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Article

Phloem-Mobile *MYB44* Negatively Regulates Expression of *PHOSPHATE TRANSPORTER 1* in Arabidopsis Roots

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Abstract: Phosphorus (P) is an essential plant macronutrient; however, its availability is often limited in soils. Plants have evolved complex mechanisms for efficient phosphate (Pi) absorption, which are responsive to changes in external and internal Pi concentration, and orchestrated through local and systemic responses. To explore these systemic Pi responses, here, we identified *AtMYB44* as a phloem-mobile mRNA, an Arabidopsis homolog of *Cucumis sativus MYB44*, that is responsive to the Pi-starvation stress. qRT-PCR assays revealed that *AtMYB44* was up-regulated and expressed in both shoot and root in response to Pi-starvation stress. The *atmyb44* mutant displayed higher shoot and root biomass, compared to wild-type plants, under Pi-starvation conditions. Interestingly, the expression of *PHOSPHATE TRANSPORTER1;2* (*PHT1;2*) and *PHT1;4* was enhanced in *atmyb44* in response to a Pi-starvation treatment. A split-root assay showed that *AtMYB44* expression was systemically regulated, under Pi-starvation conditions and, in *atmyb44*, systemic controls on *PHT1;2* and *PHT1;4* expression were moderately disrupted. Heterografting assays confirmed graft transmission of *AtMYB44* transcripts, and *PHT1;2* and *PHT1;4* expression was decreased in heterografted *atmyb44* rootstocks. Taken together, our findings support the hypothesis that mobile *AtMYB44* mRNA serves as a long-distance Pi response signal, which negatively regulates Pi transport and utilization in Arabidopsis.

Keywords: Pi-starvation stress; mobile mRNA; phloem; systemic signaling

1. Introduction

Phosphorus (P) is an essential element that is involved in various functionalities in living organisms, including plants. Therefore, it is critical to ensure proper phosphorus nutrition for optimal plant growth and development, by supplying phosphate (Pi) fertilizers to maximize crop productivity in modern agriculture systems [1,2]. However, soil-applied Pi fertilizer becomes rapidly immobilized, due to chemical reactions with cations (e.g., magnesium, calcium, aluminum, iron) in both alkaline and acidic soils. This can limit the availability to the plant of applied Pi to around 20-30% for plant use. Furthermore, Pi is a finite and non-renewable resource, therefore, it has led to increased interest in understanding the mechanisms by which plants absorb and use Pi, under limiting Pi conditions. Here, the goal is to develop elite plant lines having enhanced P acquisition and utilization efficiency traits [3–7].

Plants have evolved sophisticated adaptative mechanisms, which involve a range of physiological, morphological, biochemical, and molecular processes, to respond to low Pi conditions for efficient Pi foraging in soils [4,5,8–11]. These adaptative processes, known as phosphate starvation responses (PSRs), are categorized into local and systemic Pi responses as Pi sensing and long-distance

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signaling, and the adaptative efficiency can be determined by the PSR regulatory capacity in plants [6,10,12,13].

The local Pi responses are largely associated with traits for root growth, to perceive the Pi level in the soil and determine the root developmental fate, in response to the imposed Pi-starvation stress, whereas internal Pi concentration activates the systemic responses to integrate the Pi level information in distantly located plant tissues/organs [6,10,12,13]. Systemic Pi responses play an important role in global P homeostasis within plants, and microRNA399 (miR399) was the first identified long-distance regulatory component in Pi-stress signaling [14,15]. Under Pi-starvation conditions, miR399 is expressed in shoots and delivered into roots, via the phloem, to mediate in the degradation of *PHO2* mRNA, which encodes an E2 ubiquitin-conjugating enzyme and regulates level of high-affinity PHOSTPHATE TRANSPORTER1 (PHT1) protein in roots. Therefore, this systemic regulatory mechanism serves in control over Pi acquisition under Pi-starvation stress conditions [6,14–20].

2. Results

2.1. Identification of mobile CsMYB44 orthologs in Arabidopsis.

In a previous study, cucumber was used as a model plant to examine the role of the phloem in systemic signaling under Pi-stress conditions [29]. The phloem mRNA profiles were observed to change, dynamically, in response to an imposed Pi-stress treatment, and a range of graft-transmissible mRNAs were shown to target specific sink organs/tissues, under Pi-stress conditions [29]. The *Cucumis sativus MYB44* (*CsMYB44*, Csa6G491690), encoding a R2R3 MYB transcription factor (TF), was among these Pi-stress-induced graft-transmissible mRNAs and was delivered into sink tissue [29]. Thus, we selected *CsMYB44* to further assess its role as a long-distance signaling mRNA in response to Pi-stress.

First, to facilitate testing our hypothesis that *MYB44* plays a role in phloem-mediated systemic signaling, under Pi-starvation stress conditions, we conducted a phylogenetic analysis to identify putative *CsMYB44* orthologs in Arabidopsis. A total of 132 Arabidopsis R2R3 MYB family members were analyzed (Figure S1) and, here, the phylogenetic tree analysis revealed the presence of CsMYB44 in a specific MYB subfamily subclade (Figure 1A).

To gain insight into the regulation of the genes in this MYB subclade, we used the Arabidopsis cis-regulatory element database (https://agris-knowledgebase.org/AtcisDB) to analyze the promoter regions of *AtMYB44*, *AtMYB70*, *AtMYB73* and *AtMYB77* with the aim to identify potential *cis*-elements that might serve to control their expression under Pi-stress conditions. Numerous Pi-starvation responsive (PSR) genes are regulated by the PHOSPHATE STARVATION RESPONSE1 (PHR1) TF, a key regulator of PSR genes, and the PHR1 binding site (P1BS) is enriched in the promoter regions of many Arabidopsis Pi-responsive genes [30–32].

Our *cis*-element analysis indeed revealed that P1BS motifs were located within the promoters of *AtMYB44*, *AtMYB70* and *AtMYB73*, but not *AtMYB77* (Figure 1B). Furthermore, qRT-PCR analysis established that the transcript levels of *AtMYB44*, *AtMYB70*, *AtMYB73* and *AtMYB77* were responsive to a Pi- stress treatment, in both shoots and roots (Figure 1C-D). For *AtMYB44* and *AtMYB77*, at least a two-fold increase and decrease in transcript levels, respectively, were detected in shoots, under Pi-stress conditions (Figure 1C). By contrast, *AtMYB70* and *AtMYB73* transcript levels in the shoots were only slightly changed in response to Pi-stress (10 μM Pi) (Figure 1C). In roots, the *AtMYB44*, *AtMYB70*, *AtMYB73* and *AtMYB77* transcript levels were significantly increased under Pi-stressed conditions (10 μM Pi) (Figure 1D). Taken together, our results demonstrate that the putative CsMYB44 homologs, AtMYB44, AtMYB70, AtMYB73 and AtMYB77, are regulated in response to the imposed Pi-stress treatment. As the *AtMYB44* transcript levels increased, in both shoot and root, we focused on investigation on the role of AtMYB44 in Pi-stress signaling.

