Running title: WRKY42 regulates Pi uptake and translocation

Corresponding author: Yi-Fang Chen
E-mail address: chenyifang@cau.edu.cn
Telephone number: 8610-6273-3815
Address: College of Biological Sciences, China Agricultural University, Beijing 100193, China

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Title: WRKY42 Modulates Phosphate Homeostasis through Regulating Phosphate Translocation and Acquisition in Arabidopsis

Authors: Tong Su§, Qian Xu§, Fei-Cui Zhang§, Yun Chen, Li-Qin Li, Wei-Hua Wu, Yi-Fang Chen*

§ These three authors contributed equally to this work.
* For correspondence.

Author’s institutional affiliation: State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, National Plant Gene Research Centre (Beijing), Beijing 100193, China

Author responsible for distribution of materials: Yi-Fang Chen
(chenyifang@cau.edu.cn).

One-sentence Summary: Arabidopsis WRKY42 transcription factor modulates Pi homeostasis by regulating the expression of PHO1 and PHT1;1 to adapt to environmental changes in Pi availability

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Corresponding author with e-mail address: Yi-Fang Chen (chenyifang@cau.edu.cn)
Abstract

The *Arabidopsis* 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 demonstrates that WRKY42 regulated phosphate homeostasis in *Arabidopsis*. The *WRKY42*-overexpressing lines, similar to the *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, while the *wrky42* mutant had lower root Pi content and Pi uptake rate compared with wild-type plants. Under Pi-sufficient condition, WRKY42 positively regulated *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 via the 26S proteasome pathway. Our results demonstrated that At WRKY42 modulated Pi homeostasis by regulating the expression of *PHO1* and *PHT1;1* to adapt to environmental changes in Pi availability.
**Introduction**

Phosphorus (P) is an essential nutrient for plant growth (Rathothama, 1999) and is 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 is achieved mainly by coordination of acquisition of inorganic phosphate (orthophosphate; Pi) 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 encoded by members of the *PHT1* (phosphate transporter) gene family. There are at least nine members (PHT1;1–PHT1;9) of the PHT1 family in *Arabidopsis thaliana*, and transcripts of *PHT1;1* are the most abundant among the nine *PHT1* genes (Mudge et al., 2002). PHT1;1 and PHT1;4 play important roles in Pi uptake from soil. Under high-Pi conditions, the *pht1;1* mutants’ uptake rate was only 59–66% 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 conditions. During Pi starvation, the expression 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 PROTEIN 62 (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 post-transcription 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 (NLA) mediates PHT1;1 and PHT1;4 degradation to 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 24–44% 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), while 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 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 paper, 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 conditions. During Pi starvation, WRKY42 expression is repressed and the WRKY42 protein is degraded via a proteasome pathway, and 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 demonstrated 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 PCR (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* (*GUS*: β-glucuronidase) transgenic lines were generated. GUS staining was strong in roots (Fig. 1Ba and Bc) and weak in leaves (Fig. 1Ba and Bb). Then the expression of *WRKY42* was tested under Pi starvation. The 7-d-old wild-type seedlings were transferred to Pi-sufficient condition [Murashige and Skoog (MS) medium] or Pi-deficient condition (LP medium with 10 μM Pi) 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 *Nicotiana benthamiana* 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 Cys2His2 zinc finger motif (Eulgem et al., 2000). Both the WRKYGQK and Cys2His2 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 ABRC (Arabidopsis Biological Resource Center, http://abrc.osu.edu), and named *wrky42*. The *wrky42* mutant (Salk_121674) carried a T-DNA insertion in the third exon of *WRKY42* (Fig. 3A), and 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 the 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 medium with 1.25 mM Pi), all tested plants showed no obvious differences in phenotypes (top panel in Fig. 3D). When the 7-d-old seedlings were transferred to Pi-deficient condition (LP, low-phosphate 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 (bottom panel in Fig. 3D). During Pi starvation, the anthocyanin contents in the WRKY42-overexpressing lines (Super:WRKY42-40 and Super:WRKY42-5) and the pho1 mutant were approximately threefold that 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 the 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, since 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 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 \textit{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 \textit{PHO1} promoter were severely impaired (Fig. 5C). These data demonstrate that WRKY42 directly down-regulated \textit{PHO1} expression.

