Glutathione activates *PHT1;5* gene via WRKY75 transcription factor to regulate phosphate homeostasis in Arabidopsis

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Summary statement

Glutathione regulates Pi translocation in Arabidopsis under Pi deficiency via transcriptional activation of *AtPHT1;5* gene by the Pi-deficiency responsive *At*WRKY75 transcription factor.

Abstract

Phosphorus is a macronutrient that regulates a wide range of physiological processes, including plant growth and development. The scarcity of bioavailable inorganic phosphate (Pi) is often a constraint for plant growth in most agricultural ecosystems. Under Pi-limited conditions, plants increase Pi acquisition or alter Pi distribution within tissues by activating different Pi transporters and Pi starvation-induced genes. Glutathione (GSH), on the other hand, is known to play a critical role in regulating iron and zinc homeostasis in Arabidopsis. However, how it regulates Pi homeostasis under Pi-limited conditions remains unexplored. We found that the two GSH-deficient mutants, *cad2-1*, and *pad2-1*, were hypersensitive to Pi starvation. Under Pi-sufficient condition, the root Pi content was lower in the mutants but significantly higher in the shoot over the Col-0 plants. The pattern was reversed under Pi-deficient condition. Interestingly, among the different Pi transporters analyzed, the *AtPHT1;5* was significantly down-regulated in the two GSH-deficient mutants. Treatment with exogenous GSH could alter Pi accumulation in Col-0 and the *pht1;5* complementation lines but not in the *pht1;5* mutant. This observation confirmed the role of GSH in Pi reallocation during Pi starvation via the *At*PHT1;5 transporter. Further, promoter analysis demonstrated that GSH regulates the transcription of the *AtPHT1;5* gene. The Pi-responsive transcription factor (TF), *At*WRKY75 was identified to interact with the *AtPHT1;5* promoter, and Pi deficiency or GSH treatment enhanced its binding affinity. Together our investigation delineates that GSH regulates the *At*PHT1;5 transporter via the *At*WRKY75 TF to modulate Pi distribution under altered Pi conditions.

Introduction

Phosphorous (P) is an essential macronutrient that regulates various physiological phenomena in plants. It serves as a crucial component of nucleic acids, phospholipids, and different sugar-phosphate intermediates. Moreover, the phosphorylation of regulatory proteins and enzymes plays a critical role in modulating various signal transduction pathways (Poirier and Bucher, 2002). In plants, inorganic phosphate (Pi) is the primary source of P taken up by the roots from the soil. However, Pi is poorly available to plants due to its high reactivity with soil calcium, aluminum, or iron (Fe), leading to its precipitation or incorporation into organic compounds (Bieleski, 1973; Rodriguez and Fraga, 1999). Therefore, Pi deficiency is a severe constraint for plant growth and development. Plants have evolved intricate mechanisms to improve the uptake and utilization of Pi from the rhizosphere as well as to reallocate the internal Pi reserve for maintaining Pi homeostasis under P-limited conditions. Pi deficiency alters root system architecture (RSA), impairs photosynthesis, triggers the synthesis of acid phosphatases, ribonucleases, and organic acids, and induces anthocyanin and starch accumulation in plants (Vance et al., 2003; Yuan and Liu, 2008). Since the concentration of Pi in a plant cell is 10^2-10^3 times higher than that in the soil, plants transport Pi against the concentration gradient with the help of different low and high-affinity phosphate transporters (Shin et al., 2004). Earlier, three important Pi transporter families, *viz*. phosphate transporter (PHT) family, SYG1/Pho81/ XPR1 (SPX) domain-containing protein family, and sulfate transporter (SULTR)-like family, were reported in plants (Liu et al., 2017). Among them, the PHT family transporters, which play a crucial role in Pi acquisition and allocation, are categorized into five groups based on their subcellular localization (Liu et al., 2016). For example, PHT1 localizes on the plasma membrane, while PHT2 and PHT4 are on the chloroplast membrane. On the other hand, PHT3 and PHT5 localize on mitochondrial and vacuolar membranes, respectively.

The PHT1 family of Pi transporters comprises nine members in Arabidopsis, located on the 1, 2, 3, and 5 chromosomes (Mudge et al., 2002; Nussaume et al., 2011). Interestingly, four PHT1 family genes, *PHT1;1*, *PHT1;2*, *PHT1;3*, and *PHT1;6*, are clustered on chromosome 5, indicating a gene duplication during the evolution of the PHT1 family in the Arabidopsis genome (Mudge et al., 2002). The family functions as H^+/Pi symporters during Pi uptake from the soil and its distribution within plants (Zhang et al., 2014). The PHT1;1 plays a dominant role in Pi uptake from the rhizosphere under high Pi conditions, while PHT1;4

predominantly helps in Pi acquisition under Pi-limited conditions. Besides, PHT1;2, PHT1;3, and PHT1;5 also function in Pi uptake from soil (Misson et al., 2004; Shin et al., 2004; Nagarajan et al., 2011; Ayadi et al., 2015). Apart from Pi acquisition by roots, Pi allocation and transport to different tissues are also crucial for Pi homeostasis in plants. The PHT1;8 and PHT1;9 transporters function in root-to-shoot Pi translocation in co-operation with the PHO1, PHT1;3 and PHT1;4 (Hamburger et al., 2002; Mudge et al., 2002; Lapis-Gaza et al., 2014). Furthermore, Pi rescue from senescing leaves offers a way for recycling Pi reserves in plants. PHT1;5 is vital to the process. Similarly, the PHT1;5 transporter is implicated in Pi redistribution from source to sink tissues in response to developmental signals and Pi status in the soil (Nagarajan et al., 2011). Although Pi deprivation dramatically increases the expression of several PHT1 genes, others are expressed irrespective of the soil Pi concentration. The low Pi signal is often transduced by several Pi-responsive transcription factors (TFs) like PHOSPHATE STARVATION RESPONSE 1 (PHR1), WRKY, and MYB domain-containing families to activate specific PHT1 transporters (Rubio et al., 2001; Gu et al., 2016). Tight control of Pi transporters allows efficient Pi use in plants during Pi deficiency.

Glutathione (GSH) is a ubiquitous tripeptide that serves as a master regulator of plant growth, development, and stress response. It functions in reactive oxygen species (ROS) detoxification, redox homeostasis, and defense signaling network under various biotic and abiotic stress conditions (Noctor and Foyer, 1998; Foyer and Noctor, 2005; Foyer and Noctor, 2011; Noctor et al., 2012). In addition, GSH can impart tolerance to different metal toxicity in plants by activating phytochelatin synthesis (Ha et al., 1999; Chao et al., 2009; Bianucci et al., 2012; Seth et al., 2012; Cui et al., 2014; Jozefczak et al., 2014). Several GSH-deficient Arabidopsis mutants with varying levels of susceptibility to biotic and abiotic stress factors have been identified. Among them, the *cadmium sensitive 2-1* (*cad2-1*) mutant displays severe sensitivity to cadmium and possesses around 30% GSH content than that in wild-type plants (Howden et al., 1995). On the other hand, the *phytoalexin deficient 2-1* (*pad2-1*) mutants containing 22% GSH exhibits susceptibility to different pathogen infections (Glazebrook and Ausubel, 1994; Parisy et al., 2007).

GSH is also involved in modulating nutrient homeostasis in Arabidopsis. For example, the *zir1* mutant that contains 15% GSH displayed increased sensitivity to Fe-limited conditions (Shanmugam et al., 2012). During Fe deficiency, GSH maintains the redox

homeostasis in the cell and increases the uptake and accumulation of Fe by activating different Fe-responsive genes like *FRO2*, *FIT1*, and *IRT1* in Arabidopsis (Koen et al., 2012; Ramírez et al., 2013; Shanmugam et al., 2015). Earlier, we reported that the GSH-GSNO module regulates subcellular Fe homeostasis by modulating *At*NRAMP3, *At*NRAMP4, and *At*PIC1 transporters during Fe deficiency (Shee et al., 2022). However, the role of GSH in modulating the Pi transport or homeostasis remains unexplored. Although GSH-indole butyric acid (IBA) crosstalk was established for alteration of RSA under Pi-deprived condition, the mechanism of GSH-mediated Pi homeostasis is not elucidated (Trujilo Hernandez et al., 2020). Here we report that GSH modulates source to sink Pi transport via transcriptional activation of the *AtPHT1;5* transporter through the *At*WRKY75 TF under altered Pi conditions.

Results

GSH-deficient mutants display sensitivity to Pi deprivation

We analyzed the response of the GSH-deficient mutants, *cad2-1*, and *pad2-1*, along with the wild-type (Col-0) plants under the Pi-deprived condition. The standard Murashige and Skoog (MS) medium (1 mM KH_2PO_4; +P) was used as a Pi-sufficient medium, while the Pi-deficient medium (0 mM KH_2PO_4; 1 mM KCl; -P) lacked any Pi source. Under the Pi-sufficient condition, neither *cad2-1* nor *pad2-1* showed any phenotypic alteration compared to the Col-0 plants. In response to 7 d of Pi starvation, all three genotypes displayed smaller rosettes, shorter primary roots, and increased lateral root density compared to the Pi-sufficient condition. The *pad2-1* mutant, however, exhibited severe sensitivity to Pi deficiency with a reduced rosette diameter, lower lateral root number, and density over the Col-0 plants (Fig. 1A-E). Although the chlorophyll content dropped in all three genotypes in response to Pi deficiency, the drop was sharper in *pad2-1* (Fig. 1F). On the contrary, the increase in the anthocyanin content under Pi-deprived condition was significantly lower in both *cad2-1* and *pad2-1* compared to the Col-0 plants (Fig. 1G).