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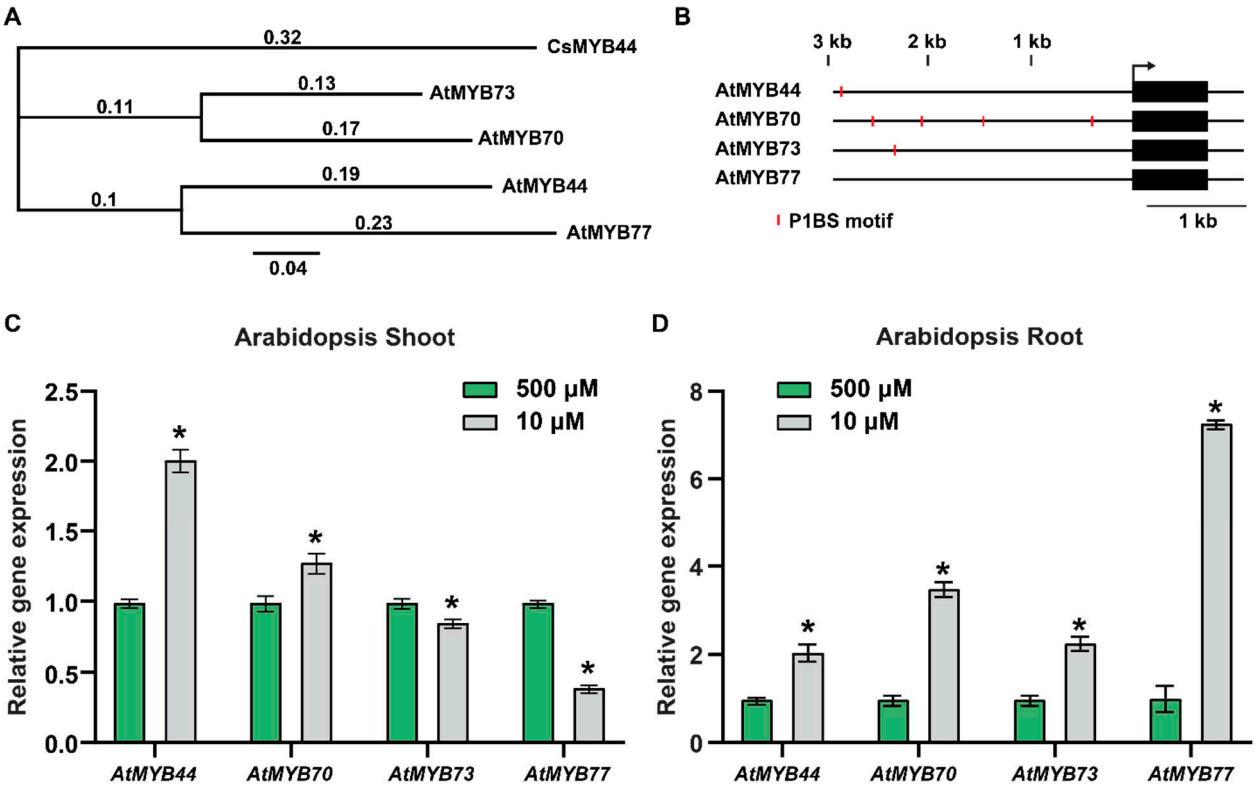

Figure 1. AtMYB44 is a potential functional homolog of CsMYB44. (A) The clade, which includes the closest MYB homologs of CsMYB44, in the phylogenic tree of the Arabidopsis MYB family, are shown in Figure S1. Numbers above the phylogenetic tree indicate posterior probabilities. (B) P1BSs, which are PHR1 binding motifs, predicted in the promoter regions of *AtMYB44*, *AtMYB70* and *AtMYB73*, but not *AtMYB77*. Red bars indicate the P1BS motifs. (C) and (D) Relative expression of *AtMYB44*, *AtMYB70*, *AtMYB73*, and *AtMYB77* in the shoot (C) and the root (D), under Pi-sufficient (500 μM) and -starvation (10 μM) conditions. Arabidopsis seedlings were transplanted onto the medium with 500 μM, or 10 μM Pi, 5 days after germination on solid medium with 500 μM Pi. Shoot and root samples of Arabidopsis plants were harvested 7 days after Pi-sufficient (500 μM), or -starvation (10 μM) treatment. Arabidopsis *Actin* was used as an internal control to normalize the qRT-PCR results. The data are presented as mean ± SD (three technical replicates and three technical repeats). Asterisks indicate significantly different values between Pi-sufficient and -starvation conditions (Student's *t*-test, $p<0.05$).

2.2. AtMYB44 is expressed in leaf and root vascular tissues under Pi-stress conditions.

To further understand the mechanism of *AtMYB44* regulation in Arabidopsis, we generated transgenic plants carrying the GFP-GUS reporter, under the control of the native *AtMYB44* promoter and analyzed the *AtMYB44* expression pattern (Figure 2). Consistent with our qRT-PCR results (Figure 1C,D), the β-glucuronidase (GUS) staining assays showed signal in both shoots and roots, and revealed stronger staining under Pi-stress (10 μM), compared with Pi-sufficient conditions (500 μM) (Figure 2A,B), consistent with induction of *AtMYB44* expression in response to Pi-stress. Here, *AtMYB44* expression was detected in leaf lamina, leaf vasculature, root vasculature, root tips of primary and lateral roots, and in lateral root primordia (Figure 2B–H). These findings were consistent with the notion that *AtMYB44* plays a role in transcriptional regulation of adaptive shoot and root development, in response to Pi-stress treatment.

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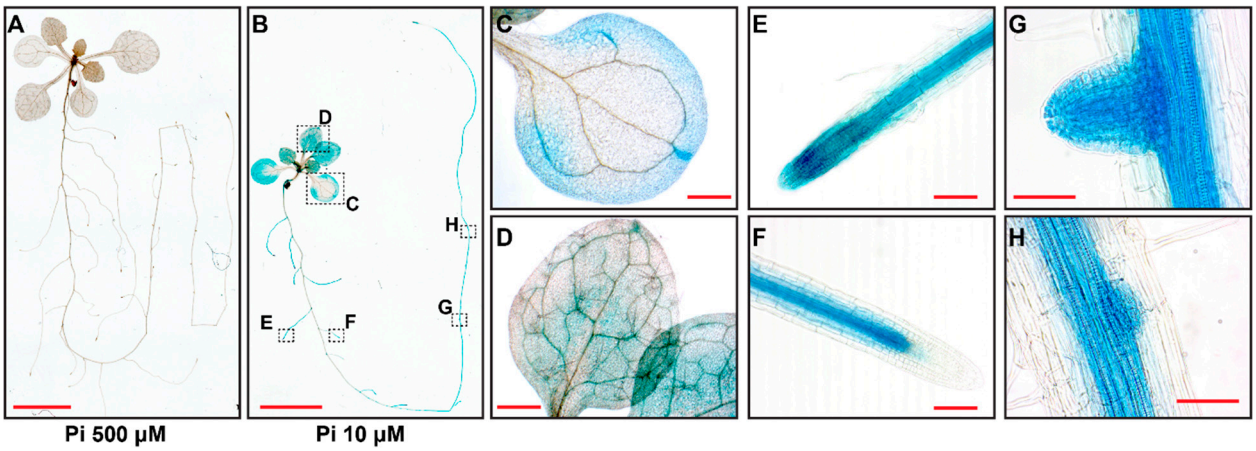