**WRKY42 and WRKY6 Have Functional Redundancy in Down-Regulating \textit{PHO1} Expression**

Our previous work demonstrated that WRKY6 negatively regulated \textit{PHO1} expression, and the \textit{PHO1} expression was repressed in the \textit{WRKY6}-overexpressing lines and elevated in the \textit{wrky6} mutant (Chen et al., 2009). We hypothesized that WRKY42 and WRKY6 had redundant functions in regulating \textit{PHO1} expression. To test this hypothesis, the \textit{wrky42 wrky6} double mutant was generated (Fig. 6A) that, when grown in Pi-sufficient conditions, had obviously higher \textit{PHO1} expression than the \textit{wrky42} mutant, \textit{wrky6} mutant and wild-type plants (Fig. 6B). Because overexpression of \textit{PHO1} enhances the shoot Pi content (Liu et al., 2012), the shoot Pi content was measured in the \textit{wrky42 wrky6} double mutant, \textit{wrky42} mutant, \textit{wrky6} mutant, and wild-type seedlings. The shoot Pi content of the \textit{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 \textit{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 \textit{WRKY42}-overexpressing lines, \textit{Super:WRKY42-40} and \textit{Super:WRKY42-5} (both had higher \textit{WRKY42} expression than
Super:WRKY42-3), contained around 2-fold 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 due to 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 32P 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 wild-type (Fig. 7B). Arsenate is an oxyanion structurally analogous to phosphate (Asher and Reay, 1979) and is taken up mainly via Pi transporter PHT1;1 (Catarecha et al., 2007). When grown on medium containing arsenate, the pht1;1 mutant showed an arsenate-tolerant phenotype, and the PHT1;1-overexpressing line was more sensitive to arsenate than wild-type plants (Supplemental Fig. S1; Catarecha et al., 2007; Wang et al., 2014). To gain further insight into the role of WRKY42 in Pi acquisition, the phenotype of WRKY42-overexpressing lines, the wrky42 mutant and wild-type seedlings were tested with arsenate. When grown on Pi-sufficient medium without arsenate [–As(V)], there were no obvious phenotypic differences among the WRKY42-overexpressing lines, the wrky42 mutant and wild-type seedlings (Fig. 7C and Supplemental Fig. S1). When grown on Pi-sufficient medium with 200 μM arsenate [+As(V)], although the toxic effect of arsenate 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 arsenate-sensitive phenotype, similar to the phenotype of PHT1;1-overexpressing lines, compared with wild-type seedlings (Fig. 7C and Supplemental Fig. S1). There were no
obvious differences between the wrky42 mutant and wild-type seedlings when grown on Pi-sufficient medium with 200 μM arsenate (Fig. 7C and 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 phosphate transporters (PHT1;1–PHT1;9) in Arabidopsis (Mudge et al., 2002) and of these expression of PHT1;1 is most highly expressed in roots when wild-type plants are grown in Pi-sufficient conditions (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 conditions. 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 due to the PHO1 repression caused by WRKY42 overexpression. To further test whether WRKY42 protein directly regulated PHT1;1 expression, transient expression experiments in tobacco (Nicotiana benthamiana) leaves were performed. The co-transformation 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 WT / 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 wild-type (WT / 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 PHT1;1 promoter was investigated using ChIP-qPCR analysis. The 7-d-old wild-type seedlings were transferred to Pi-sufficient (+P) or Pi-deficient (−P) 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, while 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 assay was also performed to detect whether WRKY42 could bind to the 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 demonstrate 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 (Fig. 5C and 9B), it was proposed that the WRKY42
protein was degraded under Pi-deficient stress. In order 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, +P) or Pi-deficient (low-phosphate medium with 10 μM Pi, –P) conditions for another 5 d. When incubated with +P total protein extract, the WRKY42 protein showed very faint degradation (Fig. 10A). When the WRKY42 protein was incubated with –P total protein extract, the WRKY42 protein was obviously degraded. This degradation of WRKY42 was inhibited by the 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 due to 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 demonstrated that the WRKY42 protein was degraded via the proteasome pathway during Pi starvation and was 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, 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 due to defective Pi loading in the xylem (Poirier et al., 1991; Chen et al., 2009). In the present study, the WRKY42-overexpressing lines showed a reduced shoot 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 demonstrate 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 arsenate-sensitive phenotype, similar to the *PHT1;1*-overexpressing line (Fig. 7 and Supplemental Fig. S1). Further 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), demonstrating 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 phosphate transporters *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 pht1:1 \( \Delta 4\Delta \) 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 to wild-type (Shin et al., 2004), indicating that PHT1;1 is the main Pi transporter under high-Pi condition. Among the 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, and 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 due to 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 that of wild-type plants, whereas the \( pho1 \) mutant had similar root Pi content with wild-type plants (Fig. 7A). And 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 obvious higher 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. Whereas 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, and the WRKY42 and WRKY6 were degraded and the repression of PHO1 by WRKY42 and WRKY6 was abolished (Figs. 10 and 6B; 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 Non-Redundant 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 ABA 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 the present 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), demonstrating 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). And the expression level of PHO1 in the wrky42 wrky6 double mutant was much higher than those in 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 \textit{PHO1} expression. In addition to negative regulation of \textit{PHO1} expression, both WRKY42 and WRKY6 activate \textit{SIRK} expression during plant senescence and pathogen defense (Robatzek and Somssich, 2002), indicating that \textit{SIRK} regulation involves these two functionally redundant WRKY transcription factors, WRKY42 and WRKY6.