To assess any possible Pi-GSH interplay, we estimated the GSH and Pi contents under Pi-sufficient and Pi-deficient conditions. Under Pi-sufficient condition, the root Pi content was significantly lower in both *cad2-1* and *pad2-1* mutants. In response to Pi deprivation, however, the *pad2-1* mutant displayed a higher root Pi accumulation over the *cad2-1* and Col-0 plants (Fig. 2A). In addition, the total root GSH content increased, but the GSH:GSSG

ratio decreased in all three genotypes when exposed to Pi starvation (Fig. 2B-C). Although the total GSH content increased and GSH:GSSG ratio decreased under the Pi-limited condition in the shoot, the Pi status displayed a contrasting pattern (Fig. 2D-F). Under the Pi-sufficient condition, the shoot Pi content was significantly higher in the mutants, while it was lower under the Pi-deficient condition.

GSH regulates the AtPht1;5 gene to modulate Pi transport

The sensitivity of the GSH-deficient mutants to Pi starvation and their altered Pi status prompted us to investigate if GSH can regulate any Pi transporter genes. To this end, we analyzed the expression of several known Pi transporters from the root and the shoot under Pi-sufficient condition and compared their relative transcript abundance between the Col-0 and the GSH-deficient mutants. Interestingly, the *AtPHT1;5* expression was significantly down-regulated in both the mutants compared to the Col-0 plants (Fig. 3A-B). Although Pi deficiency induced the *AtPHT1;5* expression in all three genotypes, its transcript abundance was significantly lower in the mutants than the Col-0 plants (Fig. 3C-D).

Further, we examined the *AtPHT1;5* expression in the Col-0 plants under exogenously altered GSH conditions. The total GSH content increased in response to exogenous GSH treatment, while the GSH biosynthesis inhibitor, buthionine sulphoximine (BSO), decreased it. However, treatment with the non-specific reducing agent, dithiothreitol (DTT), did not alter the total GSH content (Fig. 3E, H). Interestingly, the root Pi content increased significantly in response to GSH treatment but decreased when treated with BSO. The shoot, on the other hand, displayed an opposite pattern. However, the treatment with DTT failed to alter the Pi content in both the root and the shoot (Fig. 3F, I). In line with this observation, the *AtPHT1;5* expression was up-regulated in response to GSH treatment and down-regulated by BSO treatment, while DTT treatment did not alter its expression in root as well as shoot (Fig. 3G, J). Together these observations strongly suggest a GSH-mediated regulation of Pi transport through the *AtPHT1;5* transporter.

GSH modulates Pi mobilization during Pi starvation by regulating AtPht1;5 expression

To further examine the role of GSH in regulating Pi mobilization, we analyzed the response of the *pht1;5* mutant to exogenous GSH treatment. We generated complementation lines harboring the *AtPHT1;5* gene under the control of its native promoter in the *pht1;5* mutant

background and analyzed them (Fig. 4A). The Col-0, *pht1;5* mutant, and complementation lines displayed similar total GSH contents, which increased in response to Pi starvation. When treated with exogenous GSH, the total GSH content increased in all three genotypes under both Pi-sufficient and Pi-deficient conditions (Fig. 4B-C). Notably, the *pht1;5* mutant displayed altered root-to-shoot Pi allocation under both Pi-sufficient and Pi-deficient conditions. When grown under Pi-sufficient conditions, the *pht1;5* mutant accumulated lower Pi content in the root but higher Pi content in the shoot compared to the Col-0 plants. However, under Pi-deficient conditions, it displayed a higher Pi accumulation in the root but a lower Pi content in the shoot. The complementation lines, on the contrary, showed similar Pi levels as that of the Col-0 plants (Fig. 4D-E). Fascinatingly, exogenous GSH treatment could enhance Pi content in the root of the Col-0 and the complementation lines under Pi-sufficient condition but slightly reduced it during Pi starvation. In the shoot, the Pi content decreased in response to GSH supplementation under the Pi-sufficient condition, while it increased under Pi deficiency. On the contrary, exogenous GSH treatment could not alter the root and the shoot Pi contents of the *pht1;5* mutant under both Pi-sufficient and Pi-deficient conditions (Fig. 4D-E). This observation strongly suggests that GSH is involved in modulating root-to-shoot Pi allocation through the *At*PHT1;5 transporter to regulate Pi homeostasis during Pi starvation.

GSH modulates the transcriptional activation of AtPHT1;5 gene

Since GSH could modulate Pi allocation via the *AtPHT1;5* transporter, the next pertinent question was how GSH could modulate the *AtPHT1;5* expression. To gain a deeper insight, we cloned the *AtPHT1;5* promoter (*pPHT1;5*) and raised transgenic Arabidopsis lines harboring the *pPHT1;5*::*GUS* construct in the background of Col-0. A transgenic line containing the *CaMV35S::GUS* construct served as a negative control. Promoter analysis by histochemical GUS assay demonstrated that the *AtPHT1;5* promoter activity was strongly induced in response to Pi starvation, indicating its Pi-responsive nature. Interestingly, exogenous GSH treatment also significantly enhanced the GUS activity in this transgenic line indicating the role of GSH in modulating *AtPHT1;5* promoter activity (Fig. 5A). In line with this observation, the *GUS* gene expression was also strongly up-regulated in response to both Pi starvation and exogenous GSH treatment (Fig. 5B). However, both conditions did not alter the GUS activity and expression in the transgenic line harboring the *CaMV35S::GUS*

construct (Fig. 5C-D). This observation demonstrated that the GSH-mediated regulation of *AtPHT1;5* occurs at the transcriptional level.

GSH regulates AtPHT1;5 expression via the AtWRKY75 TF

GSH can not directly bind to the promoter to regulate transcription. Therefore, we were curious if any TF is involved in the process. Several WRKY and MYB TFs, like *At*WRKY6, *At*WRKY42, *At*WRKY45, *At*WRKY75, *At*MYB2, and *At*MYB62, are known to regulate Pi-responsive genes in Arabidopsis. We performed yeast one-hybrid (Y1H) assay to check if these TFs can interact with the *AtPHT1;5* promoter and regulate its transcription. We observed that the *At*WRKY42, *At*WRKY75, and *At*MYB2 could bind to the *AtPHT1;5* promoter, while others did not (Fig. 6A). Next, we wondered if all these three TFs were involved in the GSH-mediated regulation of *AtPHT1;5* under Pi-deficient condition. Interestingly, we found that among these TFs, only the *AtWRKY75* expression was down-regulated in the GSH-deficient mutants, *cad2-1*, and *pad2-1* (Fig. 6B). We further analyzed the relative transcript abundance of the *AtPHT1;5* gene in the mutants of the three TFs under Pi-sufficient and Pi-deficient conditions. Supporting our previous observation, the *AtPHT1;5* expression was induced in response to Pi starvation in the Col-0, *wrky42*, and *myb2* mutants but not in the *wrky75* mutant (Fig. 6C). This observation indicated that the *At*WRKY75 TF was essential for the Pi-responsiveness of the *AtPHT1;5* gene. The dual luciferase transactivation assay further confirmed the *AtPHT1;5* promoter activation by the *At*WRKY75 TF (Fig. 6D). We next aimed to identify the region in the *AtPHT1;5* promoter where *At*WRKY75 specifically binds. *In silico* analysis revealed the presence of three w-box (TTGAC[CT]) motifs in the *AtPHT1;5* promoter region at -99, -176, and -201 positions (Fig. S). Chromatin immunoprecipitation (ChIP) assay confirmed the binding of the *At*WRKY75 TF to the w-box regions of the *AtPHT1;5* promoter with an enhanced binding affinity in response to Pi deficiency or exogenous GSH treatment (Fig. 6E). Next, we wanted to analyze the *AtPHT1;5* promoter activity when the *At*WRKY75 TF was absent. Therefore, we expressed the *pPHT1,5::GUS* construct in the background of the *wrky75* mutant. A *CaMV35S::GUS* construct in the same background served as a negative control. We observed that neither Pi starvation nor exogenous GSH treatment could induce the *AtPHT1;5* promoter activity as evident from the histochemical GUS activity or *GUS* gene expression (Fig. 6F-G). Together these observations strongly suggest that the GSH-mediated transcriptional induction of *AtPHT1;5* occurs via the *At*WRKY75 TF.

Discussion

Over the last decade, accumulating evidence has revealed that GSH can regulate the transport and homeostasis of several nutrients like Fe and zinc in plants (Shanmugam et al., 2011; Koen et al., 2012; Ramirez et al., 2013; Shanmugam et al., 2015; Shee et al., 2021). A recent report has further demonstrated its role in regulating root hair growth under Pi deficiency via an IBA-dependent pathway (Trujilo-Hernandez et al., 2020). However, no comprehensive investigation has been conducted to date to examine the precise mechanism of how GSH regulates Pi acquisition or reallocation in plants. In this study, we discovered that GSH regulates the root and shoot Pi contents in Arabidopsis, depending on the Pi availability.