Figure 2. *AtMYB44* is expressed in response to Pi-starvation stress. Histochemical staining in 12-day-old transgenic Arabidopsis plants expressing the GFP-GUS reporter under the control of the *AtMYB44* native promoter. Transgenic Arabidopsis seeds were germinated on solid medium with 500 µM Pi (Pi-sufficient medium) for 5 days and then transplanted to medium with 500 µM, or 10 µM Pi. Arabidopsis seedlings were collected 7 days after Pi-sufficient (500 µM), or starvation (10 µM) treatment. (A) GUS expression was barely detectable in Arabidopsis seedlings, grown under Pi-sufficient (500 µM) conditions. (B) Under Pi-starvation (10 µM) conditions, GUS expression was observed in cotyledons (C), developing true leaves (D), lateral roots (E-F), and developing lateral root primordia (G-H). Dotted boxes indicate magnified regions for (C-H). Bars: 5 mm in (A-B), 500 µm in (C-D), and 50 µm in (E-H).

2.3. AtMYB70, AtMYB73 and AtMYB77 transcript levels were elevated in atmyb44 mutant, compared with Wild-type.

The *atmyb44* mutant exhibited slightly longer primary roots and a higher number of lateral roots, compared with the WT, under Pi-sufficient conditions (Figure S2A–C). However, under Pi-stress conditions, no significant difference in primary root growth and lateral root numbers were observed between WT and *atmyb44*. As expression of other *MYB* family genes, close to CsMYB44, was also responsive to imposed Pi-stress (Figure 1D), we hypothesized that other redundant MYBs could compensate in the absence of *AtMYB44* under Pi-stress conditions. To test this notion, we examined the expression level of *AtMYB70*, *AtMYB73* and *AtMYB77* in the *atmyb44* mutant background, under Pi-stress conditions, using qRT-PCR (Figure 3). Here, we confirmed that expression of *AtMYB70*, *AtMYB73* and *AtMYB77* increased (Figure 3). Enhanced transcript levels of *AtMYB70*, *AtMYB73* and *AtMYB77* were detected in *atmyb44* plants under both Pi-sufficient and stress conditions, compared with WT (Figure 3). These assays supported a role for *AtMYB70*, *AtMYB73* and *AtMYB77*, in the functional compensation of *AtMYB44* in its absence.

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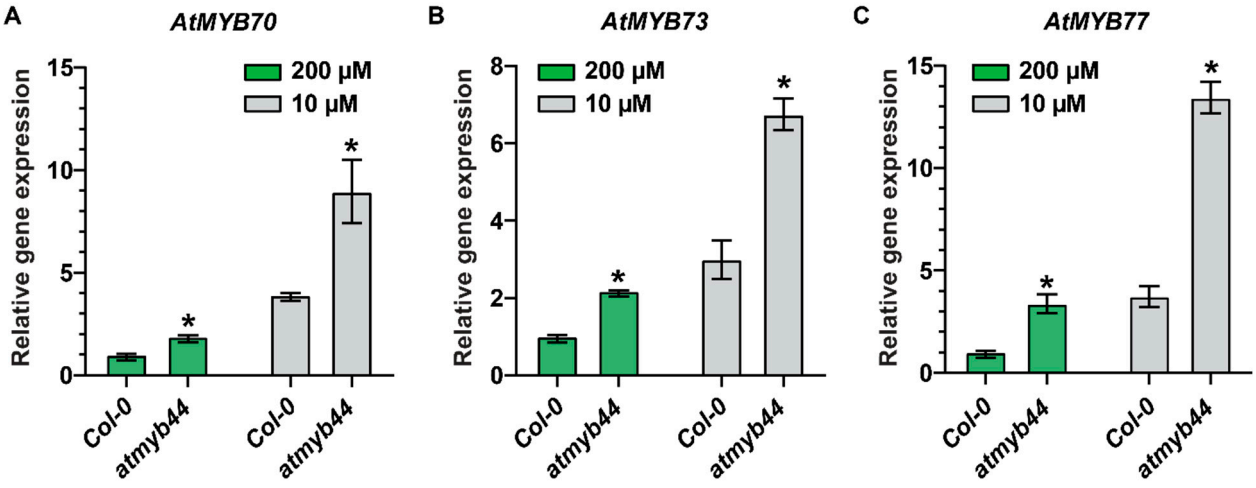

Figure 3. Transcript levels of *AtMYB70*, *AtMYB73*, and *AtMYB77* are enhanced in *atmyb44* knock-out mutants. Relative expression levels of *AtMYB70* (A), *AtMYB73* (B), and *AtMYB77* (C) in WT (Col-0) and the *atmyb44* mutant, under Pi-sufficient (200 µM) and -starvation (10 µM) conditions. Arabidopsis seedlings were transplanted onto medium with 500 µM, or 10 µM Pi, 5 days after germination on solid medium with 500 µM Pi. Arabidopsis seedlings were harvested 7 days after Pi-sufficient (500 µM), or -starvation (10 µM) treatment. Arabidopsis *Actin* was used as an internal control to normalize the qRT-PCR results. The data are presented as mean ± SD (three technical replicates and three technical repeats). Asterisks indicate significantly different values between WT (Col-0) and *atmyb44* mutant (Student's t-test, $p<0.05$).

2.4. The atmyb44 root had an elevated Pi concentration under Pi-stress conditions.

Under the Pi-stress conditions, the *atmyb44* plants did not exhibit a clear phenotypic difference in primary root growth and lateral root number, compared with WT (Figure S2). As both plant lines were grown on solid medium, which limited observation of plant developmental changes, to apply a prolonged Pi-stress, we next employed a hydroponic system to conduct phenotypic analysis of *atmyb44* and *AtMYB44* overexpression (OX) lines [33–35].

Compared to WT, higher and lower shoot biomass was observed in the *atmyb44* and *AtMYB44* OX plants, respectively, under Pi-sufficient conditions, although no significant difference in shoot biomass was detected under Pi-stress conditions (Figure 4A, Figure S3A). However, the root biomass was obviously higher in *atmyb44* than WT and *AtMYB44* OXs, under both Pi-sufficient and stress conditions (Figure 4B, Figure S3B). In *atmyb44*, the Pi concentration was higher in shoots and lower in roots, compared to the level in WT, under Pi-sufficient conditions. In contrast, the root Pi concentration in *atmyb44* was higher than WT, under Pi-stress conditions, even though no significant difference in shoot Pi concentration was detected between WT and *atmyb44* (Figure 4C,D). Taken together, these results suggested that AtMYB44 functions as a negative regulator in both shoot and root development, under Pi-sufficient conditions and that an absence of *AtMYB44* might increase Pi uptake into Arabidopsis roots.

