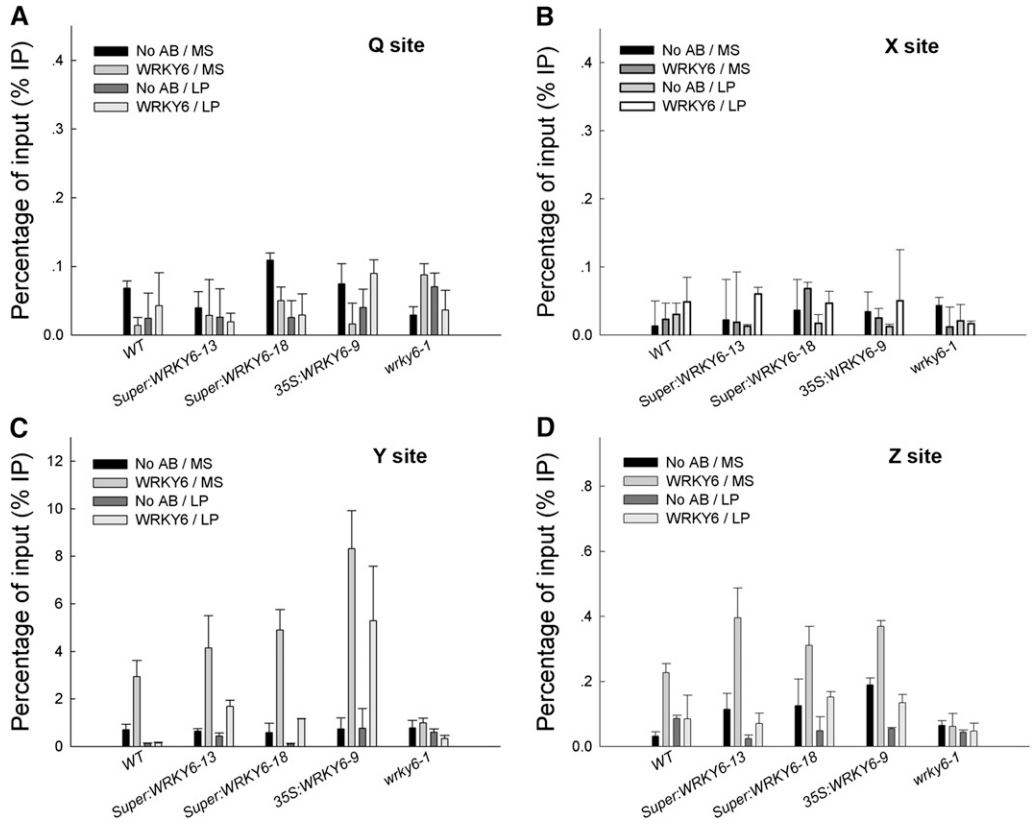

Figure 6. ChIP-qPCR Assays to Detect the Association of WRKY6 and the *PHO1* Promoter in the Tested Plants as Indicated under Pi-Sufficient (MS) and Pi-Deficient (LP) Conditions.

ChIP assays were performed with chromatin prepared from tested plants roots to analyze the binding of At WRKY6 protein to the W_Q-box (**[A]**; Q site), W_X-box (**[B]**; X site), W_Y-box (**[C]**; Y site), and W_Z-box (**[D]**; Z site) of the *PHO1* promoter in vivo. The ChIP signals with (WRKY6) and without (NoAB) addition of anti-WRKY6 serum are indicated. The experiments were repeated three times, and three replicates were included for each sample in one experiment. The data are presented as means ± SE (n = 3).

2001). Nilsson et al. (2007) reported that increased expression of the MYB-related transcription factor PHR1 resulted in enhancement in phosphate uptake in *Arabidopsis*. It is plausible to further hypothesize that one or more of these regulatory elements (which were deleted together with Q and X W-boxes in the experiments shown in Figure 4C) may directly or indirectly be involved in *PHO1* regulation.