The two GSH-deficient mutants, *cad2-1*, and *pad2-1* exhibited hypersensitivity to Pi deprivation with alterations in their RSA. Changes in RSA are an adaptive strategy for plants under Pi-limited conditions (Peret et al., 2014). Low Pi availability promoted lateral root growth over primary root by boosting lateral root density and length and limiting primary root growth by reducing cell elongation (Williamson et al., 2001; López-Bucio et al., 2002). The *pad2-1* mutant, which contains only 22% GSH, exhibited severe sensitivity to Pi deficiency with reduced lateral root density and rosette diameter compared to the wild-type. Another characteristic Pi deficiency response is a change in pigment composition, which includes chlorophyll degradation and elevated anthocyanin accumulation (Marschner, 2012; He et al., 2020). With the reduction in photosynthesis rates, the Pi-deficient plants have evolved to sustain photo-oxidative stress through anthocyanin accumulation (Hernández and Munné-Bosch, 2015). In this study, the GSH-deficient mutants displayed lower anthocyanin accumulation under Pi-deficient conditions than the wild type, indicating enhanced sensitivity. The chlorophyll loss under Pi deprivation was also maximal in the *pad2-1* mutant. These observations resemble an earlier report where the *cad2-1* displayed increased sensitivity to Pi depletion (Trujilo-Hernandez et al., 2020), indicating that the optimum GSH level is essential for adaptation under Pi-limited conditions.

Plants have various Pi transport mechanisms comprising different high- and low-affinity transporters that allow Pi acquisition and distribution within the plant. Since the source and sink relationships are continually changing depending on the developmental stage and Pi availability, the Pi transport in plants is an elaborate and complex process (Raghothama, 1999; Bucher et al., 2001; Miller et al., 2009). To analyze if GSH can regulate Pi acquisition or distribution in plants, we measured the Pi content from the GSH-deficient mutants and the

wild-type under Pi-sufficient and Pi-deficient conditions. Under Pi-sufficient conditions, both *cad2-1* and *pad2-1* mutants displayed higher Pi accumulation in shoots but lower Pi content in roots than in the wild-type. The results were, however, reversed under Pi-deficient conditions. Exogenously altering GSH content in the Col-0 plants resulted in a similar Pi distribution pattern, confirming our previous observation. These observations suggested a plausible role of GSH in modulating the Pi reallocation between roots and shoots based on the Pi availability status. Several Pi transporters are known to regulate Pi homeostasis through Pi translocation and redistribution in plants (Mudge et al., 2002; Shin et al., 2004; Nagarajan et al., 2011; Lapis-Gaza et al., 2014). Our experiments identified that GSH regulates the expression of the *AtPHT1;5* under Pi sufficient and Pi deficient conditions. Among the PHT1 members, PHT1;5 is expressed exclusively in phloem cells of older leaves, cotyledons, and flowers during Pi starvation (Mudge et al., 2002). Another genome-wide transcriptome study reported the *PHT1;5* expression in the senescent Arabidopsis leaves (van der Graaff et al., 2006). Intriguingly, PHT1;5 plays a pivotal role in transporting Pi from source to sink organs in response to developmental signals and Pi availability. During Pi starvation, the loss-of-function mutation of the *PHT1;5* gene resulted in decreased P allocation to shoots, while under Pi-replete conditions, the mutant exhibited higher shoot P content over the wild type but lower P content in the roots (Nagarajan et al., 2011). Notably, the GSH-deficient mutants in our investigation displayed a strikingly similar Pi distribution pattern with that of the *pht1;5* mutant. We hypothesized that the GSH-mediated modulation of source-to-sink Pi allocation involves the PHT1;5 transporter. To test this hypothesis, we measured the Pi content of the *pht1;5* mutants and its complementation lines under Pi-sufficient and Pi-deficient conditions in response to exogenous GSH treatment. Under both Pi-sufficient and Pi-deficient conditions, exogenous GSH altered the root and shoot Pi contents in the wild-type and complementation lines. However, the Pi content in the *pht1;5* mutants remained unchanged. These findings strongly suggest that GSH not only regulates the expression of the *AtPHT1;5* gene, but also integrates the source-to-sink Pi allocation mechanism via this transporter in accordance with the Pi availability status.

The next pertinent question is how GSH regulates the *AtPHT1;5* expression. Since GSH alters the relative transcript abundance of the transporter, transcriptional regulation is the most likely reason. Our experiments demonstrated that the *AtPHT1;5* promoter is inducible under Pi-deficient conditions. The promoter activity is also induced in response to exogenous GSH treatment indicating a GSH-mediated transcriptional regulation of the *AtPHT1;5* gene.

However, being a metabolite GSH cannot directly interact with the promoter to modulate its activity. Therefore, we were curious if any TF is involved in the process. Earlier reports suggested that GSH can activate different promoters via their TF. For example, GSH can modulate ethylene biosynthetic pathway genes via the WRKY33 TF (Datta et al., 2015). In another study, we reported that under Fe-limited conditions, GSH can regulate the NRAMP3/4 and PIC1 genes via *S*-nitrosylation of Fe-responsive bHLH TFs (Shee et al., 2022). Several TFs are reported to regulate the expression of different Pi starvation-induced genes. Previously, it was reported that the WRKY6 and WRKY42 TFs function as repressors of the Pi transporter, PHO1 (Chen et al., 2009). Under low Pi conditions, these TFs are degraded, allowing the expression of the *PHO1* gene and subsequent regulation of Pi homeostasis. WRKY75 has long been known to regulate Pi acquisition and root development in plants (Devaiah et al., 2007). Another TF, WRKY45 can directly bind to the w-box of the *PHT1;1* promoter to activate its expression under Pi-limited conditions (Wang et al., 2014). Among the different MYB TFs, MYB2 and MYB62 play a crucial role in regulating Pi starvation response. While MYB2 directly interacts with the promoter of miR399, a marker of Pi starvation response, MYB62 regulates the Pi starvation response via the gibberellic acid-mediated signaling pathway (Devaiah et al., 2009; Baek et al., 2013). In the present investigation, *At*WRKY42, *At*WRKY75, and *At*MYB2 were identified to interact with the *AtPHT;5* promoter. Among these three TFs, the *AtPHT1;5* expression was significantly lower in the *wrky7*5 mutant. This data corroborates the previous report where *AtPHT1;5* expression was diminished in the wrky75-RNAi lines under both Pi-sufficient and Pi-deficient conditions (Nagarajan et al., 2011). Moreover, the *AtWRKY75* expression was significantly down-regulated in the GSH-deficient *pad2-1* mutant, which supports the previous report where GSH-fed Col-0 leaves displayed higher WRKY75 expression over the control condition (Sinha et al., 2014). We have further demonstrated that the *At*WRKY75 binds to the w-box motifs on the *AtPHT1;5* promoter and its binding affinity increases in response to Pi starvation or GSH treatment. These data delineate that GSH induces *AtPHT1;5* expressions under Pi-deficient condition via the *At*WRKY75 TF.

In summary, it can be concluded that GSH is highly accumulated under Pi starvation to regulate the Pi translocation in plants. This regulation involves the GSH-mediated transcriptional activation of *AtPHT1;5* transporter via the Pi-responsive *At*WRKY75 TF.

Materials and Methods

Plant growth and stress treatment

Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0), that served as wild-type, and two GSH-deficient mutants, *cad2-1* (N68137), and *pad2-1* (N3804), as well as *pht1;5* mutant (N574836) were procured from Nottingham Arabidopsis Stock Centre, UK. Seeds of *myb2* (SALK_043075), *wrky42* (SALK_049063), and *wrky75* (CS121513) mutants were procured from Arabidopsis Biological Resource Centre, USA. Seeds were surface sterilized with 4% sodium hypochlorite and Tween20 and germinated on a standard MS medium. Plants were maintained in a growth chamber at 21±2°C, 60% relative humidity with the photoperiod of 16 h light/8 h dark cycles following Datta et al. (2015). For stress treatment, 7 d old seedlings were transferred to Pi sufficient (MS medium, +P) or Pi deficient (0 mM KH_2PO_4, supplemented with 1 mM KCl, -P) media and maintained for 7 d. After the stress treatment, the plants were photographed and morphological parameters, *viz*. primary root length, lateral root number, lateral root density, and rosette diameter were analyzed using Image J software.

Estimation of Pi content

Pi levels were determined following Zhou et al. (2008) from root and shoot samples colled under Pi-sufficient or Pi-deficient conditions.

Estimation of chlorophyll and anthocyanin contents

Total chlorophyll was estimated from the samples by homogenizing 200 mg tissue in 80% acetone, followed by centrifugation at 5000 g for 5 min. The total chlorophyll content was determined following Lichtenthaler (1987). Anthocyanin content was estimated according to Matsui et al. (2004). Briefly, samples were homogenized in liquid nitrogen and resuspended in 5 volumes of 45% methanol and 5% acetic acid (v/v). Following centrifugation at 5000 g for 5 min, optical density was measured at 530 nm and 637 nm using Double Beam UV-Vis 194 Spectrophotometer (U2900, Hitachi) and anthocyanin content was calculated.

