WRKY42 Modulates Phosphate Homeostasis through Regulating Phosphate Translocation and Acquisition in Arabidopsis[1][OPEN]

Tong Su[2], Qian Xu[2], Fei-Cui Zhang[2], Yun Chen, Li-Qin Li, Wei-Hua Wu, and Yi-Fang Chen*

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

The Arabidopsis (*Arabidopsis thaliana*) WRKY transcription factor family has more than 70 members, and some of them have been reported to play important roles in plant response to biotic and abiotic stresses. This study shows that WRKY42 regulated phosphate homeostasis in Arabidopsis. The *WRKY42*-overexpressing lines, similar to the *phosphate1* (*pho1*) mutant, were more sensitive to low-inorganic phosphate (Pi) stress and had lower shoot Pi content compared with wild-type plants. The *PHO1* expression was repressed in *WRKY42*-overexpressing lines and enhanced in the *wrky42 wrky6* double mutant. The WRKY42 protein bound to the *PHO1* promoter under Pi-sufficient condition, and this binding was abrogated during Pi starvation. These data indicate that WRKY42 modulated Pi translocation by regulating *PHO1* expression. Furthermore, overexpression of *WRKY42* increased root Pi content and Pi uptake, whereas the *wrky42* mutant had lower root Pi content and Pi uptake rate compared with wild-type plants. Under Pi-sufficient condition, WRKY42 positively regulated *PHOSPHATE TRANSPORTER1;1* (*PHT1;1*) expression by binding to the *PHT1;1* promoter, and this binding was abolished by low-Pi stress. During Pi starvation, the WRKY42 protein was degraded through the 26S proteasome pathway. Our results showed that AtWRKY42 modulated Pi homeostasis by regulating the expression of *PHO1* and *PHT1;1* to adapt to environmental changes in Pi availability.

Phosphorus is an essential nutrient for plant growth (Raghothama, 1999) and the main component of fertilizers to sustain modern agriculture. Approximately 70% of global cultivated land suffers from phosphate deficiency (López-Arredondo et al., 2014). Maintenance of phosphate homeostasis in plants is important for plant growth and reproduction, and it is achieved mainly by coordination of acquisition of inorganic phosphate (Pi; orthophosphate) from the soil solution, translocation of Pi from roots to shoots, and remobilization of internal Pi (Poirier and Bucher, 2002).

Pi is the only form of phosphorus that can be absorbed in plants (Chiou and Lin, 2011; López-Arredondo et al., 2014). Plants take up Pi from soil solution through phosphate transporters (PHTs) encoded by members of the *PHT1* gene family. There are at least nine members (PHT1;1–PHT1;9) of the PHT1 family in Arabidopsis (*Arabidopsis thaliana*), and transcripts of *PHT1;1* are the most abundant among nine *PHT1* genes (Mudge et al., 2002). PHT1;1 and PHT1;4 play important roles in Pi uptake from soil. Under high-Pi condition, the *pht1;1* mutants' uptake rate was only 59% to 66% of the wild type, and the Pi uptake rates of *pht1;4* mutants increased slightly (Shin et al., 2004), indicating that PHT1;1 plays an important role in Pi uptake under Pi-sufficient condition. During Pi starvation, the expressions of *PHT1;1* and *PHT1;4* are significantly induced (Muchhal et al., 1996; Karthikeyan et al., 2002; Mudge et al., 2002; Shin et al., 2004), and overexpression of *PHT1;1* increases Pi uptake in Arabidopsis (Wang et al., 2014). Several transcription factors have been reported to regulate *PHT1;1* expression. Under Pi-deficient condition, the transcription of *PHT1;1* is positively regulated by PHOSPHATE STARVATION RESPONSE1 (PHR1; Rubio et al., 2001), WRKY75 (Devaiah et al., 2007), and WRKY45 (Wang et al., 2014) and negatively regulated by MYB DOMAIN PROTEIN62 (MYB62; Devaiah et al., 2009). However, under Pi-sufficient condition, the molecular mechanism for the regulation of *PHT1;1* expression is unknown. PHT1;1 is also regulated at posttranscription level. The PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1) protein is necessary for PHT1;1 plasma membrane localization, and mutation of *PHF1* impairs the localization of PHT1;1 at the plasma membrane (González et al., 2005). Additionally, E2 conjugase PHOSPHATE2 (PHO2) modulates PHT1;1 protein degradation (Huang et al., 2013), and NITROGEN LIMITATION ADAPTATION mediates PHT1;1 and PHT1;4 degradation to

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[2] These authors contributed equally to the article.

* Address correspondence to chenyifang@cau.edu.cn.

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maintain phosphate homeostasis (Lin et al., 2013; Park et al., 2014).

Another important pathway controlling Pi homeostasis involves PHO1, which plays an important role in Pi translocation from roots to shoots (Poirier et al., 1991; Hamburger et al., 2002; Wang et al., 2004). The *pho1* mutant is deficient in loading Pi acquired by roots to the xylem vessel and only accumulates 24% to 44% as much total phosphate as wild-type plants in shoots (Poirier et al., 1991). PHO1 is located primarily in the root stelar cells and has a role in Pi efflux out of root stelar cells for xylem loading (Hamburger et al., 2002). There are 11 members of the *PHO1* gene family in the Arabidopsis genome, and only *PHO1* and *PHO1;H1* can complement the *pho1* mutant (Wang et al., 2004), indicating that PHO1 and PHO1;H1 are involved in long-distance Pi transport from roots to shoots. The increased transcript level of *PHO1;H1* during Pi starvation is mainly controlled by the PHR1 transcription factor (Stefanovic et al., 2007), whereas expression of *PHO1* is independent of PHR1 regulation (Stefanovic et al., 2007). The *PHO1* expression is directly down-regulated by the WRKY6 transcription factor under Pi-sufficient condition (Chen et al., 2009). WRKY42, a homolog of WRKY6, could bind to the *PHO1* promoter in vivo and repressed the *PHO1* promoter activity in tobacco (*Nicotiana benthamiana*; Chen et al., 2009), indicating that WRKY42 also negatively regulates *PHO1* expression. PHO2 can modulate PHO1 degradation (Liu et al., 2012).

In this article, we report that Arabidopsis WRKY42 plays important roles in modulating Pi homeostasis in Arabidopsis. WRKY42 modulates Pi uptake and translocation by directly regulating *PHT1;1* and *PHO1* expression under Pi-sufficient condition. During Pi starvation, *WRKY42* expression is repressed, and the WRKY42 protein is degraded through a proteasome pathway; then, the binding of WRKY42 to the promoters of *PHO1* and *PHT1;1* is abolished.

RESULTS

WRKY42 Encodes a Phosphate Starvation-Responsive Transcription Factor

WRKY42 is a homolog of WRKY6 in Arabidopsis (Eulgem et al., 2000), and our previous results showed that WRKY6 regulated Pi translocation (Chen et al., 2009). We wonder whether WRKY42 plays a role in Arabidopsis responses to Pi starvation. The expression pattern of *WRKY42* was first tested. Quantitative real-time (qRT)-PCR analysis showed that *WRKY42* was mainly expressed in the roots (Fig. 1A). To further confirm the expression pattern of *WRKY42*, the homozygous single-copy *ProWRKY42*:*GUS* transgenic lines were generated. GUS staining was strong in roots (Fig. 1, B, a and c) and weak in leaves (Fig. 1, B, a and b). Then, the expression of *WRKY42* was tested under Pi starvation. The 7-d-old wild-type seedlings were transferred to Pi-sufficient (Murashige and Skoog [MS] medium) or Pi-deficient (low-phosphate [LP] medium with 10 μM Pi) condition for 3 d, and then, the roots were harvested for qRT-PCR analysis. Transcription of *WRKY42* was obviously suppressed under Pi-deficient condition (Fig. 1C), indicating that WRKY42 was involved in Arabidopsis responses to Pi starvation.

The WRKY42 protein, as a transcription factor, is likely to be localized to the nucleus. To detect this, the coding region of *WRKY42* was fused with the 3′-end of the *GFP* reporter gene and expressed under the control of Super promoter (Li et al., 2001). The *GFP* gene alone under control of the Super promoter served as a control. The subcellular localization of WRKY42 was tested in a transient expression system in tobacco leaves. The WRKY42-GFP fusion protein was exclusively localized in the nucleus, and GFP alone was localized in the cytoplasm and nucleus (Fig. 2A).