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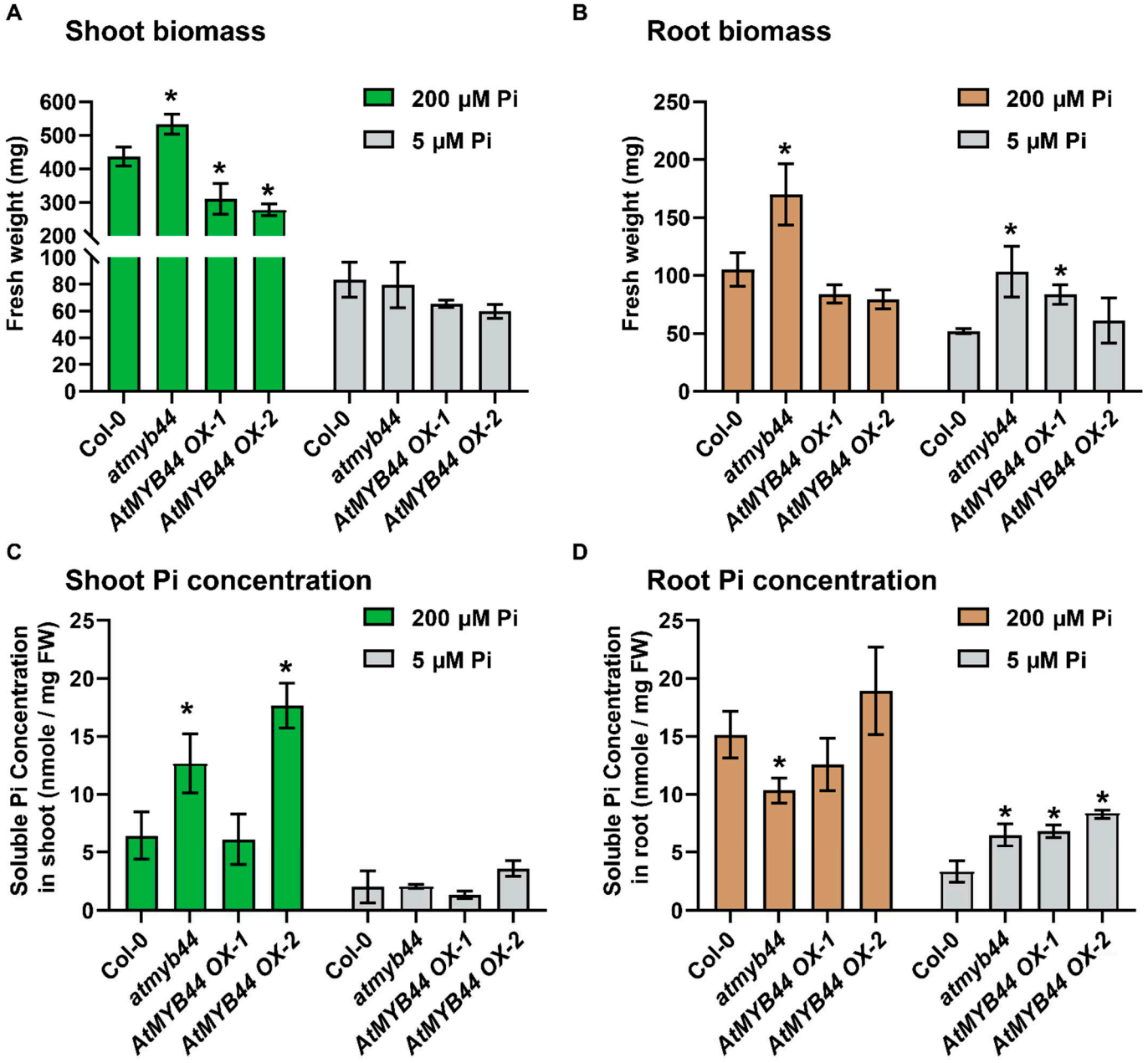

Figure 4. AtMYB44 functions in plant growth under Pi-starvation conditions. (A) Shoot and (B) root biomass measurements on 4-week-old Col-0, *atmyb44*, *AtMYB44 OX-1* and *OX2* lines, grown in a hydroponic culture system with high (200 µM) and low (5 µM) Pi concentrations. Shoot and root biomass was examined as fresh weight per Arabidopsis plant. Soluble Pi concentration in (C) shoot and (D) root of 4-week-old Col-0, *atmyb44*, *AtMYB44 OX-1* and *OX2* plants grown in a hydroponic culture system with high (200 µM) and low (5 µM) Pi concentrations. The data are presented as mean ± SD (three technical replicates and three technical repeats). Asterisks indicate significantly different values, compared with WT (Col-0) (Student's t-test, $p<0.05$).

2.5. Expression of PHT1;2 and PHT1;4 is negatively regulated by AtMYB44 in roots.

The *in-silico* analysis, using ConnecTF (https://connectf.org) [36], revealed that AtMYB44 could be involved in many gene regulatory pathways and, interestingly, bound to the promoter regions of *PHOSPAHTE TRANSPORTER 1;2* (*PHT1;2*) and *PHT1;4* as potential AtMYB44-target sites (Table S2). As we observed an enhanced level of Pi concentration in the root of *atmyb44* plants, under Pi-stress conditions (Figure 4D), and PHT1 regulates the initial uptake of Pi [6,37–44], we hypothesized that AtMYB44 regulates *PHT1* expression in roots for Pi acquisition under Pi-stress conditions.

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To test this hypothesis, we examined the expression level of *PHT1;2* and *PHT1;4* in WT, *atmyb44* and *AtMYB44* OX-1 roots, using qRT-PCR (Figure 5). The expression levels of *PHT1;2* and *PHT1;4* were much higher in *atmyb44*, under Pi-stress conditions, compared to WT (two- to four-fold higher expression; Figure 5) and the increased levels of *PHT1;2* and *PHT1;4* in *atmyb44* were diminished in *AtMYB44* OX-1 (Figure 5). We did not detect any significant differences in the expression levels of *PHT1;2* and *PHT1;4* in *AtMYB44* OX-1, compared with WT (Figure 5). This result suggested that AtMYB44 targets the promoter of *PHT1;2* and *PHT1;4*, where it acts as a negative regulator.