WRKY6 and WRKY75 May Respond to Low Pi Stress via Different Pathways

WRKY75 has been reported to play an important role in the phosphate starvation response, particularly by modulating Pi uptake and root development (Devaiah et al., 2007a). The results presented here demonstrate that WRKY6 responds to low Pi stress by regulation of *PHO1* transcription. Repression of WRKY75 expression (by RNA interference methods) resulted in a decrease of Pi uptake (Devaiah et al., 2007a), while overexpression of WRKY6 repressed *PHO1* expression and consequently reduced Pi accumulation in shoots. It is known that PHO1 functions in Pi translocation from root to shoot (Poirier et al., 1991; Hamburger et al., 2002). Considering these results together with the fact that WRKY75 did not have an effect on *PHO1* promoter activity (Figure 8B; in addition, WRKY75 did not interact with WRKY6 in the yeast two-hybrid assay), we may further hypothesize that WRKY75 and WRKY6, in response to low Pi stress, function in different regulatory pathways. Identifying the gene(s) whose expression is specifically regulated by WRKY75 as well as other possible transcription factors will help us to clarify the complex mechanisms of plant responses to low Pi stress.

Other Possible Roles of WRKY in Regulation of Pi Starvation Responsive Genes

To test if WRKY6 would play roles in regulation of other Pi starvation responsive genes, we performed comparative transcriptome analyses with various plant materials (*35S:WRKY6-9*, *wrky6-1*, and the wild type) using the Affymetrix GeneChip. As shown in Supplemental Table 2 online, among 30 low Pi response genes (Devaiah et al., 2007a; Lin et al., 2009), 11 of them showed expression changes between either wild-type and *35S:*

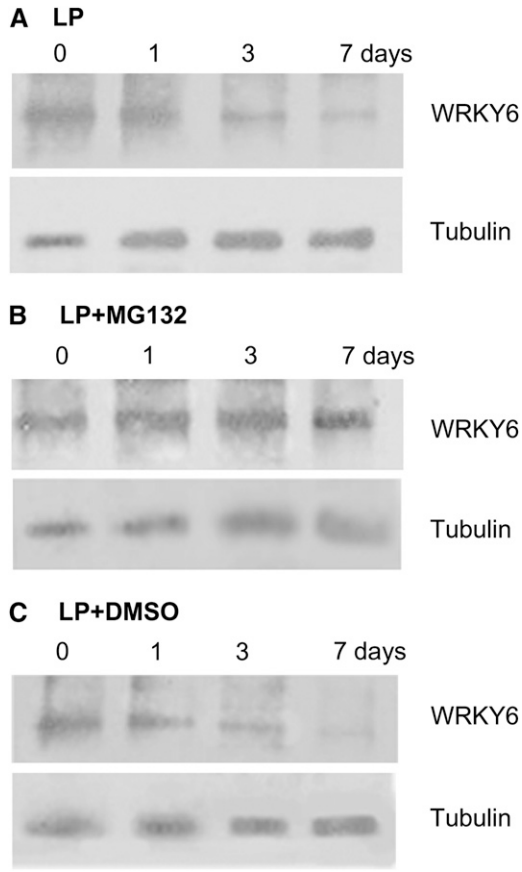

Figure 7. WRKY6 Protein Blot Analysis.

Seven-day-old wild-type seedlings were transferred to LP medium **(A)**, LP medium with 10 μM MG132 (LP+MG132) **(B)**, or LP medium with DMSO (LP+DMSO) **(C)**. The roots of seedlings were harvested for protein extraction at the indicated time. Protein extracts were analyzed by immunoblots using rabbit anti-WRKY6 serum. Tubulin levels were detected in parallel as a loading control with antitubulin antibody.

WRKY6-9 plants or the wild type and the *wrky6-1* mutant. Among the members of the *PHT1* family, *Pht1;5* and *Pht1;8* displayed transcriptional changes in *35S:WRKY6-9* plants and the *wrky6-1* mutant compared with wild-type plants (see Supplemental Table 2 online). Both *Pht1;5* and *Pht1;8* contain W-boxes in their promoters (Devaiah et al., 2007), suggesting that WRKY6 may regulate their transcription. It is known that expression of *Pht1;8* was significantly increased in the *pho2* mutant (Aung et al., 2006; Bari et al., 2006), a mutant overaccumulating Pi in leaves, suggesting a possible role of Pht1;8 in WRKY6- and PHO1-related Pi mobilization. *PS2* and *PS3*, two members of a phosphatase family, were significantly upregulated in *35S:WRKY6-9* plants and downregulated in the *wrky6-1* mutant (see Supplemental Table 2 online), suggesting that WRKY6 also might be involved in plant early responses to low Pi stress (Devaiah et al., 2007). Several low Pi responsive transcription factors listed in Supplemental Table 2 online, including PHR1, ZAT6, WRKY75, and BHLH32, did not show significant changes in their transcription either in *35S:WRKY6-9* plants or in the *wrky6-1* mutant.