As a member of the WRKY transcription factor family, WRKY42 has a highly conserved WRKYGQK motif and a characteristic Cys_2His_2 zinc finger motif (Eulgem et al., 2000). Both the WRKYGQK and Cys_2His_2 motifs are necessary for the binding affinity of WRKY proteins to the consensus sequence (C/T) TGAC(C/T), known as W-box (Eulgem et al., 2000; Rushton et al., 2010). To test whether WRKY42 protein bound to the W-box, WRKY42 was expressed in *Escherichia coli* as a fusion protein with His-tag, and an electrophoresis mobility shift assay (EMSA) was conducted with WRKY42-His fusion protein and the synthesized probes with two normal or mutant W-boxes (Fig. 2B; Lai et al., 2011). The WRKY42-His fusion protein could bind to the probe (Pchn0) with two normal W-boxes, and the binding was abolished by addition of increasing amounts of unlabeled competitors (Fig. 2C). In contrast, the WRKY42-His fusion protein could not bind to the probe (mPchn0), which has two mutant W-boxes, and the His protein alone showed no detectable binding to the W-boxes.

WRKY42 Negatively Modulates Pi Translocation

To reveal the function of WRKY42, the T-DNA insertion mutant Salk_121674 was obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu) and named *wrky42*. The *wrky42* mutant (Salk_121674) carried a transferred DNA (T-DNA) insertion in the third exon of *WRKY42* (Fig. 3A), and reverse transcription (RT)-PCR analysis showed that *WRKY42* expression was abolished in the *wrky42* mutant (Fig. 3B). In addition, *WRKY42*-overexpressing lines were generated, and expression levels of *WRKY42* in these lines were much higher than in wild-type seedlings (Fig. 3C). Of three *WRKY42*-overexpressing lines, *Super*:*WRKY42-3*, *Super*:*WRKY42-40*, and *Super*:*WRKY42-5* displayed low, medium, and high *WRKY42* expression, respectively (Fig. 3C).

Anthocyanin accumulation is one of the most striking symptoms of Pi starvation in plants (Marschner, 1995). When grown in Pi-sufficient condition (MS

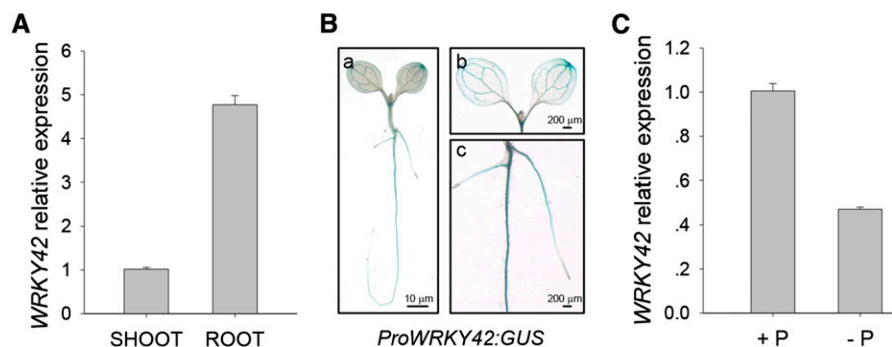

Figure 1. Expression pattern of Arabidopsis *WRKY42*. A, qRT-PCR analysis of *WRKY42* expression from shoots and roots of 10-d-old wild-type seedlings. Transcript level of *WRKY42* was quantified relative to *ACTIN2/8*. The data represent the mean values of three replicates ± SE. B, GUS staining of transgenic *ProWRKY42*:*GUS*. The *ProWRKY42*:*GUS* seedlings were germinated, grown on MS medium for 7 d, and then harvested for GUS staining (a). Details of the leaf and root of the *ProWRKY42*:*GUS* transgenic line are shown in b and c. C, qRT-PCR analysis of *WRKY42* expression in Arabidopsis under Pi starvation. Seven-day-old wild-type seedlings were transferred to Pi-sufficient condition (MS medium; +P) or Pi-deficient condition (LP medium with 10 μM Pi; −P) for 3 d; then, the roots were harvested for RNA extraction. Transcript level of *WRKY42* was quantified relative to *ACTIN2/8*. The data represent the mean values of three replicates ± SE.

medium with 1.25 mM Pi), all tested plants showed no obvious differences in phenotypes (Fig. 3D, top). When the 7-d-old seedlings were transferred to Pi-deficient condition (LP medium with 10 μM Pi) for 10 d, the *WRKY42*-overexpressing lines, particularly *Super*:*WRKY42-40* and *Super*:*WRKY42-5* (both had much higher *WRKY42* expression than *Super*:*WRKY42-3*), turned purple, similar to the *pho1* mutant, whereas the *wrky42* mutant and wild-type plants remained green (Fig. 3D, bottom). During Pi starvation, the anthocyanin contents in the *WRKY42*-overexpressing lines (*Super*:*WRKY42-40* and *Super*:*WRKY42-5*) and the *pho1* mutant were approximately 3-fold those in wild-type seedlings (Fig. 3E).

The *pho1* mutant has a defect in Pi transfer from roots to shoots, which results in reduced Pi content in shoots (Poirier et al., 1991; Hamburger et al., 2002). Therefore, a role for WRKY42 in translocating Pi was hypothesized. To test this, the shoot Pi was measured in 10-d-old *WRKY42*-overexpressing lines, *wrky42* mutant, *pho1* mutant, and wild-type seedlings grown under Pi-sufficient condition. The *WRKY42*-overexpressing lines had similarly reduced shoot Pi contents to the *pho1* mutant, and the reduced level of shoot Pi content was closely related to *WRKY42* expression (Fig. 4), indicating that WRKY42 negatively modulated Pi translocation in Arabidopsis.

WRKY42 Directly Down-Regulates *PHO1* Expression

Because *WRKY42*-overexpressing lines and the *pho1* mutant had similar low Pi-sensitive phenotypes and lower shoot Pi contents (Figs. 3 and 4), it was hypothesized that WRKY42 negatively regulated *PHO1* expression. The transcription level of *PHO1* gene was evaluated in the roots of *WRKY42*-overexpressing lines, the *wrky42* mutant, and wild-type plants, because *PHO1* is mainly expressed in roots (Hamburger et al., 2002). The transcription of *PHO1* was repressed in the *WRKY42*-overexpressing lines (Fig. 5A), and the repression level of *PHO1* expression was consistent with *WRKY42* expression in the *WRKY42*-overexpressing lines, with the strongest repression in *Super*:*WRKY42-5* and the weakest in *Super*:*WRKY42-3*.

Because WRKY42 is a typical WRKY transcription factor that can bind to W-box motif (Fig. 2C) and sequence analysis showed that there are several W-boxes within the *PHO1* promoter (Fig. 5B; Chen et al., 2009), a chromatin immunoprecipitation (ChIP) assay was used to determine whether WRKY42 could bind to the promoter of *PHO1* in vivo. The 7-d-old wild-type seedlings were transferred to Pi-sufficient (MS) or Pi-deficient (LP) medium for another 7 d, and the roots were harvested for ChIP assay. Chromatin immunoprecipitated with the anti-WRKY42 antibody was enriched in the *y* and *z* sites of the *PHO1* promoter (Fig. 5C) when wild-type seedlings were grown in Pi-sufficient condition (MS), consistent with a previous report (Chen et al., 2009). After Pi starvation treatment, the interactions between the WRKY42 and *y* or *z* sites of the *PHO1* promoter were severely impaired (Fig. 5C). These data show that WRKY42 directly down-regulated *PHO1* expression.

WRKY42 and WRKY6 Have Functional Redundancy in Down-Regulating *PHO1* Expression

Our previous work showed that WRKY6 negatively regulated *PHO1* expression, and the *PHO1* expression was repressed in the *WRKY6*-overexpressing lines and elevated in the *wrky6* mutant (Chen et al., 2009). We hypothesized that WRKY42 and WRKY6 had redundant functions in regulating *PHO1* expression. To test this hypothesis, the *wrky42 wrky6* double mutant was