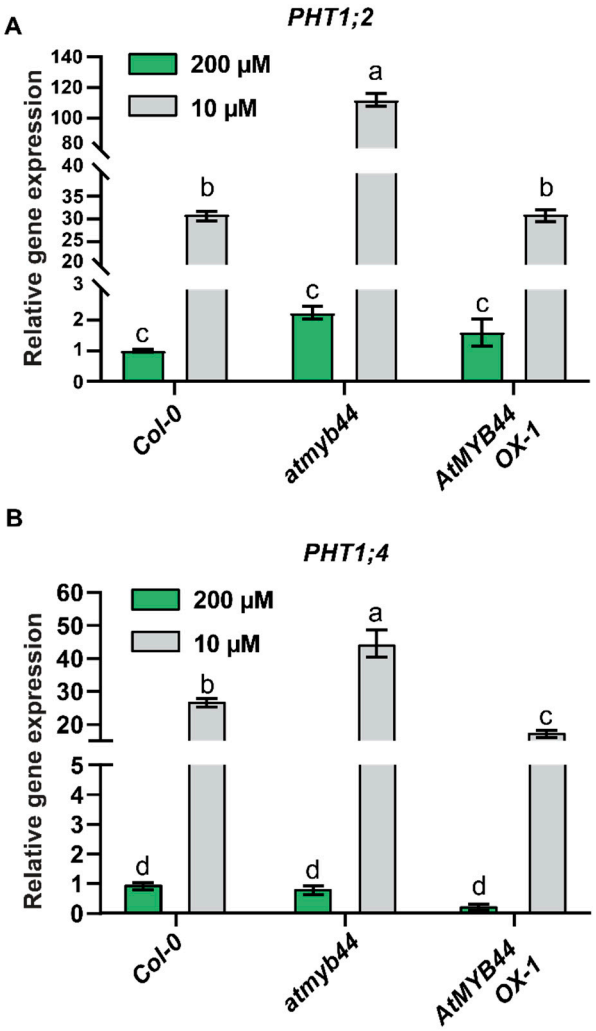

Figure 5. Expression of *PHT1* is enhanced in *atmyb44* roots. Relative expression level of *PHT1;2* (A) and *PHT1;4* (B) in WT (Col-0), *atmyb44* and AtMYB44 OX-1 roots, under Pi-sufficient (200 µM) and -starvation (10 µM) conditions. Arabidopsis seedlings were transplanted onto medium with 500 µM, or 10 µM Pi, 5 days after germination on solid medium with 500 µM Pi. Arabidopsis seedlings were harvested 7 days after Pi-sufficient (500 µM), or -starvation (10 µM) treatment. Arabidopsis *Actin* was used as an internal control to normalize the qRT-PCR results. The data are presented as mean ± SD (three technical replicates and three technical repeats). The different lowercase letters indicate significant differences in relative gene expression levels, determined by Tukey's test (p<0.05).

2.6. Mobile AtMYB44 mRNA functions as a negative regulator of PHT1;2 and PHT1;4 expression.

We demonstrated that *AtMYB44* expression responds to Pi-stress (Figure 1C,D) and AtMYB44 appears to play a role in regulating *PHT1;2* and *PHT1;4* expression, under these conditions (Figure 5). As *AtMYB44* was identified as a mobile *CsMYB44* ortholog in Arabidopsis (Figure 1A), it is plausible that *AtMYB44* could serve as a long-distance signal to exert control over Pi-starvation

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responses in Arabidopsis. To test whether *AtMYB44* acts as a systemic signal, in response to Pi-stress, we employed a split-root system to test whether *AtMYB44*-mediated gene expression is a systemic or local response, under Pi-stress conditions. The Arabidopsis root systems grown in the hydroponic culture system, were separated into two parts with each half of the root system placed into a vessel container nutrient solution with Pi (200 μM) or with no Pi (0 μM). As the control, both vessels for each half of the root system on a plant contained the same nutrient solution (CP200 for 200 μM Pi, CP0 for 0 μM) to mimic plant growth under homogenous Pi-sufficient or deficient conditions [12].

Total RNA was extracted from these roots for qRT-PCR analysis. First, we investigated regulation of *AtMYB44* expression, to see if it is part of a systemic response, after Pi-sufficient and deficient treatments were applied to different halves of the root system on individual plants area (Figure 6). As expected, no *AtMYB44* transcript was detected in *atmyb44* tissues and *AtMYB44* expression in WT was enhanced in response to the imposed Pi-stress (C0), compared with the mRNA level under Pi-sufficient conditions (CP200) (Figure 6A). In WT, a higher level of *AtMYB44* mRNA was detected in that half of a plant's root system placed in the compartment with 200 μM Pi (SP200), relative to the control (CP200). In addition, lower *AtMYB44* mRNA levels were observed for the split root system in 0 μM Pi (SP0) compared to the transcript level in the homogenously Pi-deficient control (C0) (Figure 6A). These findings support our hypothesis that *AtMYB44* serves as a systemic regulator.

Next, we examined systemic regulation of *PHT1;2* and *PHT1;4* expression in *AtMYB44* knockout mutants again using this split-root system to test whether *AtMYB44* serves as a factor in systemic *PHT1;2* and *PHT1;4* regulation. Here, the qRT-PCR analysis revealed that, consistent with the previous report [12], expression of both *PHT1;2* and *PHT1;4* in WT plants was downregulated in roots within the SP0 container, compared to *PHT1;2* and *PHT1;4* expression in CP0 in WT (Figure 6B,C). However, in *atmyb44* plants, the enhanced transcript levels of *PHT1;2* and *PHT1;4* were decreased in SP0, compared to CP0 (Figure 6B,C). Taken together, these results suggest that *AtMYB44* expression is regulated, in a systemic manner, and that other long-distance regulators might be involved in the systemic regulation of *PHT1;2* and *PHT1;4* regulation in the *atmyb44* background.

To investigate whether *AtMYB44* mRNA acts as a long-distance regulator for control over *PHT1;2* and *PHT1;4* expression, we performed micrografting assays between *atmyb44* and *AtMYB44* OX-1 lines (Figure 7A). Here, our RT-PCR analyses revealed that, as controls, *AtMYB44* expression was increased in *AtMYB44* OX-1, but not in *atmyb44*, and *AtMYB44* OX-1, and *atmyb44* were subsequently used as shoot scions or root stocks (Figure 7B). Analysis of these grafted tissues revealed that the transgene *AtMYB44* was detected in rootstocks of heterografted *AtMYB44* OX-1 (scion)/*atmyb44* (rootstock), but not in the *atmyb44* (scion)/ *AtMYB44* OX-1 (rootstock), consistent with *AtMYB44* mRNA being phloem-mobile from source to sink tissues (Figure 7B). Interestingly, the level of *PHT1;2* and *PHT1;4* expression was decreased in the rootstocks of these heterografted *AtMYB44* OX-1 (scion)/*atmyb44* (rootstock) plants, compared to the *atmyb44* homograft (Figure 7C). These data support the hypothesis that *AtMYB44* mRNA acts as a mobile, negatively acting, regulator of *PHT1;2* and *PHT1;4* expression in Arabidopsis roots.