In addition, among the genes whose expression was upregulated or downregulated by more than two times and showed relevant changes (changes in opposite direction in 35S:*WRKY6-9* plants compared with the *wrky6-1* mutant), there are a total of 25 genes (listed in Supplemental Table 3 online) whose promoters contain W-box(es). Among these genes, there are 15 genes whose transcriptions were repressed, and transcription of 10 other genes was enhanced in *35S:WRKY6-9* plants. These data indicate that, in addition to its function in plant responses to low Pi stress, WRKY6 may be involved in a broad range of transcriptional regulations related to different processes, such as senescence, pathogen defense, and wounding responses (Robatzek and Somssich, 2001, 2002).

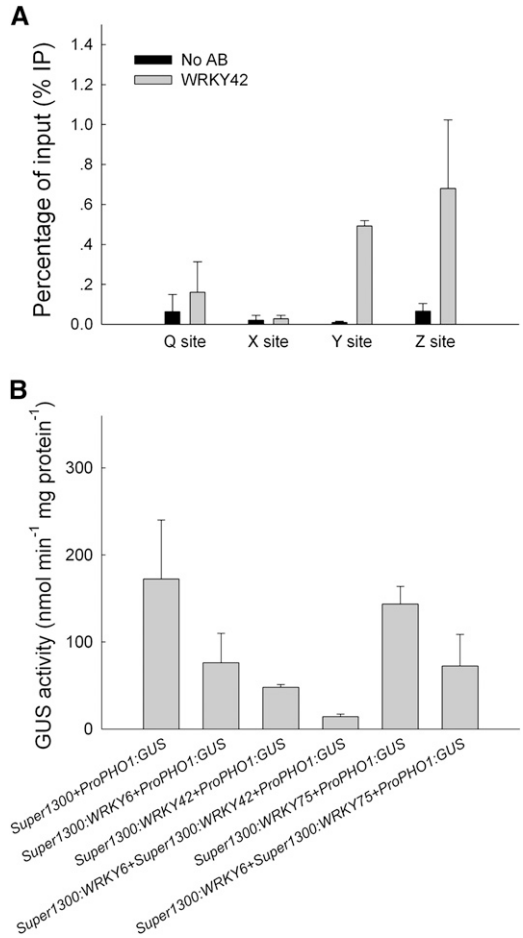

Figure 8. Suppression of *PHO1* Expression by WRKY42.

(A) ChIP-qPCR assays to detect the association between WRKY42 and W-boxes within the *PHO1* promoter in wild-type plants under normal conditions. The experiments were repeated three times, and three replicates were included for each sample in each experiment. The data are presented as means ± SE (n = 3).

(B) Transient overexpression of the ProPHO1:GUS fusion together with *Super:WRKY6*, *Super:WRKY42*, or *Super:WRKY75* in *Nicotiana benthamiana* leaves. ProPHO1:GUS fusion together with Super1300 vector was taken as the control. The data are presented as means ± SE (n = 4).

METHODS

Plant Materials and Growth Conditions

The *WRKY6* overexpression lines *35S:WRKY6-3*, *35S:WRKY6-5*, *35S:WRKY6-9*, and the *WRKY6* knockout mutant *wrky6-1* were kindly provided by Imre E. Somssich (Max-Planck-Institut, Germany; Robatzek and Somssich, 2002). The *Super:WRKY6-13* and *Super:WRKY6-18* lines were generated by cloning the coding sequence of *WRKY6* into Super1300 vector (Li et al. 2001). The *pho1* mutant was ordered from the ABRC (http://www.Arabidopsis.org/abrc/).

For phenotype tests and seed harvest, *Arabidopsis thaliana* plants were grown in a potting soil mixture (rich soils:vermiculite = 2:1, v/v) and kept in growth chambers at 22°C with illumination at 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for an 18-h daily light period. The relative humidity was ~70% (±5%).