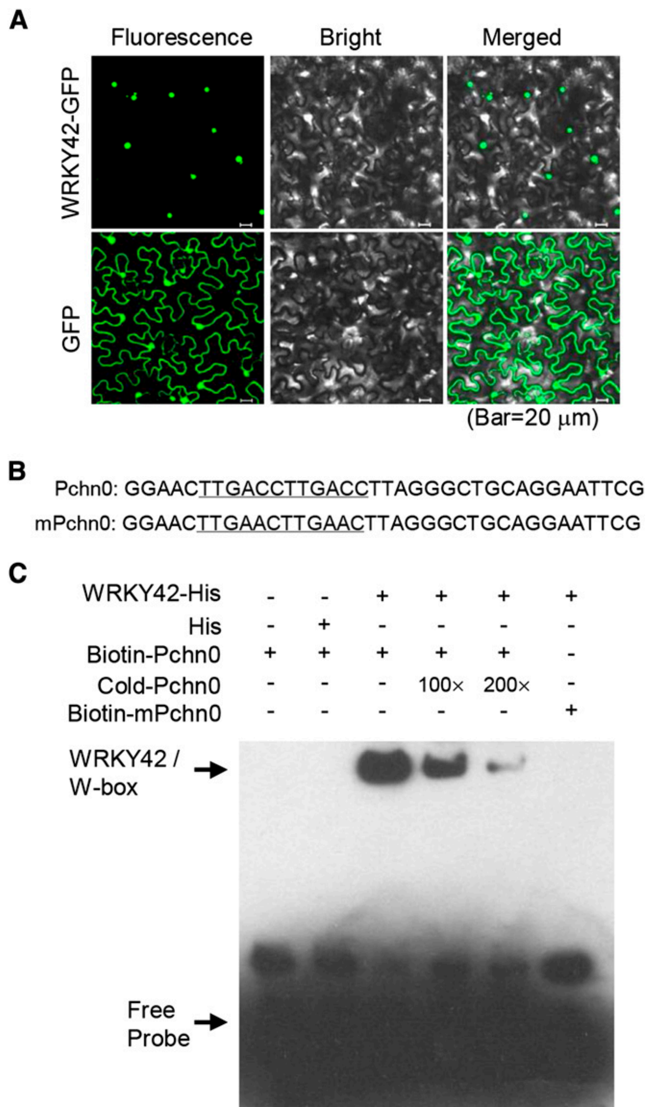

Figure 2. WRKY42 is localized in the nucleus and binds to W-box motifs. A, Subcellular localization of WRKY42-GFP fusion protein in tobacco leaves. The GFP alone was used as the control. B, Oligonucleotides used in the EMSA (C). The Pchn0 probe contains two W-box (TTGACC) sequences, and the mPchn0 probe has two mutated W-boxes (TTGAAC). The wild-type and mutated W-boxes are underlined. C, EMSA showing the binding of recombinant WRKY42 to W-box motif. The oligonucleotides (Pchn0 and mPchn0) were used as the probes. Each biotin-labeled DNA probe was incubated with recombinant WRKY42-His protein. An excess of unlabeled probe (Cold-Pchn0) was added to compete with labeled Pchn0 probe (Biotin-Pchn0). Biotin-labeled Pchn0 probe incubated with His protein served as the negative control.

generated (Fig. 6A) that, when grown in Pi-sufficient condition, had obviously higher *PHO1* expression than the *wrky42* mutant, *wrky6* mutant, and wild-type plants (Fig. 6B). Because overexpression of *PHO1* enhances the shoot Pi content (Liu et al., 2012), the shoot Pi content was measured in the *wrky42 wrky6* double mutant, *wrky42* mutant, *wrky6* mutant, and wild-type seedlings. The shoot Pi content of the *wrky42 wrky6* double mutant was higher than that in wild-type plants (Fig. 6C). These data indicate that WRKY42 and WRKY6 had redundant functions in regulating *PHO1* expression.

WRKY42 Positively Modulates Pi Uptake

In addition to shoot Pi content, the root Pi content was also measured among various plants. When grown in Pi-sufficient condition, the *WRKY42*-overexpressing lines *Super*:*WRKY42-40* and *Super*:*WRKY42-5* (both had higher *WRKY42* expression than *Super*:*WRKY42-3*) contained around 2- to 3.5-fold of the root Pi content of wild-type plants (Fig. 7A). In contrast, the *wrky42* mutant had a lower root Pi content than wild-type plants (Fig. 7A), suggesting that WRKY42 may regulate Pi uptake. The root Pi content of *pho1* mutant was also tested, and no obvious difference was found between *pho1* mutant and wild-type plants (Fig. 7A), indicating that the induced root Pi content in *WRKY42*-overexpressing lines was not because of the repression of *PHO1* caused by *WRKY42* overexpression.

The Pi uptake rate was measured to determine the effect of WRKY42 on Pi acquisition. The 10-d-old seedlings were transferred into a Pi uptake solution containing 500 μM Pi supplemented with ^{32}P orthophosphate, and Pi uptake was measured over a 4-h period. Consistent with the root Pi content, the *WRKY42*-overexpressing lines had a significantly ($P < 0.05$) higher Pi uptake rate compared with wild-type seedlings, and that of the *wrky42* mutant was lower than that of the wild type (Fig. 7B). Arsenate [As(V)] is an oxyanion structurally analogous to phosphate (Asher and Reay, 1979) and taken up mainly through Pi transporter PHT1;1 (Catarecha et al., 2007). When grown on medium containing As(V), the *pht1;1* mutant showed an As(V)-tolerant phenotype, and the *PHT1;1*-overexpressing line was more sensitive to As(V) than wild-type plants (Supplemental Fig. S1; Catarecha et al., 2007; Wang et al., 2014). To gain additional insight into the role of WRKY42 in Pi acquisition, the phenotypes of *WRKY42*-overexpressing lines, the *wrky42* mutant, and wild-type seedlings were tested with As(V). When grown on Pi-sufficient medium without As(V) [−As(V)], there were no obvious phenotypic differences among the *WRKY42*-overexpressing lines, the *wrky42* mutant, and wild-type seedlings (Fig. 7C; Supplemental Fig. S1). When grown on Pi-sufficient medium with 200 μM As(V) [+As(V)], although the toxic effect of As(V) was evident in the growth of *WRKY42*-overexpressing lines, the *wrky42* mutant, and wild-type plants, their degree of sensitivity varied. The *WRKY42*-overexpressing lines had a much more As(V)-sensitive phenotype, similar to the phenotype of *PHT1;1*-overexpressing lines, compared with wild-type seedlings (Fig. 7C; Supplemental Fig. S1). There were no obvious differences between the *wrky42* mutant and wild-type seedlings when grown on Pi-sufficient medium

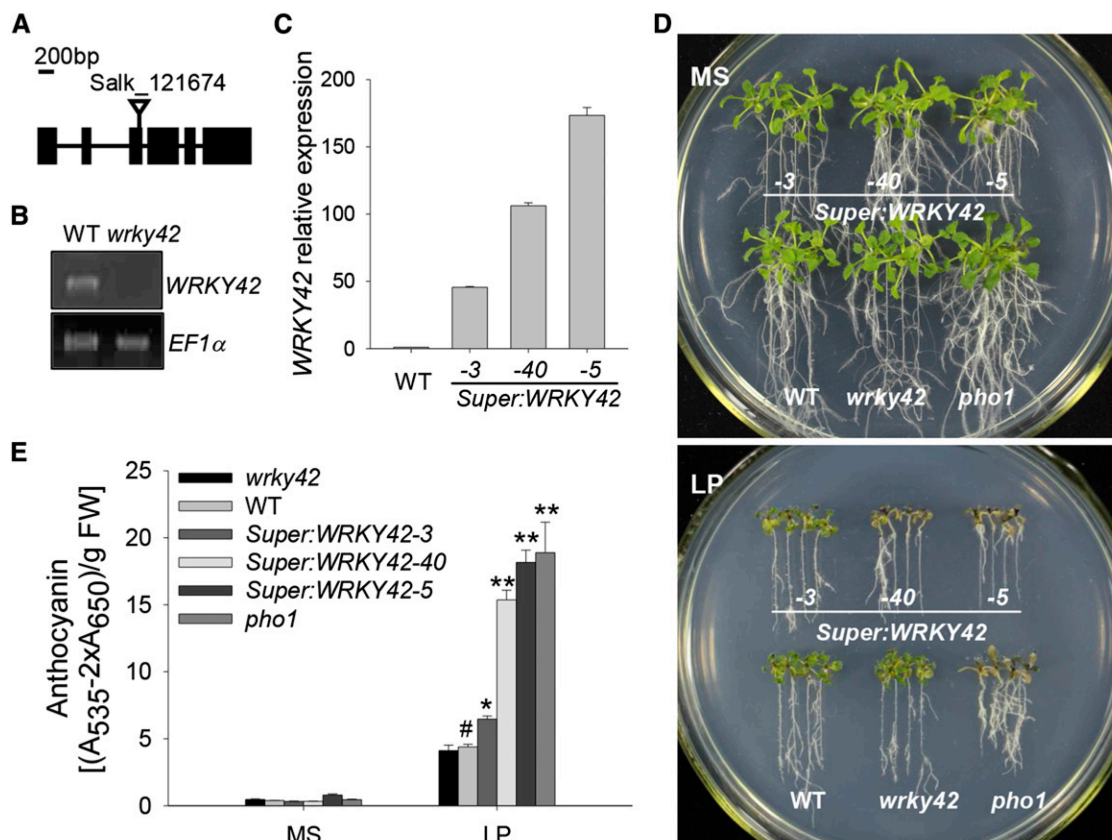

Figure 3. Phenotype tests of various plant materials. A, Diagram of the *WRKY42* gene showing the position of the T-DNA insertion. Exons (boxes), introns (lines), and the T-DNA insertion site of Salk_121674 (triangle) are indicated. B, RT-PCR analysis of *WRKY42* expression in the *wrky42* mutant (Salk_121674) and wild-type (WT) seedlings. The *EF1α* is amplified as the control. C, qRT-PCR analysis of *WRKY42* expression in the *WRKY42*-overexpressing lines (*Super*:*WRKY42-3*, *Super*:*WRKY42-40*, and *Super*:*WRKY42-5*) and wild-type plants. Transcript level of *WRKY42* was quantified relative to *ACTIN2/8*. The data represent the mean values of three replicates ± SE. D, Phenotype comparison of the *wrky42* mutant, *WRKY42*-overexpressing lines, the *pho1* mutant, and wild-type seedlings during Pi starvation. Seven-day-old seedlings were transferred to MS medium (Pi-sufficient medium with 1.25 mM Pi; top) or LP medium (low-Pi medium with 10 μM Pi; bottom) for another 10 d; then, photos were taken. E, Anthocyanin accumulation in the *wrky42* mutant, *WRKY42*-overexpressing lines, the *pho1* mutant, and wild-type seedlings during Pi starvation. Seven-day-old seedlings were transferred to MS or LP medium for another 10 d; then, the seedlings were harvested for anthocyanin content measurement. Data are shown as means ± SE (n = 3). Asterisks indicate significant differences compared with wild-type plants (paired test). FW, Fresh weight; *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control.