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Figure 6

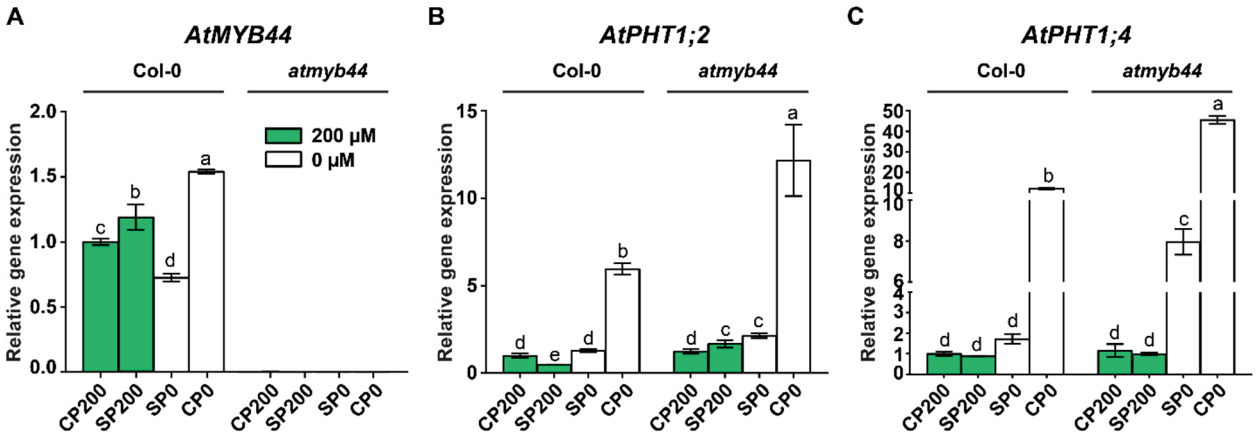

Figure 6. *AtMYB44* is a systemically regulated gene in response to an imposed Pi-starvation stress. Expression of endogenous *AtMYB44* in roots of 4-week-old WT (Col-0) and *atmyb44* plants grown in a split-root systems. CP200 and CP0 indicate homogenous Pi-sufficient (200 μM) and deficient (0 μM) treatment on roots, respectively. SP200 and SP0 indicate the half of the root system on a plant supplied with Pi-sufficient (200 μM) or deficient (0 μM) treatment, respectively. Arabidopsis roots were harvested 7 days after transferring plants into the split-root systems. (A) *AtMYB44* expression is systemically regulated. *AtMYB44* expression was examined in WT (Col-0) and *atmyb44*, using a split-root system. (B) *AtPHT1;2* and (C) *AtPHT1;4* expression in WT (Col-0) and *atmyb44*, using a split-root system. Arabidopsis *Actin* was used as an internal control to normalize the qRT-PCR results. The data are presented as mean ± SD (three technical replicates and three technical repeats). The different lowercase letters indicate significant differences in relative gene expression levels, determined by Tukey's test (p<0.05).

Figure 7

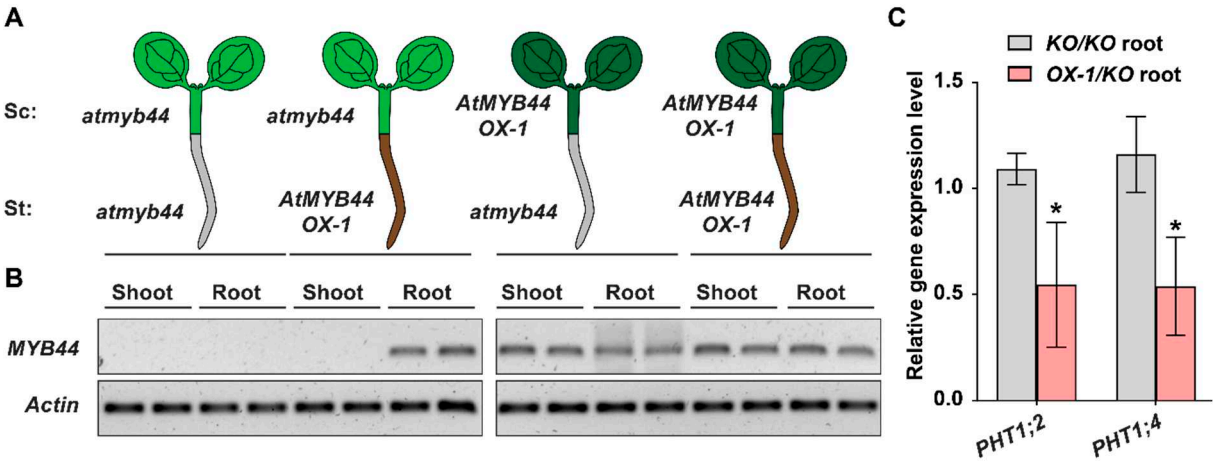

Figure 7. *AtMYB44* plays a role in systemic *PHT1;2* and *PHT1;4* regulation. (A) Schematic diagram of Arabidopsis micrografting between *atmyb44* and *AtMYB44 OX-1* seedlings. (B) *AtMYB44* is graft-transmissible from a *AtMYB44 OX-1* scion to an *atmyb44* rootstock. (C) Expression of *AtPHT1;2* and *AtPHT1;4* is decreased in the heterografted *atmyb44* rootstock. Arabidopsis *Actin* was used as an internal control to normalize the qRT-PCR results. *KO* and *OX-1* indicate *atmyb44* mutant and

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AtMYB44 OX-1 line, respectively. The data are presented as mean ± SD (three technical replicates and three technical repeats). Asterisks indicate significantly different values with homografted *atmyb44* plants. (Student's t-test, $p<0.05$).

3. Discussion

The local and systemic regulatory mechanisms acting in Pi acquisition and distribution, under Pi-stress conditions, have been extensively studied and many important genes have been identified as crucial components that regulate Pi homeostasis in plants, such as Arabidopsis and rice [4–6,34,44–47]. However, limited information is available on the nature of the systemic Pi signaling agents that control Pi homeostasis, at the whole-plant level [6,14,15,17,29,48–51]. In this study, we identified *AtMYB44* mRNA as a potential mobile systemic Pi signaling component in Arabidopsis.

3.1. AtMYB44 expression responds to an imposed Pi-stress.

In a previous study, we identified *CsMYB44* as mobile mRNA that accumulated in sink tissues, under an applied Pi-stress condition [29]. As mobile *CsMYB44* was detected in sink tissues, during the early stage of Pi-stress treatment, we hypothesized that this mobile *CsMYB44* mRNA serves as an early long-distance signaling factor for systemic Pi responses. Four homologs of CsMYB44 were identified, as potential CsMYB44 orthologs in Arabidopsis (Figure 1A, Figure S1), and their expression patterns were also responsive to an imposed Pi-stress (Figure 1B–D). As the promoter regions of *AtMYB44*, *AtMYB70* and *AtMYB73*, but not *AtMYB77*, included PIBS motifs, this suggested that *AtMYB44*, *AtMYB70* and *AtMYB73* expression could be regulated in a PHR1-dependent manners under Pi-stress conditions.

An increase in the level of *AtMYB44* expression during Pi-starvation treatment, and detection of GUS signals within the vascular tissues of both shoot and root (Figure 2), suggested that AtMYB44 could function within the vasculature in response to Pi-starvation stress. In addition, we also observed that *AtMYB44* expression was strongly detected in the root tips and lateral root primordia (Figure 2E,F). Based on our *in silico* analysis, the AtMYB44 TF could be associated with the promoter regions of many *AUXIN RESPONSE FACTORs* (*ARFs*), which contribute to control over *PHR1* expression in Arabidopsis roots under Pi-starvation conditions [52,53] (Table S2). As *AtMYB44* expression is responsive to auxin levels in the root [54], AtMYB44 may participate in auxin-dependent root development in response to Pi-stress.