Low Pi stress treatment of plants was conducted by growing seedlings on Petri dishes containing Pi-sufficient (MS) or Pi-deficient (low Pi or LP) medium. The seeds were surface sterilized with the mixed solutions of NaClO (0.5%) and Triton X-100 (0.01%) for 10 min followed by washing with sterilized distilled water four times. The sterilized seeds were first incubated on Petri dishes containing MS agar (0.8%) medium (containing 1.25 mM KH_2PO_4 and 3% sucrose) at 4°C for 2 d before germination. The seeds were germinated at 22°C under constant illumination at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the 7-d-old seedlings were transferred to LP medium. The LP medium was made by modification of MS medium such that the Pi (supplied with KH_2PO_4) concentration in LP medium was 10 μM, and agar was replaced by agarose (Promega) to avoid the contamination of phosphorous.

Quantification of Total Pi

Seven-day-old *Arabidopsis* seedlings germinated on MS medium were transferred to MS or LP medium for another 7 d, and then the roots and shoots were harvested for Pi content measurements. The samples were oven dried at 80°C for 48 h before determination of dry weight, and the samples were ashed in a muffle furnace at 300°C for 1 h and 575°C for an additional 5 h and then dissolved in 0.1 N HCl. The total Pi content in the samples was quantified as described previously (Ames, 1966).

RNA Gel Blot, RT-PCR, and Real-Time PCR Analysis

For RNA gel blot analysis, 7-d-old *Arabidopsis* seedlings germinated on MS medium were transferred to MS or LP medium for another 7 d and then the seedlings or roots were harvested for extraction of total RNA using the Trizol reagent (Invitrogen). Thirty micrograms of RNA were loaded per lane and transferred to a nylon membrane for hybridization. Gene-specific probes were amplified by PCR using *WRKY6*-specific primers (the forward primer 5′-CTTTGGCGATGTCTAGAATTGA-3′ and the reverse primer 5′-CCTCACCTACTGCTCTCGTAGG-3′) and *PHO1*-specific primers (the forward primer 5′-TACTTGATTCTTTCTTACCCTACTTCTGG-3′ and the reverse primer 5′-TCCAAGGAACGGTAACGGTACGGTCTTCACT-3′) as the templates, respectively. The probes were labeled with [α-^{32}P]dCTP using random primer labeling reagents (Pharmacia) and hybridized to RNA gel blotted onto nylon membrane. The rRNA was taken as the control.

For RT-PCR analysis, total RNA was extracted with Trizol reagent (Invitrogen) and then treated with DNase I RNase Free (Takara) to eliminate genomic DNA contamination. The cDNA was synthesized from treated RNA by SuperScript II RNase H^- reverse transcriptase (Invitrogen) using oligo$(dT)_{15}$ primer (Promega). The PCR experiments were conducted with *WRKY6*-specific primers (the forward primer 5′-ATGTTTCGTTTTCCGGTAAGTCTTGGAGGA-3′ and the reverse primer 5′-TATTGCCTATTGTCAACGTTGCTCGTTGTAACATTA-3′) and *EF1*α-specific primers (the forward primer 5′-ATGCCCCAGGACATCGTGATTTCAT-3′ and the reverse primer 5′-TTGGCGGCACCCTTACGTGGATCA-3′). *EF1*α was used as a quantitative control.

For real-time PCR analysis, total RNA extraction was performed as described above, and the RNA was treated with DNase I RNase Free (Takara) to eliminate genomic DNA contamination. The cDNA was synthesized from total RNA by SuperScript II RNase H^- reverse transcriptase (Invitrogen) using Radom Hexamer Primer (Promega). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems; P/N 4368577) on a 7500 Real Time PCR System machine (Applied Biosystems) following the manufacturer's protocols. The PCR amplification was performed at 95°C for 15 s and 60°C for 1 min. Relative quantitative results were calculated by normalization to 18S rRNA. qPCR was conducted with *WRKY6*-specific primers (the forward primer 5′-TAGTCACGACGGGATGATGA-3′ and the reverse primer 5′-ATTAGGAGGCGGAGGTGAGT-3′) and *PHO1*-specific primers (the forward primer 5′-TGGTTCTCCGGAACAAGAAC-3′ and the reverse primer 5′-TGACTTCAAGTGACGCCAAG-3′).