with 200 μM As(V) (Fig. 7C; Supplemental Fig. S1). Together, these data indicate that overexpression of *WRKY42* enhanced Arabidopsis Pi accumulation.

WRKY42 Directly Up-Regulates *PHT1;1* Expression

There are nine PHTs (PHT1;1–PHT1;9) in Arabidopsis (Mudge et al., 2002); of these, expression of *PHT1;1* is most highly expressed in roots when wild-type plants are grown in Pi-sufficient condition (Mudge et al., 2002), and overexpression of *PHT1;1* enhances Arabidopsis Pi uptake (Wang et al., 2014). Therefore, we examined expression of *PHT1;1* in roots of *WRKY42*-overexpressing lines, the *wrky42* mutant, and wild-type plants under Pi-sufficient condition. Transcription of *PHT1;1* was obviously elevated in the *WRKY42*-overexpressing lines (*Super*:*WRKY42-40* and *Super*:*WRKY42-5*) and repressed in the *wrky42* mutant compared with wild-type plants (Fig. 8A). The *PHT1;1* expression was also tested in the *pho1* mutant. The expression level of *PHT1;1* in the *pho1* mutant was similar to that in wild-type plants (Fig. 8B), indicating that the *PHT1;1* induction in *WRKY42*-overexpressing lines was not caused by the *PHO1* repression caused by *WRKY42* overexpression. To further test whether WRKY42 protein directly regulated *PHT1;1* expression, transient expression experiments in tobacco leaves were performed. The cotransformation of *Super*:*WRKY42* with *PHT1;1* promoter-driving GUS reporter gene (*ProPHT1;1*:*GUS*; Wang et al., 2014) resulted in enhanced GUS activity (Fig. 8C), indicating that

WRKY42 positively regulated *PHT1;1* expression in vivo. In addition, we crossed the *ProPHT1;1:GUS* line (Wang et al., 2014) with the *WRKY42*-overexpressing lines (*Super:WRKY42-5* and *Super:WRKY42-40*) and wild-type plants and obtained the *Super:WRKY42-5/ProPHT1;1:GUS*, *Super:WRKY42-40/ProPHT1;1:GUS*, and wild-type/*ProPHT1;1:GUS* plants, respectively. The root GUS staining showed that the *PHT1;1* expression was promoted in the *WRKY42*-overexpressing lines (*Super:WRKY42-5/ProPHT1;1:GUS* and *Super:WRKY42-40/ProPHT1;1:GUS*) compared with that in the wild type (the wild type/*ProPHT1;1:GUS*) under Pi-sufficient condition (Fig. 8D). These data indicated that WRKY42 positively regulated *PHT1;1* expression.

Promoter sequence analysis showed that there were several W-boxes within the *PHT1;1* promoter (Fig. 9A; Martín et al., 2000; Wang et al., 2014); thus, we hypothesized that WRKY42 directly regulates *PHT1;1* expression by binding to the W-box within the *PHT1;1* promoter. The in vivo interaction between WRKY42 and the W-box motifs within the *PHT1;1* promoter was investigated using ChIP-quantitative PCR (qPCR) analysis. The 7-d-old wild-type seedlings were transferred to Pi-sufficient (+phosphorus) or Pi-deficient (−phosphorus) medium for another 7 d, and then, the roots were harvested for ChIP-qPCR assay. When wild-type plants were grown in Pi-sufficient condition, the chromatin immunoprecipitated with the anti-WRKY42 antibody was enriched in the P2 fragment of the *PHT1;1* promoter, whereas no interaction was observed between WRKY42 and the *PHT1;1* promoter containing P1, P3, or P4 fragments (Fig. 9B). During Pi starvation, the interaction between WRKY42 and the P2 fragment within the *PHT1;1* promoter was abolished (Fig. 9B). Furthermore, the EMSA was also performed to detect whether WRKY42 could bind to the

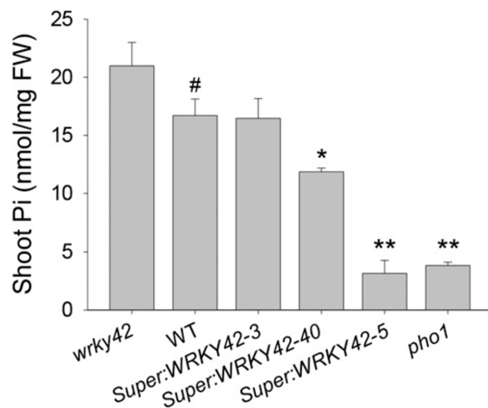

Figure 4. Shoot Pi content measurement in various plant materials. The shoot Pi contents of 10-d-old *wrky42* mutant, *WRKY42*-overexpressing lines, *pho1* mutant, and wild-type (WT) seedlings grown in Pi-sufficient condition. Data are shown as means ± SE ($n = 4$). Asterisks indicate significant differences compared with wild-type plants (paired test). FW, Fresh weight; *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control.

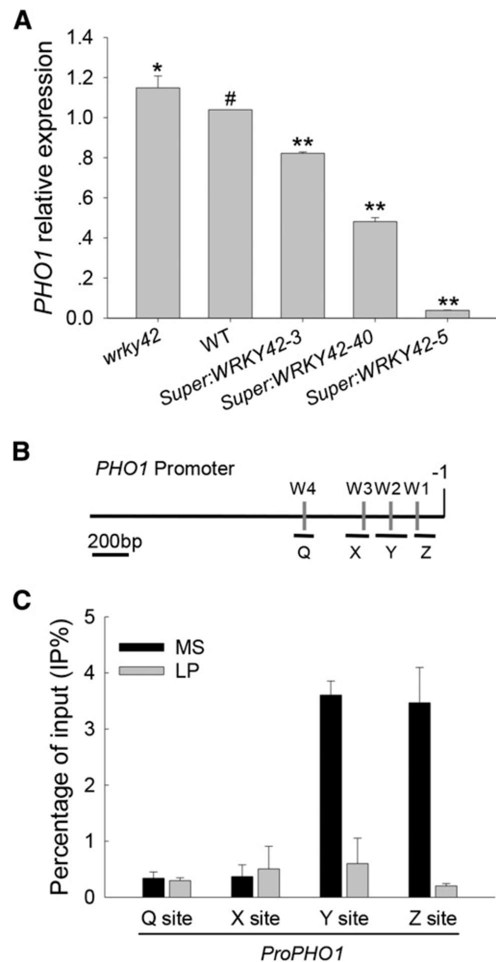

Figure 5. WRKY42 down-regulates *PHO1* expression and binds to the *PHO1* promoter. A, qRT-PCR analysis of *PHO1* expression in the *wrky42* mutant, *WRKY42*-overexpressing lines, and wild-type (WT) plants. All plants were germinated and grown on MS medium for 10 d; then, the roots were harvested for RNA extraction. Transcript level of *PHO1* was quantified relative to *ACTIN2/8*. The data represent the mean values of three replicates ± SE. Asterisks indicate significant differences compared with the wild type (paired test). *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control. B, Diagram of the *PHO1* promoter showing the relative positions of the W-boxes. The adenine residue of the translational start codon ATG was assigned position +1, and the numbers flanking the sequences of the *PHO1* promoter fragments were counted based on this number. 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. C, ChIP-qPCR assay to detect the association between WRKY42 and the *PHO1* promoter. Seven-day-old seedlings were transferred to Pi-sufficient (MS) or Pi-deficient (LP) condition for another 7 d; then, the roots were harvested for ChIP-qPCR. Chromatins were immunoprecipitated with anti-WRKY42 antibody, and the amount of indicated DNA in immune complex was tested by qRT-PCR. The ratio of immunoprecipitation DNA over the input was presented as the percentage of input (IP%). The experiments were repeated three times, and three replicates were included for each sample in each experiment. The data are presented as means ± SE ($n = 3$).