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

In conclusion, our genetic, physiological, and biochemical approaches showed WRKY42 played important roles in phosphate homeostasis. The WRKY42 transcription factor regulated the expression of \textit{PHT1;1} and \textit{PHO1} to adapt environmental changes in Pi availability (Fig. 11). Under Pi-sufficient conditions, WRKY42 repressed the \textit{PHO1} expression and positively regulated \textit{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 Col-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 ABRC (http://www.arabidopsis.org/abrc).

The *Arabidopsis* 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, 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 arsenate treatment, the sterilized seeds were plated on 1/2 MS medium or 1/2 MS medium with 200 μM arsenate [As(V)].

**Phosphate Content and Phosphate Uptake Assay**

The *Arabidopsis* plants were germinated and grown on MS medium for 10 d, and 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 ³²P 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, and 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 1132-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-mediated transformation (Agrobacterium strain GV3101) using the floral-dip method (Clough and Bent, 1998), and 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). EF1α was used as a quantitative control.

The primers used are listed in Supplemental Table 1 online.

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* GV1301, respectively. 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 *Nicotiana benthamiana***

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* strain GV3101 separately. For every infiltration sample, *Super:LUC* was added as an internal control. *Agrobacterium* cells were harvested by centrifugation and suspended in induction buffer to OD$_{600}$ of 0.4. After 2 hr at 22°C, *Agrobacterium* cells were infiltrated into leaves of 7-week-old *Nicotiana benthamiana* leaves, and the infiltration ratio of *Super:WRKY42 and ProPHT1;1* or *pCAMBIA1300-ProSuper* and *ProPHT1;1* was 9:1(v/v). After infiltration for 36 hr, 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/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 *E. 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, and 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 1 online. Three independent experiments were performed with similar results.
Data are mean values of three replicates ± SE from one experiment.

**EMSA Assay**

The EMSA assay 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 1 online). 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 medium (+P) or LP (–P) medium for 5 d, and 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 MgCl₂, 4 mM PMSF, 5 mM DTT 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 –P plants, the final concentration was 10 μM. The 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 were separated on a 10% 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 1:5000 dilution (Miltenyi).

Accession numbers
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
Supplemental Figure 1. Arsenate tolerance phenotype test.
Supplemental Table 1. Primers used in this study.

AUTHOR CONTRIBUTIONS
Y.-F.C. and W.-H.W. designed the research. T.S., Q.X, F.-C.Z., Y.C., Y.-F.C. and L.-Q.L. performed research and analyzed data. Y.-F.C., T.S. and F.-C.Z. wrote the article. W.-H.W. revised the article.
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Figure legends

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 and 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, and 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.