Antibody Generation and ChIP-qPCR Assay

The whole coding sequences were amplified by PCR using *WRKY6*-specific primers (the forward primer 5′-cccgggCCCGGGATGGACAGAGGAGGTCT-3′ and the reverse primer 5′-ctcgagCTCGAGCTATTGATTTTTGTTGTTTC-3′) and *WRKY42*-specific primers (the forward primer 5′-ggatccGGATCCATGTTTCGTTTTCCGGTAAG-3′ and the reverse primer 5′-gagctcGTCGACTCTTATTGCCTATTGTCAAC-3′). The lowercase letters represent the restriction sites. The whole coding sequences of At *WRKY6* and *WRKY42* were cloned into pGEX-4T-2 (Pharmacia). The reconstructed pGEX-4T-2 plasmid containing *WRKY6* or *WRKY42* was then transformed into *Escherichia coli* strain BL21 (DE3) to express proteins by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 18°C for 8 h. The resulting glutathione *S*-transferase (GST) fusion proteins were purified using Glutathione Sepharose 4B (Pharmacia). Protein concentrations were determined using the Bio-Rad protein assay kit. The polyclonal antibodies against WRKY6 or WRKY42, generated by inoculation of rabbits, were purified using the Amino Link Plus Immobilization Kit (Pierce).

The 7-d-old seedlings germinated on MS medium were transferred to MS or LP medium for another 7 d and then the roots were harvested for ChIP experiments. The chromatin samples for ChIP experiments were obtained following the methods by Saleh et al. (2008). The roots of plants seedlings were first cross-linked by formaldehyde, and the purified cross-linked nuclei were then sonicated to shear the chromatin into suitably sized fragments. The antibody that specifically recognizes the recombinant WRKY6-GST or WRKY42-GST was used to immunoprecipitate DNA/protein complexes from the chromatin preparation. The DNA in the precipitated complexes was recovered and analyzed by qPCR methods. Following the methods described by Haring et al. (2007), qPCR analysis was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems; P/N 4368577) in a 20-μL qPCR reaction on the 7500 Real Time PCR System machine (Applied Biosystems) following the manufacturer's protocols. The chosen primer combinations (see Supplemental Table 4 online) can amplify fragments of 150 to 200 bp within the promoter of *PHO1*. To ensure the reliability of ChIP data, the input sample and no-antibody (NoAB) control sample were analyzed with each primer set. The results were quantified with a calibration line made with DNA isolated from cross-linked and sonicated chromatin.

Vector Construction and *Arabidopsis* Transformation and GUS Assays

PHO1 promoter variants, including the full-length promoter (2282 bp), the promoter containing two W-boxes (Y and Z, 1141 bp), and the promoter without W-box (454 bp), were amplified by PCR from *Arabidopsis*

genomic DNA and cloned into the transformation vector pCAMBIA1381 at the *Sal*I and *Pst*I restriction sites, respectively. All primer sequences used for vector constructions are listed in Supplemental Table 5 online. All tested *Arabidopsis* plants (Col-0, *35S:WRKY6-9*, and the *wrky6-1* mutant) were transformed by these three constructed vectors, respectively, using the *Agrobacterium tumefaciens*–mediated gene-transfer procedure (Clough and Bent, 1998). All transgenic lines used in this study are T3 homozygous plants with single copy insertion. The GUS staining and GUS activity measurements were performed as described previously (Xu et al., 2006a).

SDS-PAGE and Protein Gel Blot Analysis

For MG132 treatment, 10 mM stock solution was made by dissolving MG132 (Calbiochem) in DMSO, and the final MG132 concentration was 10 μM. For the control of MG132 treatment, 1/1000 DMSO was added to the medium. The 7-d-old seedlings germinated on MS medium were transferred to low Pi (LP), low Pi with 10 μM MG132 (Calbiochem) (LP+MG132), or low Pi with DMSO (LP+DMSO) medium, and then the roots were harvested at the indicated time for immunoblot analysis. Total proteins were extracted according to the method of Saleh et al. (2008), and 50 μg proteins of each sample were separated on a 10% SDS-PAGE and analyzed by protein gel blot according to Towbin et al. (1979). Mouse anti-tubulin (Sigma-Aldrich) (with 1:4000 dilution) and rabbit anti-WRKY6 serum (with 1:10,000 dilution) were used as the primary antibodies, and goat anti-mouse (or anti-rabbit) peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson) (with 1:8000 dilution) was used as the secondary antibody. The membranes were visualized using a Super-Signal West Femto Trial Kit (Themor) following the manufacturer's instructions.