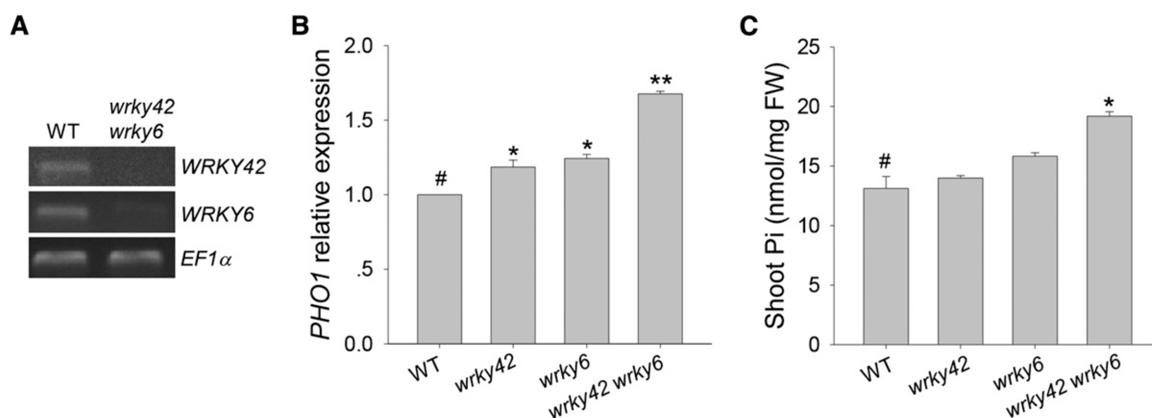

Figure 6. Loss of function of WRKY42 and WRKY6 enhanced *PHO1* expression and shoot Pi content. A, RT-PCR analysis of *WRKY42* and *WRKY6* expression in the *wrky42 wrky6* double mutant and wild-type (WT) plants. The *EF1α* is amplified as the control. B, qRT-PCR analysis of *PHO1* expression in the *wrky42* mutant, *wrky6* mutant, *wrky42 wrky6* double mutant, and wild-type plants. Transcript level of *PHO1* was quantified relative to *ACTIN2/8*. Each data bar represents the means ± SE ($n = 3$). Asterisks indicate significant differences compared with the wild type (paired test). *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control. C, The shoot Pi content of 17-d-old *wrky42* mutant, *wrky6* mutant, *wrky42 wrky6* double mutant, and wild-type seedlings grown on Pi-sufficient medium. Data are shown as means ± SE ($n = 3$). Asterisks indicate significant differences compared with the wild type (paired test). FW, Fresh weight; *, $P < 0.05$; #, wild-type plants were used as a control.

P2 fragment of the *PHT1;1* promoter in vitro. The WRKY42-His fusion protein could bind to P2 within the *PHT1;1* promoter, and the binding was effectively reduced by adding increasing amounts of unlabeled competitors with the same P2 sequence (Fig. 9C). In contrast, the WRKY42-His fusion protein could not bind to the mutation probe (mP2), which has two mutated W-boxes (Fig. 9C). As the negative control, the His protein alone did not bind to the *PHT1;1* promoter (Fig. 9C). These data show that WRKY42 positively regulated *PHT1;1* expression.

WRKY42 Is Degraded during Phosphate Starvation

Because the interaction between WRKY42 and the promoters of *PHO1* or *PHT1;1* was abolished during Pi starvation (Figs. 5C and 9B), it was proposed that the WRKY42 protein was degraded under Pi-deficient stress. To determine the relationship between the WRKY42 degradation and Pi status, the cell-free degradation analysis was conducted. The recombinant WRKY42-His protein was purified from *E. coli* and incubated with the total protein extracts from the 7-d-old wild-type seedlings cultured under Pi-sufficient (MS medium with 1.25 mM Pi; +phosphorus) or Pi-deficient (LP medium with 10 μM Pi; −phosphorus) condition for another 5 d. When incubated with +phosphorus total protein extract, the WRKY42 protein showed very faint degradation (Fig. 10A). When the WRKY42 protein was incubated with −phosphorus total protein extract, the WRKY42 protein was obviously degraded. This degradation of WRKY42 was inhibited by the carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), a 26S proteasome inhibitor (Fig. 10A), indicating that Pi starvation induced the proteasome-dependent degradation of WRKY42.

To further confirm the degradation of WRKY42 during Pi starvation in vivo, the *Super*:*WRKY42-GFP* and *Super*:*GFP* transgenic lines were generated. The 7-d-old *Super*:*WRKY42-GFP* and *Super*:*GFP* seedlings were transferred to Pi-sufficient (MS) or Pi-deficient (LP) medium and then harvested at the indicated time for protein gel-blot analysis using anti-GFP. The WRKY42 protein decreased much more rapidly in *Super*:*WRKY42-GFP* exposed to Pi starvation compared with Pi-sufficient condition (Fig. 10B). To further confirm that reduction of WRKY42 protein level was caused by the proteasome-dependent degradation in vivo, the 7-d-old *Super*:*WRKY42-GFP* seedlings were also transferred to LP medium with 10 μM MG132. The addition of MG132 clearly inhibited WRKY42 degradation under Pi starvation condition (Fig. 10B). *Super*:*GFP* was used as a control, and no GFP degradation was detected in Pi-deficient or -sufficient condition (Fig. 10B). Taken together, these data showed that the WRKY42 protein was degraded through the proteasome pathway during Pi starvation and stabilized by abundant Pi.

DISCUSSION

WRKY42 Is a Key Regulator in Phosphate Homeostasis in Plants

Phosphate plays important roles in regulation of many biochemical and physiological processes and is an essential building block of cell components. The intracellular concentration of Pi in plants is tightly regulated to maintain Pi homeostasis. To achieve this,

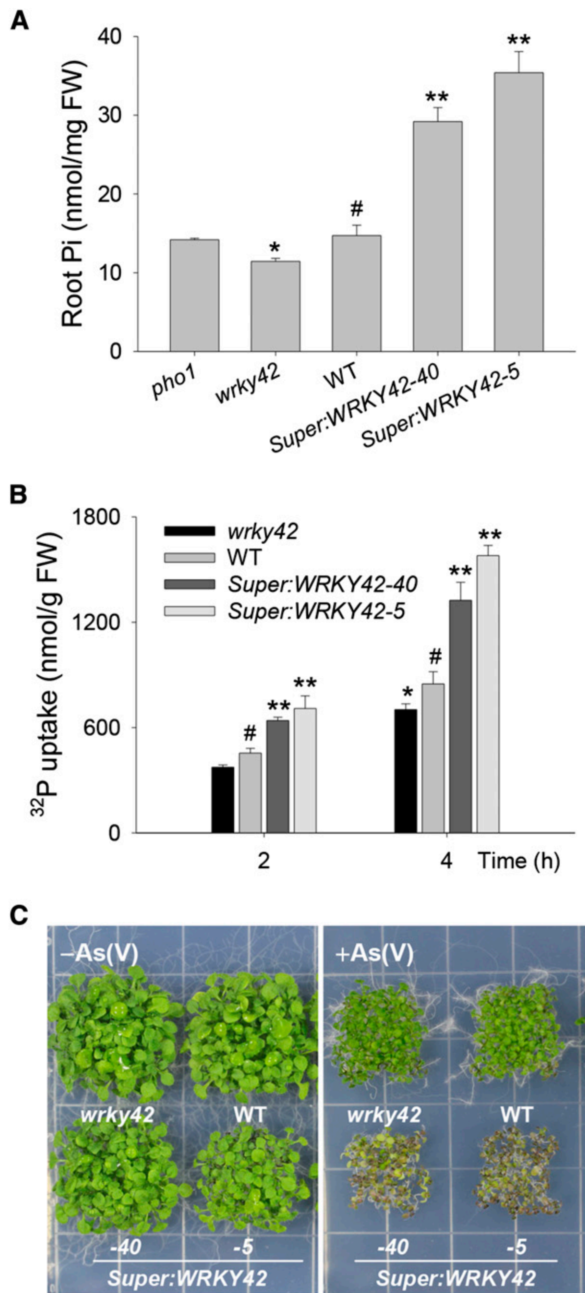

Figure 7. Overexpression of *WRKY42* enhances Pi acquisition. A, The root Pi contents of 10-d-old *wrky42* mutant, *WRKY42*-overexpressing lines, and wild-type (WT) seedlings grown on Pi-sufficient medium. Data are shown as means ± SE ($n = 4$). Asterisks indicate significant differences compared with the wild type (paired test). B, Pi uptake was monitored over a 4-h period in 10-d-old *wrky42* mutant, *WRKY42*-overexpressing lines, and wild-type seedlings. Data are shown as means ± SE ($n = 3$). Asterisks indicate significant differences compared with wild-type plants (paired test). C, As(V) tolerance phenotype of plants germinated and grown on one-half-strength MS medium with [+As(V)] or without [−As(V)] 200 μM As(V) for 20 d. FW, Fresh weight; *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control.