AtMYB44, AtMYB70, AtMYB73 and AtMYB77 share high structural similarity and participate in root system development [24,55,56]. Interestingly, we detected enhanced expression of *AtMYB70*, *AtMYB73*, and *AtMYB77* in *atmyb44* plants, compared with WT (Figure 3). It was earlier proposed that AtMYB44 is a functional paralog of AtMYB73 and AtMYB77 for auxin-mediated lateral root growth and development [55]. Even though the functional redundancy of AtMYB70 with AtMYB44, in root growth has not yet been examined, it is implicit that AtMYB70, AtMYB73 and AtMYB77 are redundant with AtMYB44 to coordinate adaptive root development, under Pi-stress conditions.

Like a previous report [25], the *atmyb44* root appears to have slightly enhanced root growth performance under both Pi-sufficient and -stress conditions, compared to WT (Figures 4A,B, S2 and S3). One proposed AtMYB44 function is as a negative regulator in abscisic acid (ABA) responses, which are involved in root growth inhibition [25,57,58]. AtMYB44 interacts with PYRABACTIN RESISTANCE 1-LIKE 8 (PYL8), identified as an ABA receptor, to regulate early ABA signaling and promote lateral root growth [25,55]. ABA signaling can play a role in enhancing the promoter activity of various auxin-responsive genes [58]. Although limited information is available regarding the role of ABA in Pi-starvation responses [59], it is plausible that AtMYB44 might inhibit ABA responses and activate auxin signaling to enhance root development in response to the imposed Pi-stress. However, *AtMYB44* OX lines did not show a clearly opposite phenotype with the *atmyb44* line, under Pi-stress (Figures 4A,B, S2 and S3). Although this result could not be explained with the role of AtMYB44 in Pi-starvation signaling, as AtMYB44 has been proposed to be involved in multiple signaling pathways, ectopic expression of *AtMYB44* might result in aberrant ABA and auxin signaling to establish unidentified molecular responses, under these Pi-stress conditions.

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3.2. AtMYB44 mRNA acts as a systemic Pi signaling factor to negatively regulate root Pi transport systems.

It was earlier reported that MYB TFs are involved in the control over Pi-starvation responses in plants [28,45,60–62]. Arabidopsis MYB62, induced in response to Pi-stress, appears to function as a negative regulator for PSR gene expression and be involved in gibberellic acid biosynthesis [61]. Another MYB-like TF, REGULATOR OF LEAF INCLINATION 1 (RLI1), interacts with SPX1 (for Syg1/Pho81/XPR1) and the SPX1-RLI1 complex appears to block RLI1 locating to the promoter regions of RLI1 target genes, which are involved in elongation of lamina join cells in rice [63,64]. Additionally, potato MYB44 functions as a negative regulator for *PHOSPHATE1* (*PHO1*) expression, whose protein is engaged in Pi transport, from root to shoot, through Pi loading into the xylem [28].

In this study, we provide insight into the function of AtMYB44 as a negative regulator in systemic responses to Pi-stress. Enhanced *PHT1;2* and *PHT1;4* expression and elevated soluble Pi concentration were detected in *atmyb44* plants, compared to WT (Figures 4C,D and 5). It is noticeable that the soluble Pi concentration was higher in shoots and lower in roots of the *atmyb44* mutant, under Pi-sufficient conditions, compared with WT (Figure 4C,D). This suggests that, in Arabidopsis, AtMYB44 plays a negative role in root-to-shoot Pi transport under Pi-sufficient condition. Hence, Pi translocation through the xylem might be enhanced in *atmyb44* plants.

Interestingly, under Pi-stress conditions, although a similar level of soluble Pi concentration was detected in the shoots of WT and *atmyb44*, its level was higher in *atmyb44* roots, and this result seems to be correlated with increased root biomass (Figures 4 and S2). Additionally, shoot biomass of *atmyb44* was significantly elevated under sufficient Pi, but not Pi-limiting conditions (Figure 4). It is possible that AtMYB44 functions in roots as a negative regulator of *PHT1;2* and *PHT1;4* (Figure 5), thus, enhanced levels of PHT1;2 and PHT1;4 would increase the efficiency of Pi uptake and plant growth performance in *atmyb44* plants under Pi-stress conditions. Taken together, it appears that AtMYB44 might play a negative role in Pi transport from root to shoot, under Pi-sufficient conditions, but during a Pi-stress treatment, it might act as a negative regulator on Pi uptake in Arabidopsis.

The split-root assays demonstrated that *AtMYB44* expression is systemically regulated by Pi-stress (Figure 6A). Consistent with a previous study [12], systemic responses of *PHT1;2* and *PHT1;4* were observed and, interestingly, such long-distance regulation was partially disrupted in the *atmyb44* mutant background (Figure 6B,C). The mobility of *AtMYB44* mRNA appeared to reduce the level of *PHT1;2* and *PHT1;4* transcript abundance in roots (Figure 7). In this regard, various RNA species, including mRNAs, small interfering RNAs, non-coding RNAs, mRNAs etc., have been shown to be translocated through the phloem and some mobile RNA molecules can act as signaling agents in plant development and physiology [14,29,49,50,65–71]. For example, *GIBBERELLIC ACID-INSENSITIVE* mRNA is transported from the source to sink tissues and contributes to regulating leaf development [72,73]. Additionally, potato tuberization is regulated by transport of *SP6A* mRNA from potato leaves to the underground stolon [74,75]. The shoot-derived *INDOLEACETIC ACID18* (*IAA18*), *IAA28* and *TRANSLATIONALLY CONTROLLED TUMOUR PROTEIN* (*TCTP*) traffic into the roots to regulate the lateral root development [76–78].

In our study, we propose that mobile *AtMYB44* mRNA serves as a long-distance phloem-based signal in the Pi-stress response (Figure 7). Although *AtMYB44* lacked modified base 5-methylcytosine (m5C), CU- or tRNA-like motifs, which have been characterized as contributing to phloem mRNA mobility [79–81], our heterografting assay established the mobility of *AtMYB44* mRNA in Arabidopsis (Figures 7 and S4). As AtMYB44 and CsMYB44 protein have not been detected in the phloem exudate [82,83], it is likely that *AtMYB44* mRNA and not protein serves as the signaling agent in Pi homeostasis. Although mobile *AtMYB44* mRNA is involved in regulating *PHT1* expression, in roots, the regulatory mechanism underlying its phloem mobility, in Pi acquisition and utilization, remains to be elucidated. Based on previous studies [80,81], the shoot derived *AtMYB44* mRNA could be transported through the phloem and then translated in the targeted root tissues to regulate Pi transport.