Figure 2. WRKY42 is Localized in the Nucleus and Binds to W-box Motifs.

(A) Subcellular localization of WRKY42–GFP fusion protein in Nicotiana benthamiana leaves. The GFP alone was used as the control.

(B) Oligonucleotides used in the EMSA assay (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 assay 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.

Figure 3. Phenotype Tests of Various Plant Materials.
(A) Diagram of the WRKY42 gene showing the position of 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 seedlings (WT). 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 (WT). 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 panel) or LP medium (low-Pi medium with 10 μM Pi, bottom panel) for another 10 d, and 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, and 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): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as a control (#).

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 seedlings grown in Pi-sufficient conditions. Data are shown as means ± SE (n = 4). Asterisks indicate significant differences compared with wild-type plants (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the 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 plants. All plants were germinated and grown on MS medium for 10 d, and 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 wild-type (paired test): *, P < 0.05. Wild-type plants (WT) 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, and then the roots were harvested for ChIP-qPCR. Chromatins were immunoprecipitated with anti-WRKY42 antibody, and amount of indicated DNA in immune complex was tested by qRT-PCR. The ratio of IP 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 plants. The *EF1α* is amplified as the control.
(B) qRT-PCR analysis of PHO1 expression in the wrky42 mutant, wrky6 mutant and 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 wild-type (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the 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 wild-type (paired test): *, P < 0.05. Wild-type plants (WT) were used as a control (#).

**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 seedlings grown on Pi-sufficient medium. Data are shown as means ± SE (n = 4). Asterisks indicate significant differences compared with wild-type (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the control (#).

(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 mean ± SE (n = 3). Asterisks indicate significant differences compared with wild-type plants (WT) (paired test): *, P < 0.05; **, P < 0.01.

(C) Arsenate tolerance phenotype of plants germinated and grown on 1/2 MS medium with [+As(V)] or without [–As(V)] 200 µM arsenate for 20 d.

**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 plants. The plants were germinated and grown on MS medium for 10 d, and 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): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the control (#).

(B) qRT-PCR analysis of \textit{PHT1;1} expression in the roots of the \textit{pho1} mutant and wild-type plants. Transcript level of \textit{PHT1;1} was quantified relative to \textit{ACTIN2/8}. Each data bar represents the means ± SE (n = 3).

(C) Transient overexpression of \textit{WRKY42} fused to \textit{ProPHT1;1:GUS} in \textit{Nicotiana benthamiana} leaves. Each data bar represents the means ± SE (n = 5). Asterisks indicate significant differences: *, P < 0.05; # is the control.

(D) GUS staining showing the expression patterns of \textit{PHT1;1} in the \textit{WRKY42}-overexpression lines and wild-type plants. The plants were germinated and grown on MS medium for 7 days and then harvested for GUS staining.

Figure 9. \textit{WRKY42} Binds to the \textit{PHT1;1} Promoter.

(A) Diagram of the \textit{PHT1;1} promoter region showing the relative positions of the W-boxes (gray rectangles), and 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 \textit{PHT1;1} promoter fragments were counted based on this number.

(B) ChIP-qPCR assay to detect the association between \textit{WRKY42} and the \textit{PHT1;1} promoter. Seven-day-old seedlings were transferred to Pi-sufficient (MS) or Pi-deficient (LP) conditions for another 7 d, and then the roots were harvested for ChIP-qPCR assay with anti-WRKY42. The ratio of IP DNA over the input was presented as the percentage of input (% IP). The data are presented as means ± SE (n = 3).

(C) EMSA assay to analyze the binding of \textit{WRKY42} to P2 fragment of \textit{PHT1;1} promoter. Each biotin-labeled DNA probe was incubated with His–\textit{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.
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, and then the seedlings were harvested for protein extraction. The plant protein extracts were incubated with recombinant WRKY42-His for the indicated time, and 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.