plants have evolved a series of strategies, such as enhancing Pi acquisition and remobilizing internal Pi (Raghothama, 1999; Vance et al., 2003). Arabidopsis *PHO1* encodes a membrane protein and is involved in Pi loading from roots to shoots (Hamburger et al., 2002). The *pho1* mutant has lower shoot Pi (Poirier et al., 1991) and shows a low Pi-sensitive phenotype caused by defective Pi loading in the xylem (Poirier et al., 1991; Chen et al., 2009). In this study, the *WRKY42*-overexpressing lines showed a reduced shoot

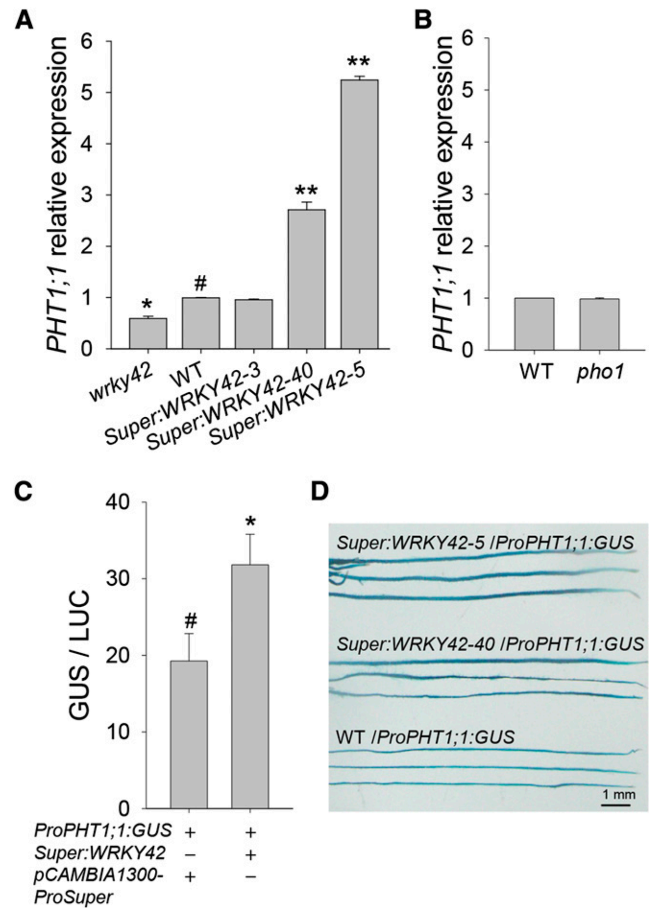

Figure 8. WRKY42 positively regulates *PHT1;1* expression. A, qRT-PCR analysis of *PHT1;1* expression in the roots of the *WRKY42*-overexpressing lines, *wrky42* mutant, and wild-type (WT) plants. The plants were germinated and grown on MS medium for 10 d; then, the roots were harvested for RNA extraction. Transcript level of *PHT1;1* was quantified relative to *ACTIN2/8*. Each data bar represents the means ± SE ($n = 3$). Asterisks indicate significant differences compared with wild-type plants (paired test). B, qRT-PCR analysis of *PHT1;1* expression in the roots of the *pho1* mutant and wild-type plants. Transcript level of *PHT1;1* was quantified relative to *ACTIN2/8*. Each data bar represents the means ± SE ($n = 3$). C, Transient overexpression of *WRKY42* fused to *ProPHT1;1*:*GUS* in tobacco leaves. Each data bar represents the means ± SE ($n = 5$). Asterisks indicate significant differences. D, GUS staining showing the expression patterns of *PHT1;1* in the *WRKY42*-overexpression lines and wild-type plants. The plants were germinated, grown on MS medium for 7 d; then, harvested for GUS staining. *, $P < 0.05$; **, $P < 0.01$; #, wild-type plants were used as a control.

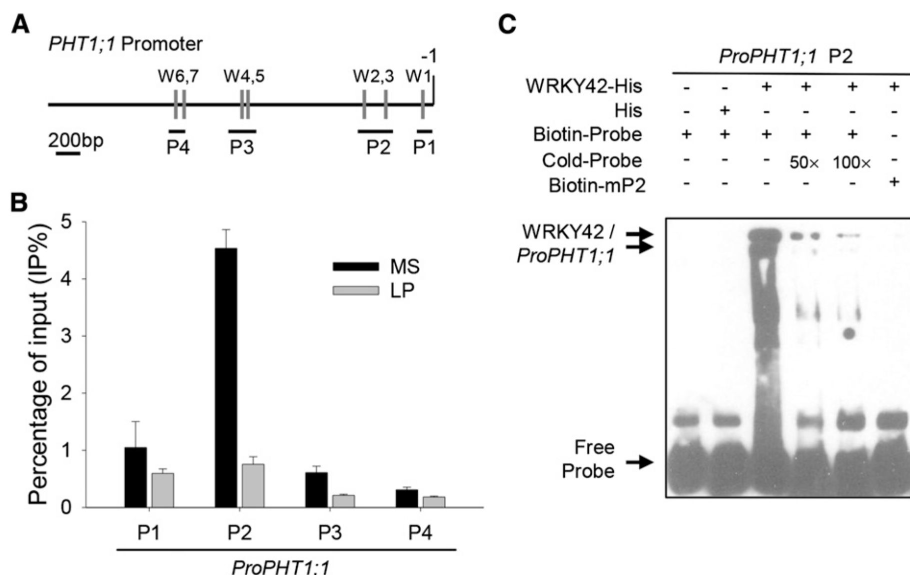

Figure 9. WRKY42 binds to the *PHT1;1* promoter. A, Diagram of the *PHT1;1* promoter region showing the relative positions of the W-boxes (gray rectangles) and the relative positions and sizes of the different PCR-amplified fragments (black lines under the W-boxes). The adenine residue of the translational start codon ATG was assigned position +1, and the numbers flanking the sequences of the *PHT1;1* promoter fragments were counted based on this number. B, ChIP-qPCR assay to detect the association between WRKY42 and the *PHT1;1* promoter. Seven-day-old seedlings were transferred to Pi-sufficient (MS) or Pi-deficient (LP) condition for another 7 d; then, the roots were harvested for ChIP-qPCR assay with anti-WRKY42. The ratio of immunoprecipitation DNA to the input was presented as the percentage of input (IP%). The data are presented as means ± SE (n = 3). C, EMSA to analyze the binding of WRKY42 to P2 fragment of the *PHT1;1* promoter. Each biotin-labeled DNA probe was incubated with His-WRKY42 protein. An excess of unlabeled probe was added to compete with labeled promoter sequence. Biotin-labeled probe incubated with His protein served as the negative control.

Pi and low Pi-sensitive phenotype, similar to the *pho1* mutant (Figs. 3 and 4), suggesting that WRKY42 played a role in regulating Pi translocation. As a typical WRKY transcription factor, WRKY42 directly bound to the W-boxes within the *PHO1* promoter and repressed *PHO1* expression under Pi-sufficient condition (Fig. 5). These data show that the WRKY42 transcription factor negatively regulated Pi translocation.

Interestingly, our data also showed that WRKY42 positively regulated Pi acquisition. Overexpression of *WRKY42* enhanced Pi uptake and root Pi content, and *WRKY42*-overexpressing lines showed an As(V)-sensitive phenotype, similar to the *PHT1;1*-overexpressing line (Fig. 7; Supplemental Fig. S1). Additional molecular results showed that WRKY42 up-regulated *PHT1;1* expression by binding to the promoter of *PHT1;1* (Figs. 8 and 9), and overexpression of *PHT1;1* significantly enhanced plant Pi uptake (Wang et al., 2014), showing that WRKY42 modulated Pi uptake by directly up-regulating *PHT1;1* expression. There are nine *PHT1* family genes in Arabidopsis (Mudge et al., 2002), and PHTs PHT1;1 and PHT1;4 play significant roles in Pi acquisition from both low- and high-Pi environments (Shin et al., 2004). During growth under high-Pi condition, the *pht1:1Δ4Δ* double mutant shows a 75% reduction in Pi uptake capacity relative to wild-type plants and results in significantly reduced shoot Pi contents (Shin et al., 2004). Similar to the *pht1:1Δ4Δ* double mutant, the *pht1;1-1* mutant showed a reduction in shoot Pi content compared with wild-type plants, whereas the shoot Pi content of the *pht1;4* mutant was not significantly different from the wild type (Shin et al., 2004), indicating that PHT1;1 is the main Pi transporter under high-Pi condition. Among nine *PHT1* genes, *PHT1;1* has the highest transcription level (Mudge et al., 2002), and *PHT1;1*-overexpressing lines show a high-Pi uptake rate (Wang et al., 2014), suggesting that the transcription regulation of *PHT1;1* is an important mechanism for Pi acquisition in a high-Pi environment; this regulation of *PHT1;1* expression is at least partially by WRKY42.