Transient Expression Assays in *Nicotiana benthamiana*

The constructs of ProPHO1:GUS with Super1300, *Super1300:WRKY6*, or *Super1300:WRKY42* were transformed into *A. Agrobacterium* (GV3101). *Agrobacterium* cells were harvested by centrifugation and suspended in the solutions containing 10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 200 μM acetosyringone to an optical density (600 nm) of 0.7, incubated at room temperature for 4 h, and then used to infiltrate leaves of *N. benthamiana* using a needle-free syringe. The GUS activity of the infiltrated leaves was quantitatively determined.

Yeast Two-Hybrid Assay

The *WRKY6* full-length cDNA sequence was amplified with *WRKY6*-specific primers (the forward primer 5′-GCcatatgATGGACAGAG-GATGGTCTGG-3′ and the reverse primer 5′-GCgtcgacCTATT-GATTTTTGTTGTTTCCTTCG-3′) and cloned into pGBK-T7 vector at *Nde*I and *Sal*I sites. The lowercase letters in the primers indicate restriction sites. Two-hybrid screening and assays were performed as described (Kohalmi et al., 1997). Strain AH109 (BD Biosciences) of *Saccharomyces cerevisiae* was used in all yeast two-hybrid experiments.

Microarray Analysis

For microarray analysis, the seeds of *WRKY6* overexpression line 35S:*WRKY6-9*, *WRKY6* knockout mutant *wrky6-1*, and wild-type (Col-0) plants were germinated at 22°C under constant illumination at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and grown for 7 d on MS medium (control conditions), and the seedlings were used for total RNA extraction using the Trizol reagent (Invitrogen). Microarray experiments were conducted once, and three arrays for different plant materials were hybridized according to the Affymetrix GeneChip Expression Analysis Manual (http://www.affymetrix.com). The scanned arrays were analyzed first with Affymetrix GCOS 1.0 (MAS 5.0) software to generate detection calls and normalized using Affymetrix GCOS software, and the TCT value was set to 100. When analyzing the transcriptionally changed genes, the signal ratio between two plant materials was calculated to represent the fold change of this gene for its transcription in the corresponding process, and the fold change P value for each gene was obtained at the same time using GCOS software. A gene was considered to be transcriptionally changed when it was upregulated or downregulated more than onefold. The genes with P values < 0.05 and fold changes > 1 are included in Supplemental Table 3 online.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GeneBank/EMBL database under the following accession numbers: *WRKY6* (At1g62300), *WRKY42* (At4g04450), *WRKY75* (At5g13080), *PHO1* (At3g23430), and *EF1α* (At5g60390). Microarray data from this article have been deposited with the National Center for Biotechnology Information Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE18273.

Supplemental Data

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

Supplemental Figure 1. Phenotype Test of *WRKY6*-Overexpressing Lines.

Supplemental Figure 2. Pi Content Measurements in Various Plant Materials.

Supplemental Figure 3. Phenotype Comparison of the *wrky6-1* Mutant, *35S:WRKY6-9*, and Wild-Type Plants under a Short-Day (10-h-Light/14-h-Dark) Condition.

Supplemental Table 1. Yeast Two-Hybrid Results with At WRKY6 as the Bait.

Supplemental Table 2. Expression of Pi Starvation Responsive Genes in the *35S:WRKY6-9* Plant and the *wrky6-1* Mutant.

Supplemental Table 3. Genes with W-Boxes Whose Expression Was Up- or Downregulated More Than Twofold in the *35S:WRKY6-9* Line (OE) and the *wrky6-1* Mutant (KO) Compared with Wild-Type Plants.

Supplemental Table 4. Primers Used for ChIP-qPCR Experiments.

Supplemental Table 5. Primers Used for the ProPHO1:GUS Constructs.

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