Estimation of total GSH content and determination of GSH:GSSG ratio

Reduced GSH content was estimated by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) method as described by Anderson (1985). Briefly, 200 mg of tissue was homogenized in 5% sulphosalicylic acid and centrifuged at 12000 g for 20 min. The supernatant was allowed to react with 50 µM DTNB in reaction buffer [0.1 M phosphate buffer (pH 7) and 0.5 mM EDTA] for 5 min and optical density was measured at 412 nm using a Double Beam UV-Vis

Spectrophotometer (U2900, Hitachi). For estimation of total GSH, 0.4 mM NADPH and 1 U glutathione reductase were added and incubated for 20 min. The optical density was measured at 412 nm. The amount of GSSG was determined by subtracting the amount of reduced GSH from the total GSH.

RNA extraction and quantitative-RT PCR analysis

Trizol method was used for isolation of total RNA from samples. The complementary DNA (cDNA) was prepared using iScript™ cDNA Synthesis Kit (Bio-rad) as per manufacturer's protocol. Quantitative PCR amplification was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-rad) using iTaq™ Universal SYBR® Green Supermix (Bio-rad) and gene specific primers. *AtActin* 2 was used as a reference gene for normalization.

Chemical treatment of seedlings

15 d old seedlings were separately treated with freshly prepared 100 µM GSH or 1 mM BSO for 72 h or with 5 mM DTT for 24 h as standardized before (Datta et al., 2015).

Vector construction and raising of Arabidopsis transgenic lines

The genomic DNA was extracted from Col-0 plants following CTAB method. The 1500 bp sequence upstream of the +1 site of *AtPHT1;5* gene was PCR amplified using specific primers and cloned into *pCAMBIA1304* vector between *BglII* and *BamHI* restriction enzyme (RE) sites to generate *pPHT1;5*::*GUS* construct. The coding sequences of the *AtPHT1;5* and *AtWRKY75* genes were PCR amplified from cDNA using gene specific primers. The amplified *AtPHT1;5* gene was cloned into *pBI121* vector between *BamHI* and *SacI* RE sites to produce the *CaMV35S*::*PHT1;5* construct. The PCR amplified *AtPHT1;5* promoter was further sub-cloned into the recombinant *CaMV35S*::*PHT1;5* vector between *SphI* and *BamHI* RE sites to generate *pPHT1;5::PHT1;5* construct. The amplified *AtWRKY75* gene was cloned into *pVYCE* vector under control of the *CaMV35S* promoter between *SpeI* and *BamHI* RE sites to generate *CaMV35S::WRKY75-HA* construct. The recombinant *pPHT1;5*::*PHT1;5* construct was used for *Agrobacterium*-mediated genetic transformation of *pht1;5* mutant to raise the complementation lines whereas *CaMV35S::WRKY75-HA* construct was transformed into Col-0 plants to develop *AtWRKY75* overexpression lines following floral dip transformation method (Clough and Bent, 1998). *pPHT1;5*::*GUS* recombinant construct was similarly transformed into *wrky75* mutant. The vector control lines harboring the

CaMV35S::GUS construct was generated in the background of Col-0 as well as *wrky75* mutant. The putative transformants were selected by antibiotic screening (kanamycin for *pPHT1;5*::*GUS*, and *pPHT1;5*::*PHT1;5* whereas hygromycin for *CaMV35S::WRKY75-HA* and *CaMV35S::GUS*) and positive plants were selected by selectable marker specific PCR amplification. The selected plants were grown up to T_2 generation in a growth chamber as described above.

Histochemical GUS assay

The 7 d old seedlings from transgenic lines harboring the *pPHT1;5*::*GUS*, and *CaMV35S::GUS* constructs (in the background of Col-0 and *wrky75* mutant) were maintained under +P or -P conditions or treated with exogenous GSH as described above. The samples were then infiltrated with GUS staining solution (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% [v/v] Triton X-100, 100 mM phosphate buffer, pH 7.0, and 10 mM EDTA) and histochemical GUS assay was performed following Jefferson et al. (1987). Samples were washed with 70% alcohol and photographed.

Yeast one hybrid (Y1H) analysis

The PCR amplified promoter sequence of *AtPHT1;5* gene was cloned into *pAbAi* vector (Takara) between the *SacI* and *KpnI* RE sites following manufacturer's protocol. The cds of *AtWRKY6*, *AtWRKY42*, *AtWRKY45*, *AtWRKY75*, *AtMYB2*, and *AtMYB62* genes were amplified by PCR using gene specific primers and cloned into *pGADT7-Rec* vector (Takara). The recombinant constructs were transformed into Y1H gold strain (Takara). The transformed yeast strains were grown on SD/-Leu and SD/-Leu/Aureobasidin A media (Takara) and Y1H analysis was performed using Matchmaker® Gold Yeast One-hybrid screening system (Takara) according to the manufacturer's protocol. The interaction between p53-pAbAi was used as positive control and pAbAi alone was considered as negative control.

Dual luciferase transactivation assay

The promoter region of *AtPHT1;5* was cloned into *pGreen-LUC008* vector between *SpeI* and *KpnI* RE sites to drive the expression of firefly luciferase gene expression. The construct constitutively expressing Renilla luciferase (*REN*) gene and harbouring *pPHT1;5::LUC* cassette was used as reporter. The *CaMV35S::WRKY75-HA* construct was considered as effector. *Agrobacterium* strains harboring the recombinant constructs (effecter and reporter)

were mixed in 1:1 ratio (v/v) and used for *Agrobacterium*-mediated infiltration of *Nicotiana tabaccum* leaves (Sahid et al., 2020). For negative control, *Agrobacterium* strain harboring the effector construct and empty *pGreen-LUC008* vector were mixed into 1:1 ratio (v/v) and used for infiltration (Zhang et al., 2015). After incubation for 48 h at dark, infiltrated leaves were harvested and total protein was isolated. 1 µg protein was used for dual luciferase transactivation assay using Dual-Luciferase® Reporter Assay System (Promega) following manufacturer's protocol. The luminescence was measured using the Varioskan LUX luminometer (Thermo scientific) and relative luciferase activity was calculated using the relative LUC/REN ratio.

CHIP-qPCR analysis

The *CaMV35S::WRKY75-HA* harboring transgenic line was used for ChIP assay following Cheng et al. (2020) with slight modifications. Briefly, 7 d old seedlings were maintained under +P or -P conditions or treated with exogenous GSH as described above. 800 mg tissue from each sample was harvested, washed with milliQ water, cross-linked with 1% (v/v) formaldehyde solution under vacuum for 10 min, and homogenized in liquid nitrogen. The chromatin complex was isolated, and sheared by sonication (Hielscher Ultrasonic processor, UP200S). The complex was then incubated with 2 µg of anti-HA antibody (Sigma). The precipitated DNA was recovered and ChIP qPCR was performed using EpiQuik™ Chromatin Immunoprecipitation (ChIP) Kit (Epigentek). For the negative control, non-immune IgG antibody was used. The real time PCR was performed in triplicate using CFX96 Touch™ Real-Time PCR Detection System (Bio-rad) using iTaq™ Universal SYBR® Green Supermix (Bio-rad) and two sets of w-box specific primers of *AtPHT1;5* promoter. The chromatin without any antibody treatment was used as input DNA. The %input was calculated from Ct values.

In silico promoter analysis

The presence of w-box motif was identified in the promoter region of *AtPHT1;5* using PlantPan 3.0 (plantpan.itps.ncku.edu.tw/promoter.php) and MEME suite 5.3.3 (https://memesuite.org/).

Statistical analysis

The data was statistically analyzed using GraphPad Prism version 8.3.0 software (GraphPad Software, San Diego, California USA). The variation of morphological and biochemical parameters, as well as relative transcript abundance, were evaluated using one-way ANOVA (Fig. 3A-B,E-J, 5) or two-way ANOVA (Fig. 1, 2, 3C-D, 4, 6B-C, E, G) followed by Dunnett's multiple comparison tests. To determine the difference between two sets of data, statistical significance at $P \leq 0.05$ was used. Details of statistical tests, replicates, sample size, and the significance levels of P-values were indicated in respective figure legends. The data was provided as mean ± standard error of mean (SEM).

Conflict of interest

Authors declare no conflict of interest.

Author Contributions

RD and SP conceived and designed the research plan; RS performed most of the experiments; DS performed *in silico* analysis and Y1H analysis, SS generated the constructs, RD and SP analyzed the data and prepared the manuscript.

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Figure Legends

FIGURE 1 Response of GSH-deficient mutants to Pi starvation. 7 d old MS grown seedlings of Col-0, *cad2-1* and *pad2-1* were exposed to +P and -P conditions for 7 d and analyzed. (A) Representative images of plants grown under +P and –P conditions, (B) rosette diameter, (C) primary root length, and (D) lateral root number (E) lateral root densit,y (F) chlorophyll content, and (G) anthocyanin content. The experiment was independently repeated thrice and results were represented as mean±SEM (n=15). Statistical differences between the genotypes were analyzed by two-way ANOVA followed by Dunnett's multiple comparison test and statistical significance were denoted by asterisks at P<0.05 (*), P< 0.001 (***), and P< 0.0001 (****).

FIGURE 2 Estimation of Pi content, total GSH and GSH:GSSG ratio under +P and -P conditions. 7 d old MS grown Col-0, *cad2-1* and *pad2-1* seedlings were exposed to -P condition for 7 d and analyzed. Control plants were maintained in the MS medium for the entire period. (A) Root Pi content, (B) total root GSH content, (C) root GSH:GSSG, (D) shoot Pi content, (E) total shoot GSH content, and (F) shoot GSH:GSSG ratio. The experiment was independently repeated thrice and results were represented as mean±SEM (n=9). Statistical differences between the genotypes were analyzed by two-way ANOVA followed by Dunnett's multiple comparison test and statistical significances were denoted by asterisks at P<0.05 (*), P<0.01 (**), P< 0.001 (***), and P< 0.0001 (****).