It was also hypothesized that the enhanced *PHT1;1* expression in *WRKY42*-overexpressing lines was partially caused by the Pi depletion in the aerial part because of the repression of *PHO1* by *WRKY42* overexpression. The root Pi content results showed that the root Pi contents of *WRKY42*-overexpressing lines were higher than those of wild-type plants, whereas the *pho1* mutant had similar root Pi content with wild-type plants (Fig. 7A). Also, the rates of root Pi uptake were similar between the *pho1* mutant and wild-type plants (Poirier et al., 1991). These data indicated that the disruption of *PHO1* could not enhance plant Pi uptake. The expression of *PHT1;1* in *WRKY42*-overexpressing lines was obviously higher

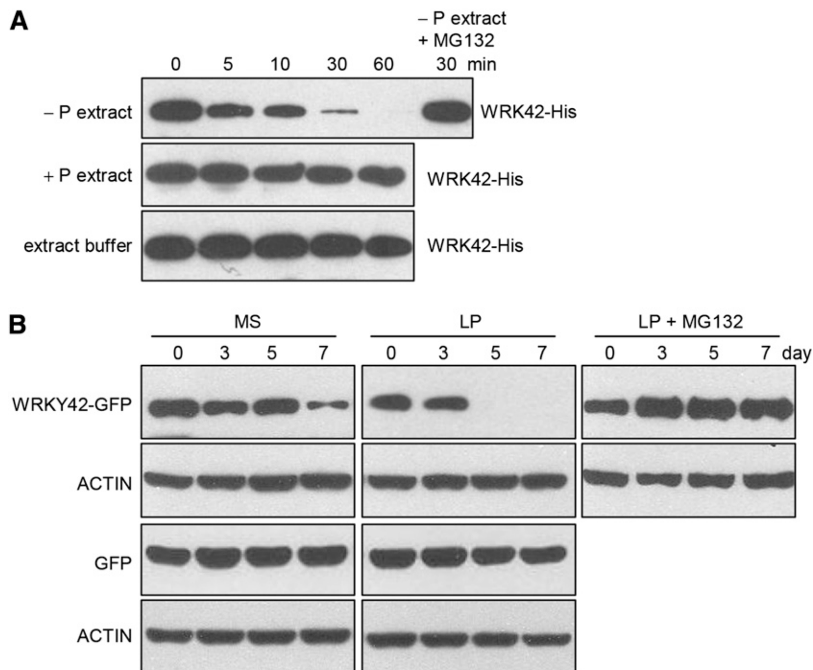

Figure 10. WRKY42 is degraded during Pi starvation. A, Cell-free degradation assay. Seven-day-old wild-type seedlings were transferred to Pi-sufficient medium (+P) or Pi-deficient medium (−P) for another 5 d; then, the seedlings were harvested for protein extraction. The plant protein extracts were incubated with recombinant WRKY42-His for the indicated time; then, WRKY42 abundance was determined by immunoblotting with anti-His. B, Immunoblot analysis of WRKY42 protein. Seven-day-old *Super:WRKY42-GFP* and *Super:GFP* transgenic seedlings were transferred to MS medium, LP medium, or LP medium with 10 μM MG132 (LP + MG132), and the seedlings were harvested at the indicated time for protein extraction. Protein extracts were analyzed by immunoblots using anti-GFP. Actin was used as the loading control.

than that in wild-type plants (Fig. 8A), and the expression level of *PHT1;1* was similar between the *pho1* mutant and wild-type plants (Fig. 8B), suggesting that the enhanced *PHT1;1* expression in *WRKY42*-overexpressing lines was independent of *PHO1* disruption.

During Pi starvation, transcription of *WRKY42* was repressed (Fig. 1C), and the WRKY42 protein was degraded in a proteasome-dependent manner (Fig. 10), indicating that WRKY42 regulated the expression of *PHO1* and *PHT1;1* under Pi-sufficient condition. The expression of *PHT1;1* was obviously induced during Pi starvation (Muchhal et al., 1996; Karthikeyan et al., 2002; Mudge et al., 2002; Shin et al., 2004), suggesting that other transcription factor(s) up-regulated *PHT1;1* expression under low-Pi stress. Previous reports showed that the MYB transcription factor PHR1 and the WRKY transcription factor WRKY45 modulated the increased expression of *PHT1;1* during Pi starvation (Rubio et al., 2001; Wang et al., 2014), indicating that the expression level of *PHT1;1* was precisely regulated by different transcription factors according to Pi availability. During Pi starvation, the *PHO1* expression was induced, the WRKY42 and WRKY6 were degraded, and the repression of *PHO1* by WRKY42 and WRKY6 was abolished (Figs. 6B and 10; Chen et al., 2009), suggesting that the induced expression of *PHO1* during Pi starvation was at least partially dependent on the degradation of WRKY42 and WRKY6.

WRKY42 and WRKY6 Have Redundant and Nonredundant Functions during Different Arabidopsis Physiological Processes

WRKY proteins are plant-specific transcription factors, with over 70 members in the Arabidopsis WRKY family. Previous reports showed that WRKY transcription factors have redundant functions, such as WRKY18, WRKY40, and WRKY60, in response to microbial pathogens (Xu et al., 2006) as well as abscisic acid signaling (Shang et al., 2010) and WRKY3 and WRKY6 responses to herbivory (Skibbe et al., 2008). A previous report showed that WRKY6 can directly down-regulate *PHO1* expression by binding to the *y* and *z* sites within the *PHO1* promoter (Chen et al., 2009). In this study, WRKY42 was a negative regulator of *PHO1* expression. Overexpression of *WRKY42* repressed *PHO1* expression, and WRKY42 bound to the *y* and *z* sites within the *PHO1* promoter (Fig. 5C), showing that WRKY42 directly down-regulated *PHO1* expression. The *PHO1* expression was enhanced in the *wrky42* or *wrky6* single mutants compared with wild-type plants (Fig. 6B). Additionally, the expression

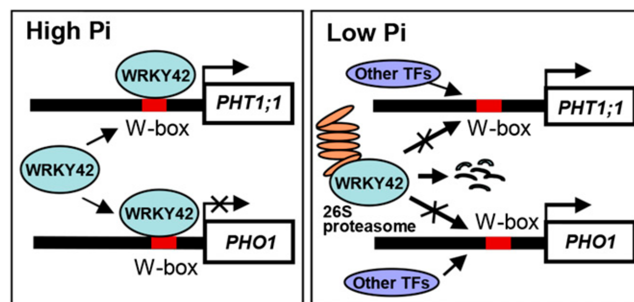

Figure 11. Hypothetical model of the WRKY42/PHO1/PHT1;1-regulatory pathway in plants regulating Pi homeostasis. Under high-Pi condition, the WRKY42 directly represses *PHO1* expression and activates *PHT1;1* expression by binding to the W-box motifs within the promoters of *PHO1* and *PHT1;1* to maintain phosphate homeostasis. Under low-Pi stress, the WRKY42 protein is degraded; then, the regulation of *PHO1* and *PHT1;1* by WRKY42 ceased. TF, Transcription factor.

level of *PHO1* in the *wrky42 wrky6* double mutant was much higher than that in the wild type or single mutant (Fig. 6B), and the shoot Pi content of the *wrky42 wrky6* double mutant was also elevated (Fig. 6C), similar to *PHO1*-overexpressing lines (Liu et al., 2012). Thus, the two WRKY transcription factors, WRKY42 and WRKY6, have redundant roles in Arabidopsis Pi translocation by down-regulating *PHO1* expression. In addition to negative regulation of *PHO1* expression, both WRKY42 and WRKY6 activate *Senescence-Induced Receptor-Like Kinase* (*SIRK*) expression during plant senescence and pathogen defense (Robatzek and Somssich, 2002), indicating that *SIRK* regulation involves these two functionally redundant WRKY transcription factors, WRKY42 and WRKY6.