Figure 11. Hypothetical Model of WRKY42/PHO1/PHT1;1-Regulatory Pathway in Plant 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, in order to maintain phosphate homeostasis. Under low Pi stress, the WRKY42 protein is degraded, and then the regulation of PHO1 and PHT1;1 by WRKY42 ceased.
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 and 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, and 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.
Figure 2. WRKY42 is Localized in the Nucleus and Binds to W-box Motifs.

(A) Subcellular localization of WRKY42–GFP fusion protein in *Nicotiana benthamiana* leaves. The GFP alone was used as the control.

(B) Oligonucleotides used in the EMSA assay (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 assay 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.
Figure 3. Phenotype Tests of Various Plant Materials. (A) Diagram of the WRKY42 gene showing the position of 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 seedlings (WT). 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 (WT). 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 panel) or LP medium (low-Pi medium with 10 μM Pi, bottom panel) for another 10 d, and 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, and 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): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as a control (#).
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 seedlings grown on MS medium. Data are shown as means ± SE (n = 4). Asterisks indicate significant differences compared with wild-type plants (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the 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 plants. All plants were germinated and grown on MS medium for 10 d, and then the roots were harvested for RNA extraction. The data represent the mean values of three replicates ± SE. Asterisks indicate significant differences compared with wild-type (paired test): *, P < 0.05. Wild-type plants (WT) were used as a control (#).

(B) Diagram of the PHO1 promoter region showing the number and relative position of the W-boxes (gray rectangles), and relative position and size 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 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) conditions for another 7 d, and then the roots were harvested for ChIP-qPCR. Chromatin were immunoprecipitated with anti-WRKY42 antibody, and amount of indicated DNA in immune complex was tested by qRT-PCR. The ratio of IP 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 plants. The EF1α is amplified as the control.

(B) qRT-PCR analysis of PHO1 expression in the wrky42 mutant, wrky6 mutant and 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 wild-type (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the 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 wild-type (paired test): *, P < 0.05. Wild-type plants (WT) were used as a control (#).
Figure 7. Overexpression of WRKY42 Enhances Pi Acquisition.

(A) The root Pi contents of 10-d-old *pho1* mutant, *wrky42* mutant, *WRKY42*-overexpressing lines and wild-type seedlings grown on MS medium. Data are shown as means ± SE (n = 4). Asterisks indicate significant differences compared with wild-type (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the control (#).

(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 mean ± SE (n = 3). Asterisks indicate significant differences compared with wild-type plants (WT) (paired test): *, P < 0.05; **, P < 0.01.

(C) Arsenate tolerance phenotype of plants germinated and grown on 1/2 MS medium with [+As(V)] or without [–As(V)] 200 μM arsenate for 20 d.
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 plants. The plants were germinated and grown on MS medium for 10 d, and then the roots were harvested for RNA extraction. Each data bar represents the means ± SE (n = 3). Asterisks indicate significant differences compared with wild-type plants (paired test): *, P < 0.05; **, P < 0.01. Wild-type plants (WT) were used as the control (#).

(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 Nicotiana benthamiana leaves. Each data bar represents the means ± SE (n = 5). Asterisks indicate significant differences: *, P < 0.05; # is the control.

(D) GUS staining showing the expression patterns of PHT1;1 in the WRKY42-overexpression lines and wild-type plants. The plants were germinated and grown on MS medium for 7 days and then harvested for GUS staining.
Figure 9. WRKY42 Binds to the PHT1;1 Promoter.
(A) Diagram of the PHT1;1 promoter region showing the number and relative position of the W-boxes (gray rectangles), and 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) conditions for another 7 d, and then the roots were harvested for ChIP-qPCR assay with anti-WRKY42. The ratio of IP DNA over the input was presented as the percentage of input (% IP). The data are presented as means ± SE (n = 3).
(C) EMSA assay to analyze the binding of WRKY42 to P2 fragment of PHT1;1 promoter. Each biotin-labeled DNA probe was incubated with WRKY42–His 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.
Figure 10. WRKY42 is Degraded during Pi Starvation.

(A) Cell-free degradation assay. Seven-day-old wild-type seedlings were transferred to MS medium (+P) or LP medium (−P) for another 5 d, and then the seedlings were harvested for protein extraction. The plant protein extracts were incubated with recombinant WRKY42-His for the indicated time, and 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.
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