FIGURE 3 Expression analyses of different Pi transporters under altered GSH conditions. 14 d old MS grown Col-0, *cad2-1*, and *pad2-1* plants were analyzed for the expression pattern of various Pi transporter genes. Expression of different Pi-transporters from root (A) and shoot (B) tissues was depicted as heat map. Relative transcript abundance of *AtPHT1;5* gene was analyzed from root (C) and shoot (D) of 7 d old Col-0, *cad2-1*, and *pad2-1* seedlings grown under +P and -P conditions for additional 7 d. 14 d old MS grown Col-0 plants were treated with 100 μM GSH, or 1 mM BSO, or 5 mM DTT solutions and analyzed. Control plants were maintained in half strength MS medium for the entire duration. (E) Total root GSH content, (F) root Pi content, (G) relative transcript abundance of *AtPHT1;5* gene in roots, (H) total shoot GSH content, (i) shoot Pi content, and (j) relative transcript abundance of *AtPHT1;5* gene in shoots. Results were represented as mean±SEM (n=3). Statistical differences between the genotypes or treatments were analyzed by one-way ANOVA (A,B, E-J) or two-way ANOVA (C-D) followed by Dunnett's multiple comparison

test and statistical significance was denoted by asterisk at P<0.05 (*), P<0.01 (**), P< 0.001 (***), and P< 0.0001 (****).

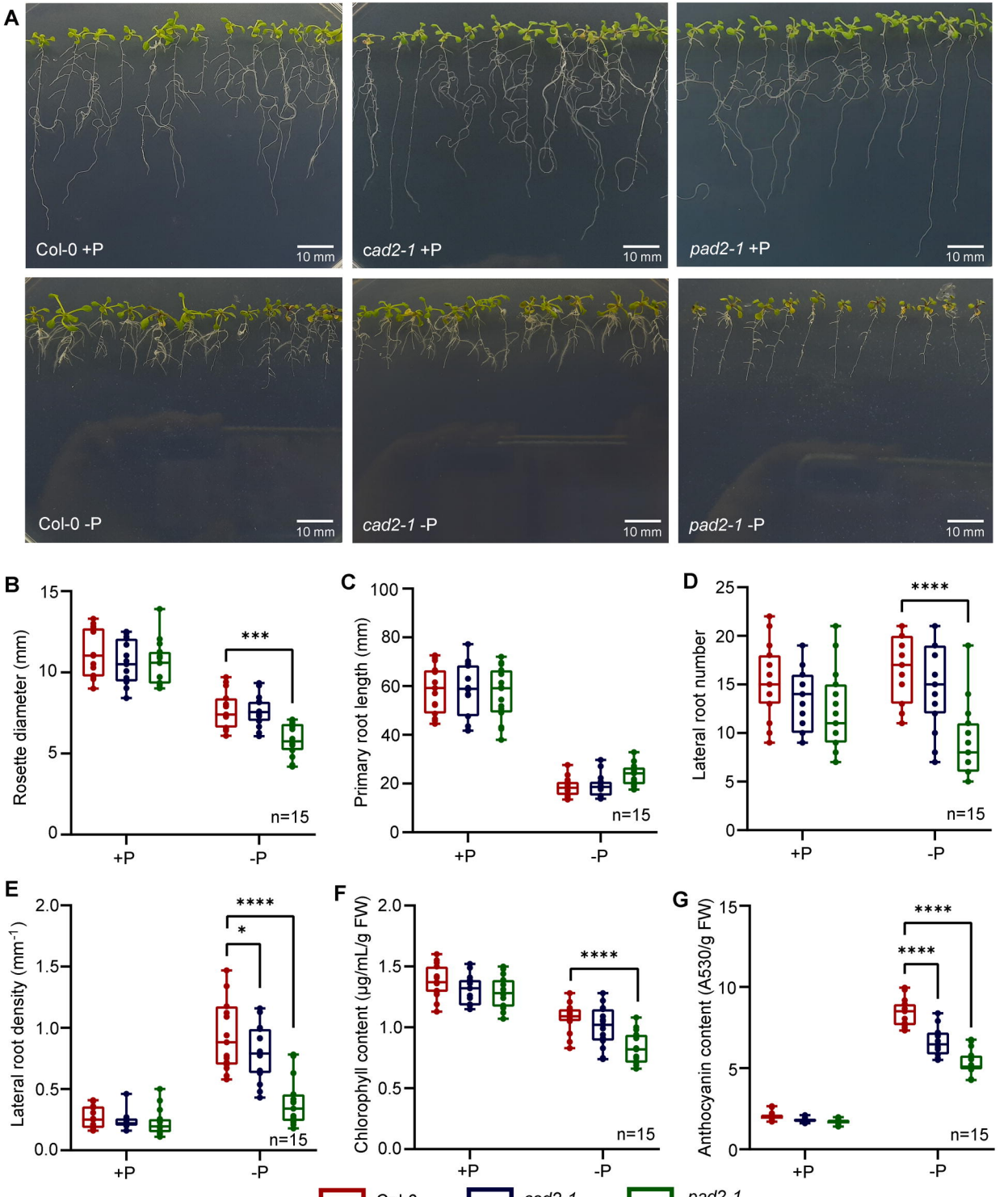
FIGURE 4 Response of *pht1;5* mutant and complementation lines to exogenous GSH under Pi-sufficient and Pi-deficient conditions. 7 d old MS grown seedlings of Col-0, *pht1;5* mutant and complementation (*pht1;5/pPHT1;5::PHT1;5*) lines were treated with 100 μM GSH, exposed to +P and -P conditions for 7 d and analyzed. (A) Representative images of plants grown under +P, +P+GSH, -P, and –P+GSH conditions, (B) total root GSH content, (C) total shoot GSH content, (D) root Pi content, and (E) shoot Pi content. The experiment was independently repeated thrice and results were represented as mean±SEM (n=9). Statistical differences between the genotypes were analyzed by two-way ANOVA followed by Dunnett's multiple comparison test and statistical significance was denoted by asterisks P<0.01 (**), and P< 0.0001 (****). Scale bar: 1 cm

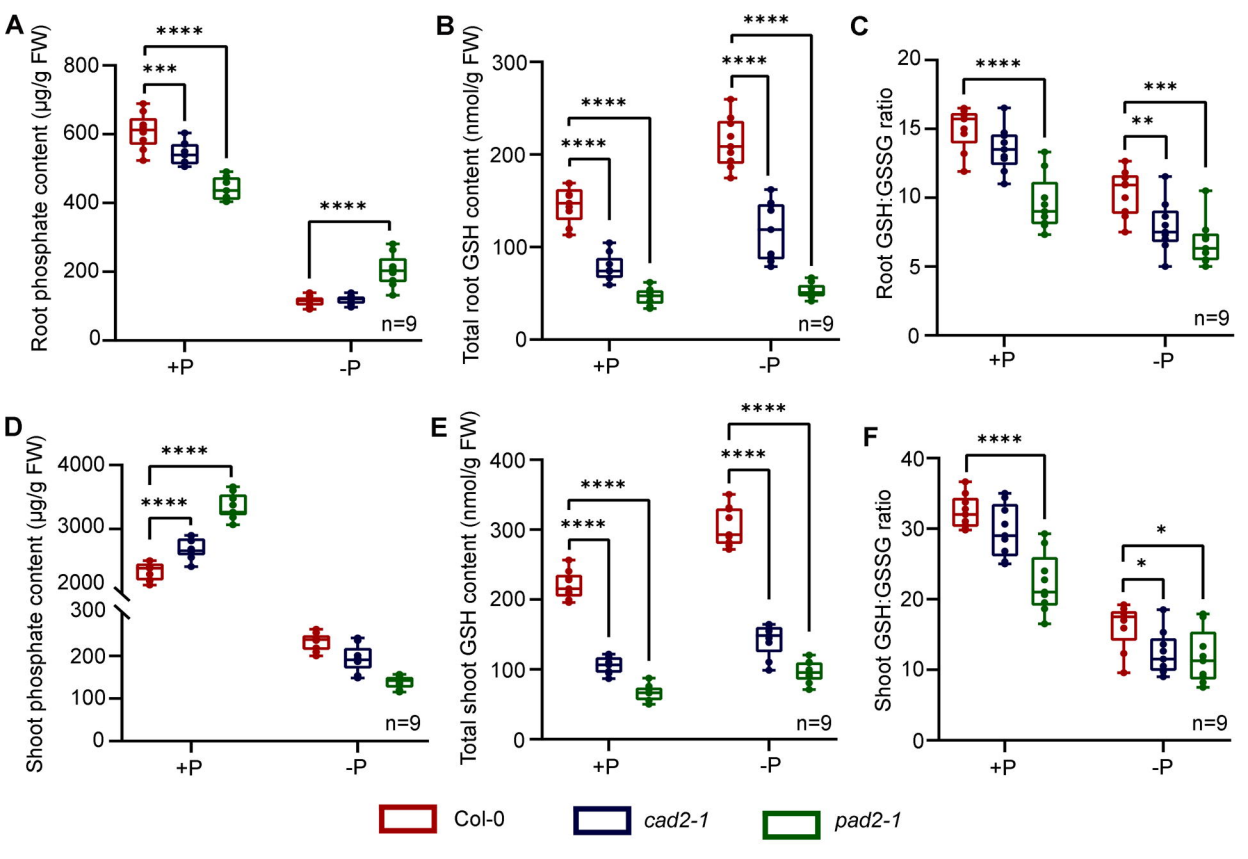
FIGURE 5 Promoter analyses of the *AtPHT1;5* gene. The seedlings of transgenic lines harboring *pPHT1;5::GUS* construct and the vector control (*35S::GUS*) lines were exposed to +P, –P, and +P+GSH conditions and used for promoter analysis by histochemical GUS assay and qRT-PCR analysis for *GUS* gene expression. (A) Representative images of histochemical GUS staining of transgenic line harboring *pPHT1;5::GUS* construct, (B) relative transcript abundance of *GUS* (*uidA*) gene of the same, (C) representative images of histochemical GUS staining of vector control line harboring the *35S::GUS* construct (negative control), and (D) relative transcript abundance of *GUS* (*uidA*) gene of the same. The experiment was independently repeated thrice. For relative transcript abundance, results were represented as mean±SEM (n=3). Statistical differences between the treatments were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test and statistical significance of relative gene expression was denoted by different asterisks at P<0.01 (**), and P< 0.001 (***). Scale bar: 0.5 cm