WRKY42 and WRKY6 have nonredundant functions in Arabidopsis Pi acquisition. In this study, *PHT1;1* expression was elevated in the *WRKY42*-overexpressing lines and repressed in the *wrky42* mutant compared with wild-type plants (Fig. 8), and WRKY42 could bind to the *PHT1;1* promoter (Fig. 9), showing that WRKY42 directly up-regulated *PHT1;1* expression. In contrast, expression of *PHT1;1* in *WRKY6*-overexpressing lines was similar to that of wild-type plants (data not shown). Although WRKY6 does not modulate *PHT1;1* expression under Pi-sufficient condition, WRKY6 is responsible for *PHT1;1* repression under As(V) stress (Castrillo et al., 2013). When grown in the presence of As(V), *WRKY6-GFP*-overexpressing lines show an As(V)-tolerant phenotype compared with wild-type plants, and expression of *PHT1;1* is repressed relative to the wild type (Castrillo et al., 2013). However, when grown on medium with As(V), *WRKY42*-overexpressing lines showed an As(V)-sensitive phenotype, similar to the *PHT1;1*-overexpressing line (Fig. 7C; Supplemental Fig. S1), indicating that WRKY42 was not involved in repressing *PHT1;1* expression under As(V) stress. Together, although both WRKY42 and WRKY6 can regulate *PHT1;1* expression, their mechanisms are different. WRKY42 activated *PHT1;1* expression under Pi-sufficient condition, and WRKY6 repressed *PHT1;1* transcription under As(V) stress.

In conclusion, our genetic, physiological, and biochemical approaches showed that WRKY42 played important roles in phosphate homeostasis. The WRKY42 transcription factor regulated the expression of *PHT1;1* and *PHO1* to adapt environmental changes in Pi availability (Fig. 11). Under Pi-sufficient condition, WRKY42 repressed the *PHO1* expression and positively regulated *PHT1;1* expression. During Pi starvation, WRKY42 was degraded, and then, regulation of *PHO1* and *PHT1;1* by WRKY42 ceased.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type plants were the Columbia-0 ecotype. The *Super*:*PHT1;1*, *pho1*, and *pht1;1* plants used in the study were described previously (Chen et al., 2009; Wang et al., 2014). The *WRKY42* T-DNA insertion mutant Salk_121674 (referred to as the *wrky42* mutant) and the *WRKY6* T-DNA insertion mutant Salk_012997 (the *wrky6* mutant) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc).

The Arabidopsis (*Arabidopsis thaliana*) seeds were surface sterilized and cold treated at 4°C 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 illumination of 100 μmol m^{-2} s^{-1} for a 16-h daily light period unless otherwise indicated.

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

For As(V) treatment, the sterilized seeds were plated on one-half-strength MS medium or one-half-strength MS medium with 200 μM As(V).

Phosphate Content and Phosphate Uptake Assay

The Arabidopsis plants were germinated and grown on MS medium for 10 d; then, the shoots and roots were harvested for Pi content measurement. The Pi content in the samples was quantified as described previously (Ames, 1966; Chiou et al., 2006). For the Pi uptake assay, 10-d-old seedlings grown on MS medium were transferred to the Pi uptake solution containing 500 μM Pi supplemented with 0.2 μCi ^{32}P orthophosphate. A group of 15 seedlings was used as one biological sample.

Anthocyanin Measurement

The 7-d-old seedlings were transferred to MS or LP medium for another 10 d; then, the seedlings were harvested for anthocyanin measurement. Anthocyanin was determined as described by Lu et al. (2014).

Plasmid Construction and Plant Transformation

To construct *Super*:*WRKY42*, the coding sequence of *WRKY42* was cloned into the modified *pCAMBIA1300-ProSuper* vector under the control of the Super promoter (Li et al., 2001). To construct *Super*:*WRKY42-GFP*, the coding sequence of *WRKY42* was fused in frame to the GFP in the modified *pCAMBIA1300-GFP* plasmid. To construct *ProWRKY42*:*GUS*, a 1,132-bp DNA fragment of the region upstream from the *WRKY42* coding sequence was cloned into the *pCAMBIA1381* vector. All constructs were introduced into Arabidopsis by *Agrobacterium* sp.-mediated transformation (*Agrobacterium* sp. strain GV3101) using the floral dip method (Clough and Bent, 1998); then, the single-copy transgenic lines were obtained.

qRT-PCR and RT-PCR

qRT-PCR was performed using SYBR Green PCR Master Mix (Life Technologies) on a 7500 Real Time PCR System (Applied Biosystems) following the manufacturer's protocol. Relative quantitative results were calculated by normalization to *Actin2/8*.

For RT-PCR assay, the total RNA was extracted from the *wrky42* mutant, *wrky42 wrky6* double mutant, and wild-type plants, and then, the expression of *WRKY42* or *WRKY6* was determined by RT-PCR as described by Chen et al. (2009). *Elongation Factor EF1α* (*EF1α*) was used as a quantitative control.

The primers used are listed in Supplemental Table S1.

Subcellular Localization

For the subcellular localization assay, *WRKY42* fused to GFP was cloned into a modified *pCAMBIA1300*:*GFP* vector, resulting in a *WRKY42*:*GFP* construct. The plasmids were transformed into *Agrobacterium* sp. GV1301. The transient expression assays were conducted as described by Chen et al. (2009). Fluorescence of GFP in the transformed leaves was imaged using a confocal laser-scanning microscope (LSM510; Carl Zeiss).

Transient Expression Assays in Tobacco

The transient GUS expression assays were performed as described (Chen et al., 2009). The constructs *ProPHT1;1*:*GUS*, *Super*:*WRKY42*, and *pCAMBIA1300-ProSuper* were transformed into *Agrobacterium* sp. strain GV3101 separately. For every infiltration sample, *Super*:*Luciferase* (*LUC*) was added as an internal control. *Agrobacterium* sp. cells were harvested by centrifugation

and suspended in induction buffer to an optical density at 600 nm of 0.4. After 2 h at 22°C, *Agrobacterium* sp. cells were infiltrated into 7-week-old tobacco (*Nicotiana benthamiana*) leaves, and the infiltration ratio of *Super*:*WRKY42* to *ProPHT1;1* or *pCAMBIA1300-ProSuper* to *ProPHT1;1* was 9:1 (v/v). After infiltration for 36 h, leaf discs were harvested for GUS and LUC proteins extraction. The GUS and LUC activities of the infiltrated leaves were quantitatively determined, and the GUS to LUC ratio was used to quantify the promoter activity.

ChIP-qPCR Assay

To generate the anti-WRKY42 antibody, the whole coding sequence of WRKY42 was cloned into the *pET30a* vector. The recombinant WRKY42-His protein was expressed in *Escherichia coli* and purified. The polyclonal anti-WRKY42 antibody was generated by inoculating a mouse with the recombinant WRKY42. For ChIP-qPCR assay, 7-d-old seedlings were transferred to MS or LP medium for another 7 d; then, the roots were harvested for ChIP assay. The ChIP-qPCR assay was conducted as previously described (Chen et al., 2009; Feng et al., 2014), and the primers used are listed in Supplemental Table S1. Three independent experiments were performed with similar results. Data are mean values of three replicates ± SE from one experiment.

EMSA

The EMSA was conducted using a LightShift Chemiluminescent EMSA Kit (Pierce) following the manufacturer's protocol. The recombinant WRKY42-His protein and His protein were purified from *E. coli*. The fragments of the *PHT1;1* promoters were obtained by PCR using biotin-labeled or unlabeled primers (Supplemental Table S1). Biotin-unlabeled fragments of the same sequences were used as competitors, and the His protein alone was used as the negative control.

Protein Extraction and Cell-Free Degradation

Seven-day-old Arabidopsis seedlings were transferred to MS (+phosphorus) or LP (−phosphorus) medium for 5 d; then, the seedlings were harvested and ground into fine powder in liquid nitrogen. Total proteins were extracted in degradation buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 4 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 10 mM ATP as described by Wang et al. (2009). The total protein concentration was determined by Bio-Rad protein assay. The total protein extracts prepared were adjusted to equal concentrations in the degradation buffer for each assay. Then, exogenous MG132 was added to the total proteins extracted from −phosphorus plants, and the final concentration was 10 μM; 250 ng of recombinant WRKY42-His protein was incubated in 20-μL extracts (containing 50 μg of total proteins) for the individual assays. The extracts were incubated at 22°C, and samples were taken at indicated times for determination of WRKY42 protein abundance by immunoblots with anti-His.

Immunoblot Analysis

Total proteins were extracted according to Saleh et al. (2008), and 80 μg of proteins of each sample was separated on a 10% (w/v) SDS-PAGE and transferred to polyvinylidene fluoride membranes. MG132 treatment was conducted as described by Chen et al. (2009). WRKY42-GFP and GFP proteins were detected by anti-GFP at 1:5,000 dilution (Miltenyi).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *WRKY42* (At4g04450), *PHO1* (At3g23430), *PHT1;1* (At5g43350), *WRKY6* (At1g62300), *ACT2* (At3g18780), *ACT8* (At1g49240), and *EF1α* (At5g60390).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. As(V) tolerance phenotype test.

Supplemental Table S1. Primers used in this study.

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