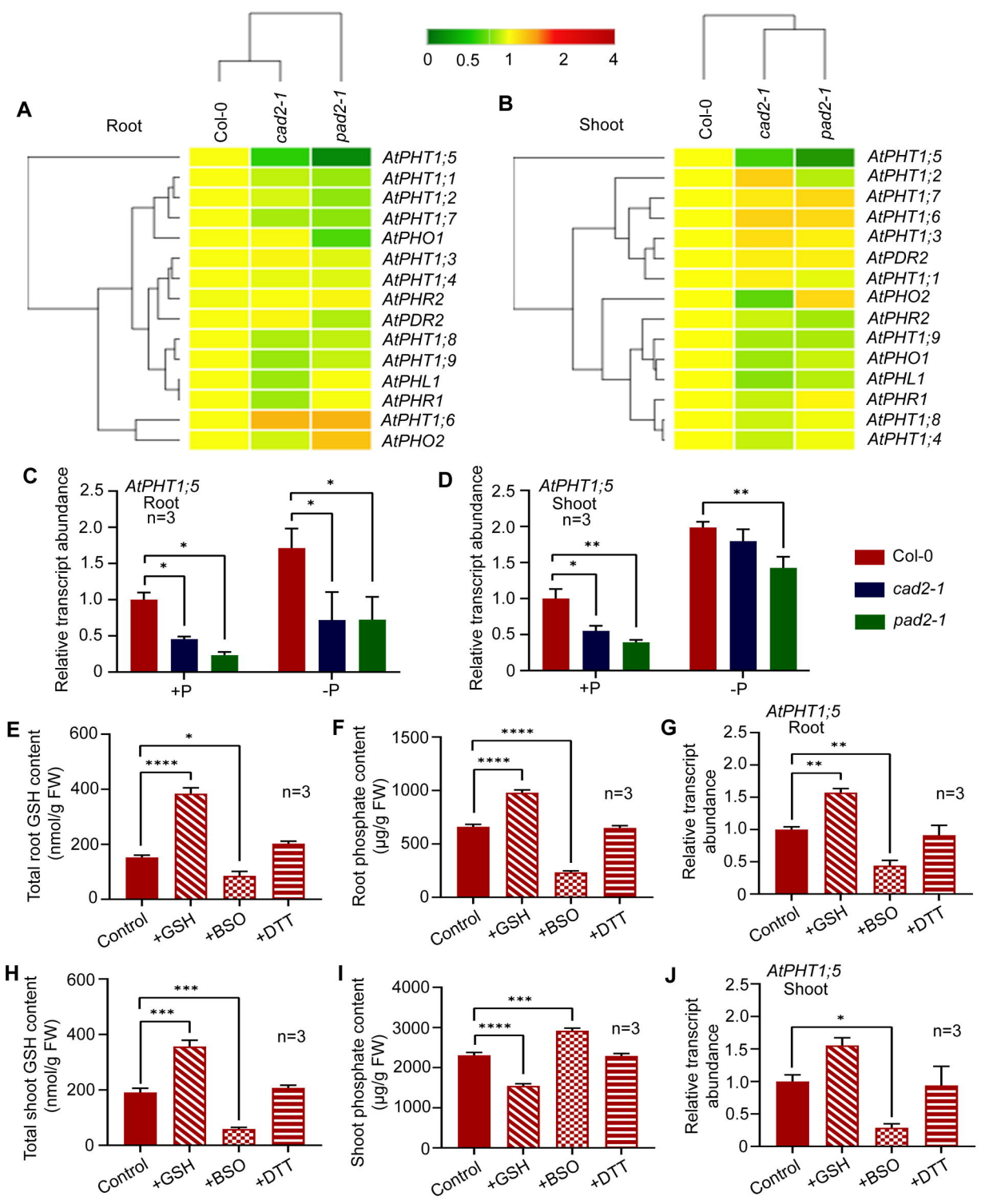
FIGURE 6 Identification of TFs interacting with *AtPHT1;5* promoter and its regulation by GSH. (A) Y1H analysis for interaction of the Pi-responsive TFs, *At*WRKY6, *At*WRKY42, *AtWRKY45*, *At*WRKY75, *At*MYB2, and *At*MYB62 with the *AtPHT1;5* promoter. (B) Relative transcript abundance of *AtMYB2*, *AtWRKY42* and *AtWRKY75* genes in the Col-0, *cad2-1* and *pad2-1* plants, (C) relative transcript abundance of *AtPHT1;5* gene in the Col-0, *wrky42*, *wrky75*, and *myb2* mutants grown under +P and –P condition. (D) Dual luciferase transctivation assay for *At*WRKY75-*AtPHT1;5* promoter. (E) ChIP-qPCR analysis for

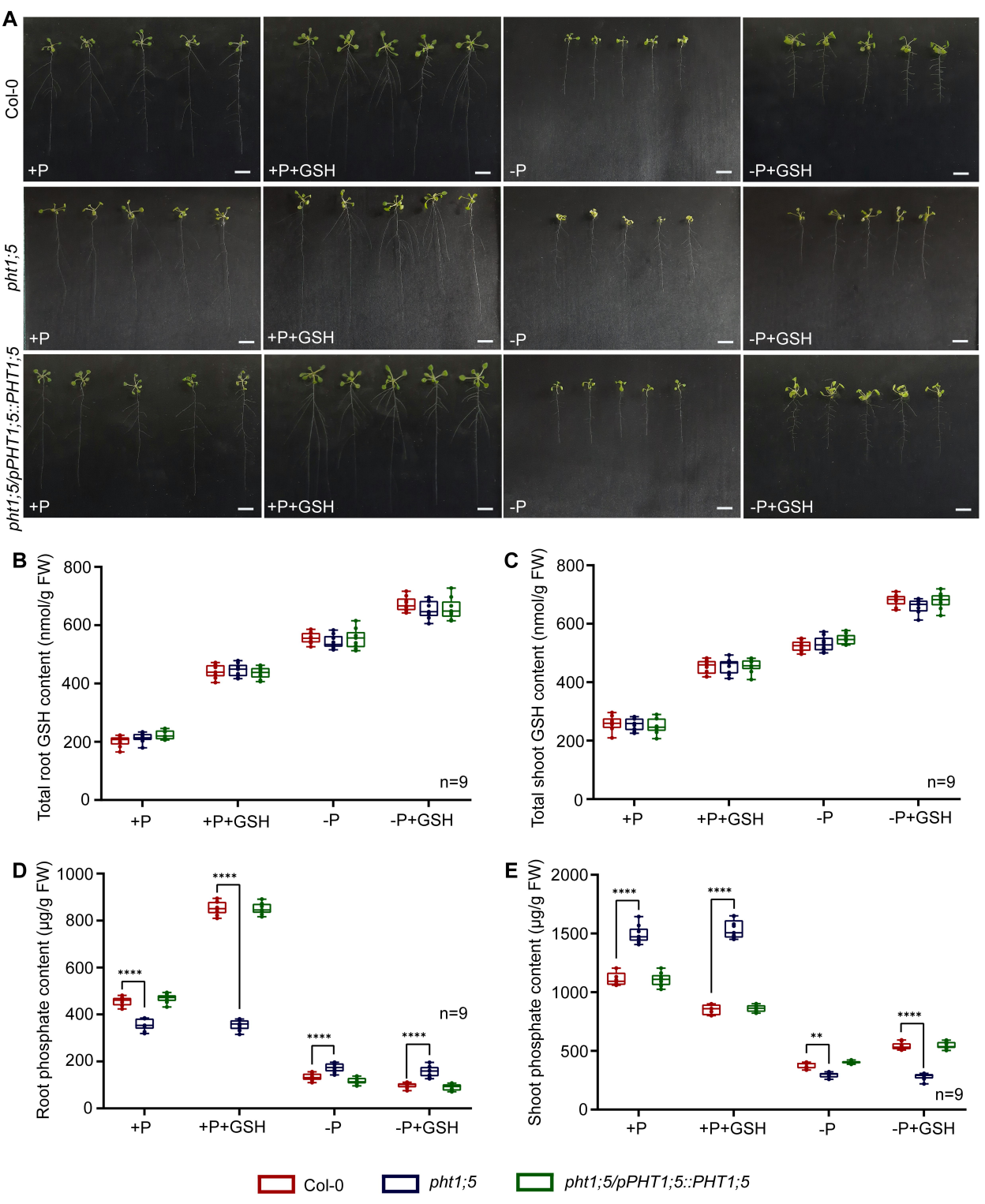
binding of *At*WRKY75 to the AtPHT1;5 promoter under +P, -P, and +P+GSH conditions. The position of the w-box motifs along with primer positions are marked on the promoter. (F) Promoter analysis of the *AtPHT1;5* gene in the *wrky75* mutant background. Representative images of histochemical GUS staining of the transgenic *wrky75/pPHT1;5::GUS* and *wrky75/35S::GUS* (vector control) lines grown under +P, -P and +P+GSH conditions. (G) Relative transcript abundance of *GUS* (*uidA*) gene of the same. The experiment was independently repeated thrice. For relative transcript abundance, results were represented as mean±SEM (n=3). Statistical differences between the treatments were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test and statistical significance of relative gene expression was denoted by different asterisks at $P<0.01$ (**), and $P< 0.001$ (***).

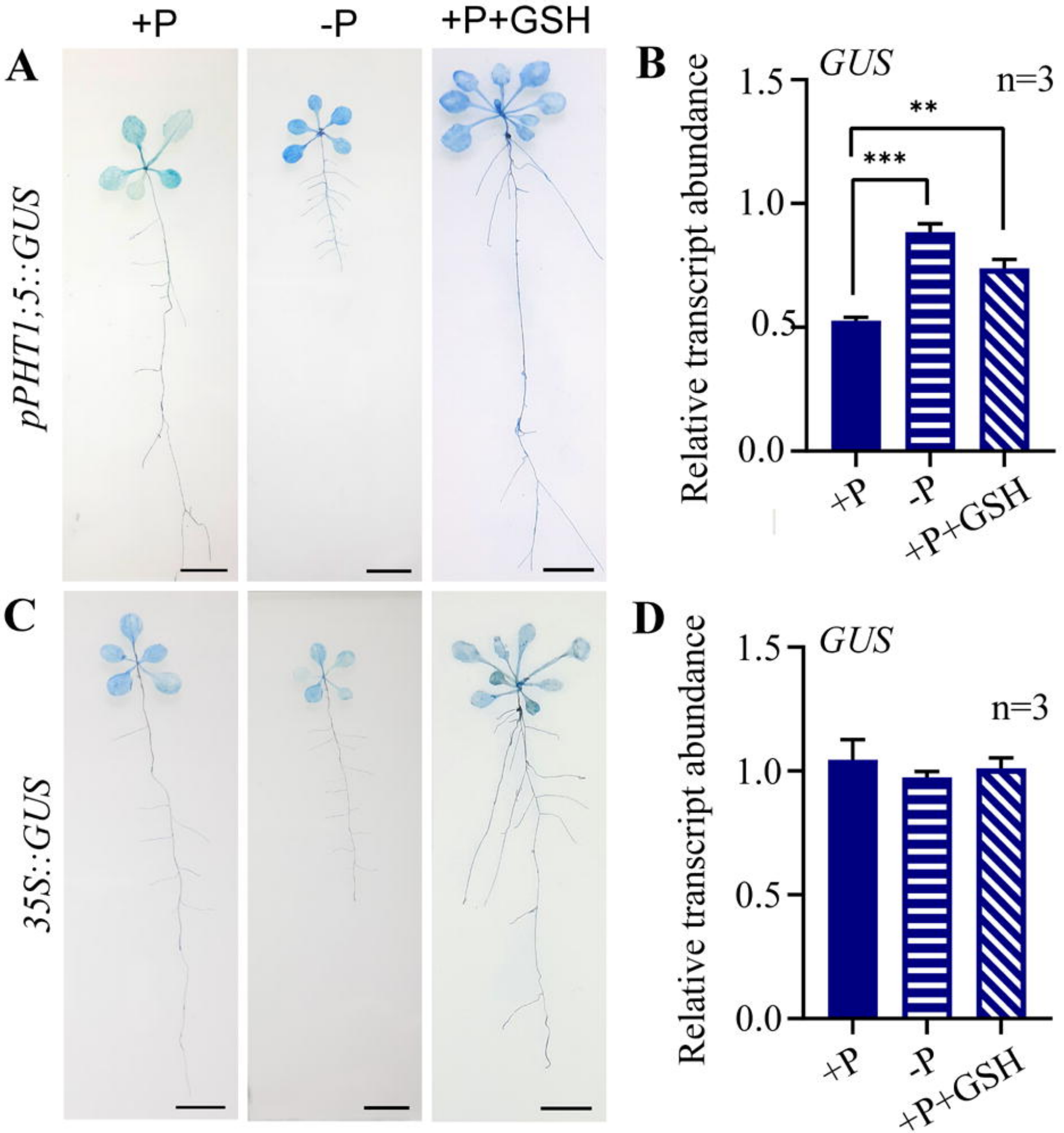
FIGURE 7 Proposed model for GSH mediated regulation of Pi translocation under Pi-limited condition. Pi deficiency leads to the accumulation of GSH in cells which then activates the Pi transporter, *AtPHT1;5* and increase source to sink Pi distribution. This GSH mediated regulation involves transcriptional induction of the *AtPHT1;5* gene via the Pi responsive *At*WRKY75 TF.

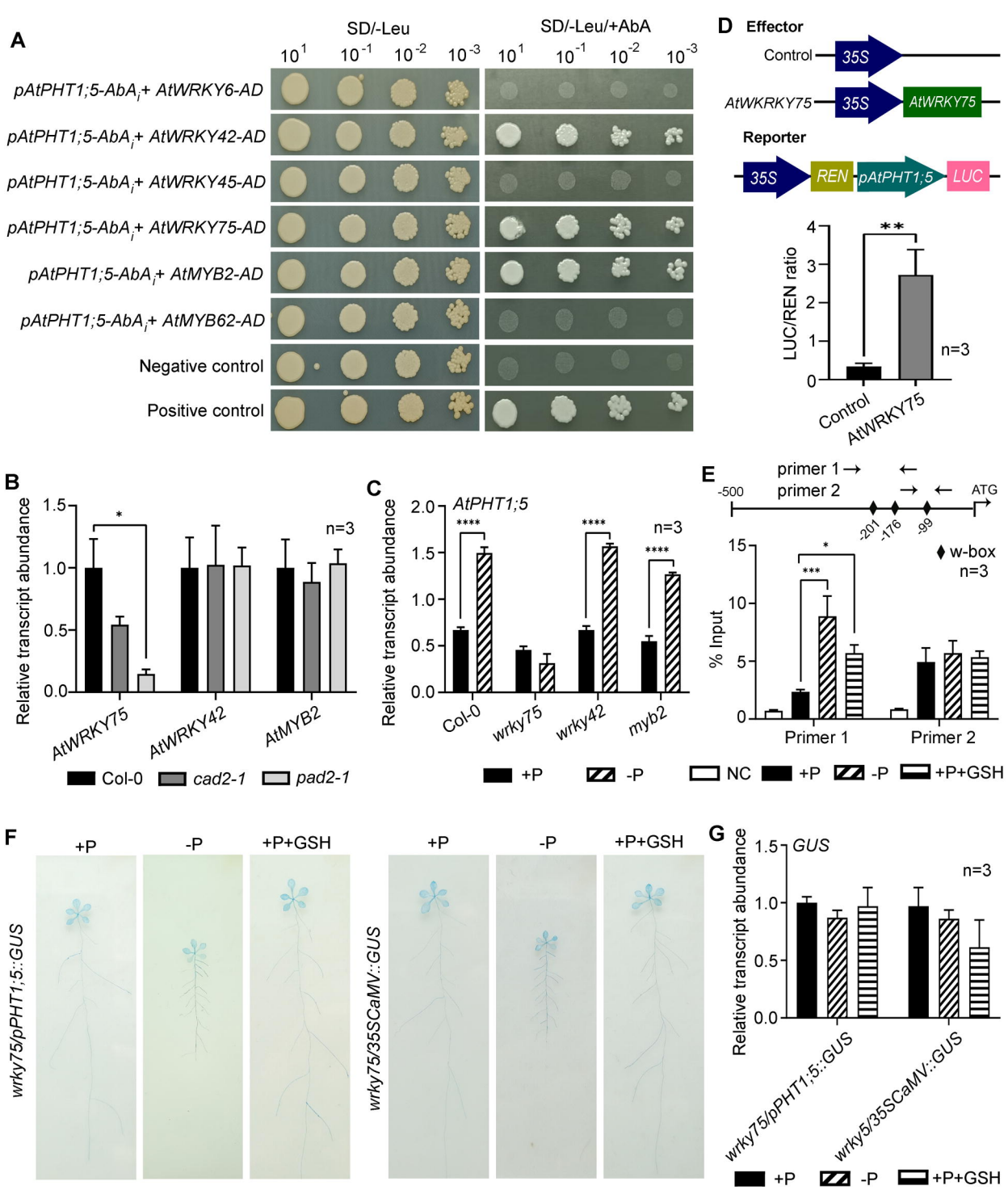

A
Col-0 +P
10 mm
cad2-1 +P
10 mm
pad2-1 +P
10 mm
Col-0 -P
10 mm
cad2-1 -P
10 mm
pad2-1 -P
10 mm
B
Rosette diameter (mm)
***
n=15
+P
-P
C
Primary root length (mm)
n=15
+P
-P
D
Lateral root number
****
n=15
+P
-P
E
Lateral root density (mm-1)
****
*
n=15
+P
-P
F
Chlorophyll content (µg/mL/g FW)
****
n=15
+P
-P
G
Anthocyanin content (A530/g FW)
****
****
n=15
+P
-P
Col-0
cad2-1
pad2-1

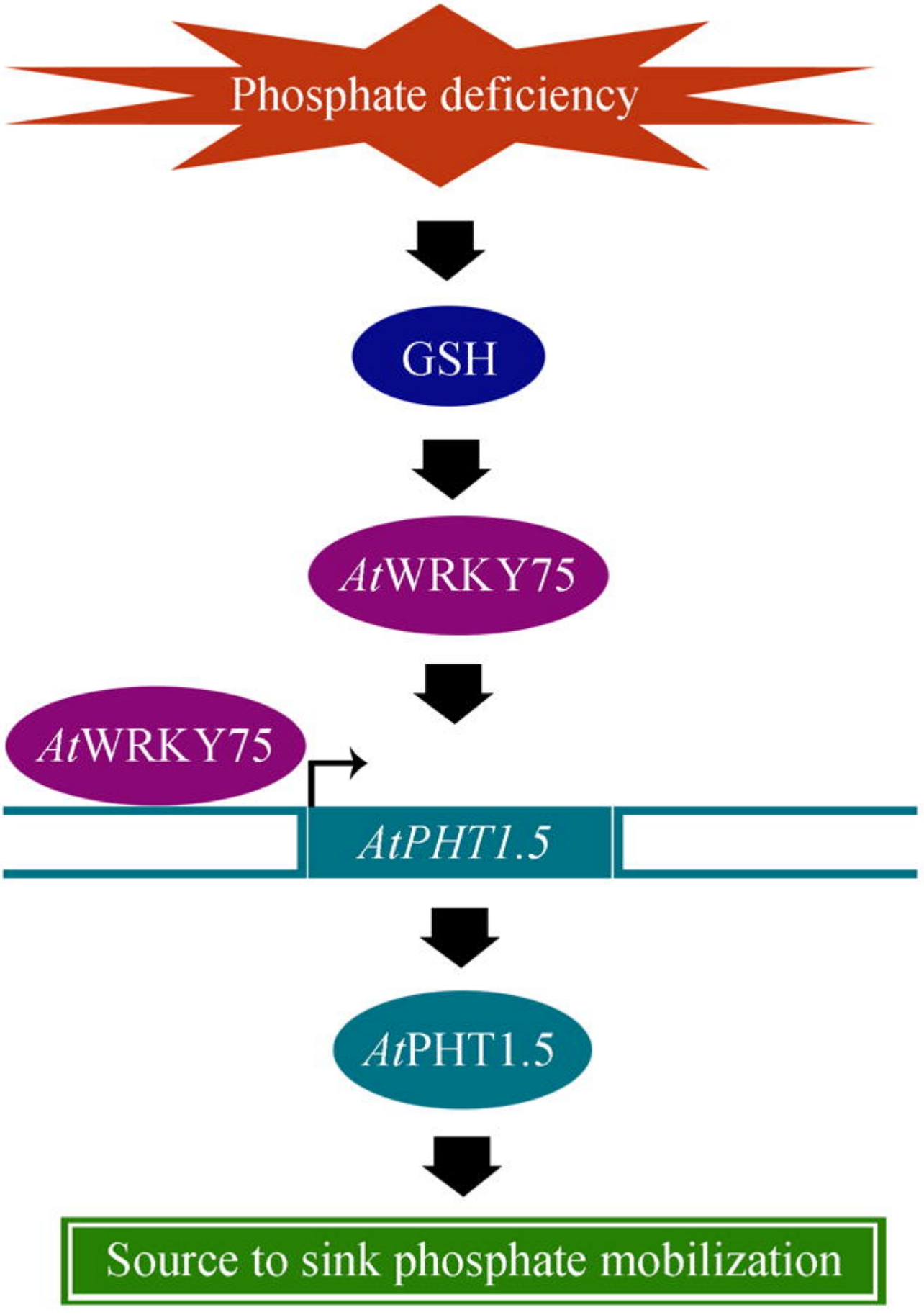

A
Root phosphate content (µg/g FW)
800
600
400
200
0
****
***
****
n=9
+P
-P
B
Total root GSH content (nmol/g FW)
300
200
100
0
****
****
****
****
n=9
+P
-P
C
Root GSH:GSSG ratio
20
15
10
5
0
****
**
***
n=9
+P
-P
D
Shoot phosphate content (µg/g FW)
4000
3000
2000
300
200
100
0
****
****
n=9
+P
-P
E
Total shoot GSH content (nmol/g FW)
400
300
200
100
0
****
****
****
****
n=9
+P
-P
F
Shoot GSH:GSSG ratio
40
30
20
10
0
****
*
*
n=9
+P
-P
Col-0
cad2-1
pad2-1


A
Root
Col-0
cad2-1
pad2-1
AtPHT1;5
AtPHT1;1
AtPHT1;2
AtPHT1;7
AtPHO1
AtPHT1;3
AtPHT1;4
AtPHR2
AtPDR2
AtPHT1;8
AtPHT1;9
AtPHL1
AtPHR1
AtPHT1;6
AtPHO2
0 0.5 1 2 4
B
Shoot
Col-0
cad2-1
pad2-1
AtPHT1;5
AtPHT1;2
AtPHT1;7
AtPHT1;6
AtPHT1;3
AtPDR2
AtPHT1;1
AtPHO2
AtPHR2
AtPHT1;9
AtPHO1
AtPHL1
AtPHR1
AtPHT1;8
AtPHT1;4
C
AtPHT1;5
Root
n=3
Relative transcript abundance
+P
-P
D
AtPHT1;5
Shoot
n=3
Relative transcript abundance
+P
-P
Col-0
cad2-1
pad2-1
E
Total root GSH content (nmol/g FW)
n=3
Control
+GSH
+BSO
+DTT
F
Root phosphate content (μg/g FW)
n=3
Control
+GSH
+BSO
+DTT
G
AtPHT1;5
Root
Relative transcript abundance
n=3
Control
+GSH
+BSO
+DTT
H
Total shoot GSH content (nmol/g FW)
n=3
Control
+GSH
+BSO
+DTT
I
Shoot phosphate content (μg/g FW)
n=3
Control
+GSH
+BSO
+DTT
J
AtPHT1;5
Shoot
Relative transcript abundance
n=3
Control
+GSH
+BSO
+DTT


A
Col-0
pht1;5
pht1;5/pPHT1;5::PHT1;5
+P
+P+GSH
-P
-P+GSH
B
Total root GSH content (nmol/g FW)
C
Total shoot GSH content (nmol/g FW)
D
Root phosphate content (µg/g FW)
E
Shoot phosphate content (µg/g FW)
n=9
****
**
Col-0
pht1;5
pht1;5/pPHT1;5::PHT1;5


+P
-P
+P+GSH
A
pPHT1;5::GUS
B
GUS
n=3
Relative transcript abundance
1.5
1.0
0.5
0.0
**
***
+P
-P
+P+GSH
C
35S::GUS
D
GUS
n=3
Relative transcript abundance
1.5
1.0
0.5
0.0
+P
-P
+P+GSH


A
SD/-Leu
SD/-Leu/+AbA
10^1 10^-1 10^-2 10^-3
pAtPHT1;5-AbAi+ AtWRKY6-AD
pAtPHT1;5-AbAi+ AtWRKY42-AD
pAtPHT1;5-AbAi+ AtWRKY45-AD
pAtPHT1;5-AbAi+ AtWRKY75-AD
pAtPHT1;5-AbAi+ AtMYB2-AD
pAtPHT1;5-AbAi+ AtMYB62-AD
Negative control
Positive control
D
Effector
Control
35S
AtWKRKY75
AtWRKY75
Reporter
35S
REN
pAtPHT1;5
LUC
LUC/REN ratio
**
n=3
Control
AtWRKY75
B
Relative transcript abundance
*
n=3
AtWRKY75
AtWRKY42
AtMYB2
Col-0
cad2-1
pad2-1
C
AtPHT1;5
Relative transcript abundance
****
n=3
Col-0
wrky75
wrky42
myb2
+P
-P
E
primer 1
primer 2
-500
ATG
-201
-176
-99
w-box
n=3
% Input
***
*
Primer 1
Primer 2
NC
+P
-P
+P+GSH
F
+P
-P
+P+GSH
wrky75/pPHT1;5::GUS
wrky75/35SCaMV::GUS
G
GUS
Relative transcript abundance
n=3
wrky75/pPHT1;5::GUS
wrky5/35SCaMV::GUS
+P
-P
+P+GSH


Phosphate deficiency
GSH
AtWRKY75
AtWRKY75
AtPHT1.5
AtPHT1.5
Source to sink phosphate mobilization