Our study showed enhanced *AtMYB44* expression, under Pi-stress conditions, and a negative role for AtMYB44 in *PHT1* expression in Arabidopsis roots (Figure 8). As an increase in both *AtMYB44* and *PHT1* expression was detected under Pi-stress, this raises the question as to why plants

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would enhance *AtMYB44* expression, to repress the Pi transport system under Pi-stress conditions, even though one would expect these plants should increase their Pi uptake capacity, to adapt under limited Pi input. Mobile *AtMYB44* mRNA might play a role in fine-tuning the regulation of Pi homeostasis, in response to Pi-stress, for control over energy balance between adaptive plant development and Pi uptake (Figure 8). Our current findings provide insight into plant Pi-stress regulatory pathways and further studies will be required to reveal the molecular mechanism by which such mobile *AtMYB44* mRNA effect the ability of the plant to acquire and utilize Pi during Pi-stress conditions. Such information would be of value in genetic engineering of crops for improved yield performance under reduced Pi fertilizer applications.

Figure 8

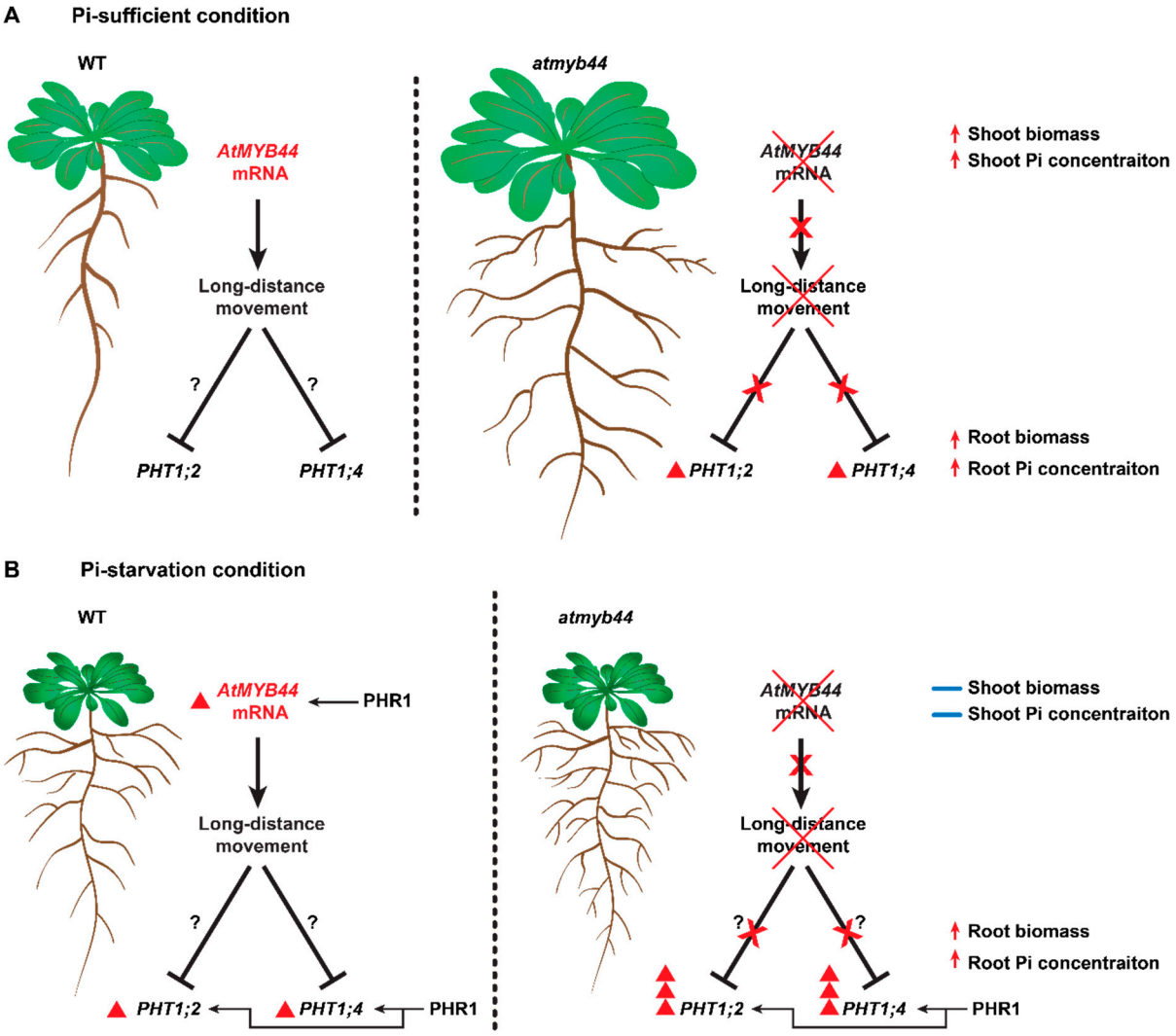

Figure 8. Schematic model of mobile *AtMYB44*-mediated *PHT1;2* and *PHT1;4* regulation in Arabidopsis. (A) The mobile *AtMYB44* transcript is long-distantly transported from source leaves to roots and serves as a systemic signal to negatively regulate the expression of Pi transporters, *PHT1;2* and *PHT1;4*, in Arabidopsis roots. In *atmyb44* mutant, *AtMYB44* mRNA is absent, thereby diminishing inhibition of *PHT1;2* and *PHT1;4* expression in roots. It results in increases of biomass and soluble Pi concentration in both shoots and roots of *atmyb44* mutant, compared to WT. (B) Under Pi-starvation conditions, PHR1 recognizes the promoter region of *AtMYB44* and *PHT1;2*/*PHT1;4* in shoots and roots, respectively, to induce those expressions. As *AtMYB44* expression is abolished in *atmyb44* mutant, *PHT1;2* and *PHT1;4* expression is more enhanced in *atmyb44* roots, compared to WT, due to absence of the negative regulatory factor, mobile *AtMYB44* mRNA, in those expression. Red darts

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and arrows indicate the increased level of designated gene expression and traits. Blue bars indicate the similar level of designated trait between WT and *atmyb44* under Pi-starvation conditions.

4. Materials and Methods

4.1. Plant materials

Arabidopsis plants were grown in controlled environment chambers under long-day conditions (16 h light/8 h dark, 120-150 μmol m^{-2} s^{-1} of photosynthetically active radiation), at temperatures of 22° C day/18°C night [84,85]. The T-DNA knock-out line (SALK_039074 [*atmyb44*]) for AT5G67300 was obtained from the Arabidopsis Biological Resource Center (ABRC). The genotypes were confirmed using PCR analysis with appropriate primer sets (Table S1).

4.2. Growth conditions and Pi-stress treatments

Arabidopsis plant lines were grown on solid medium (pH 5.7), in sand, or in a hydroponic culture system, as described previously [29,84,86,87]. Briefly, Arabidopsis seeds were sterilized, germinated on solid medium with 500 μM $NH_4H_2PO_4$ and then, at 7 days after germination (DAG), they were transferred onto fresh medium or medium with $(NH_4)_2SO_4$ that was partially, or completely replaced, $NH_4H_2PO_4$. For hydroponically grown Arabidopsis plants, the tip of a 10 μL pipette was cut and then filled with 0.7% (w/v) agar, upon which sterilized seeds were sown, prior to setting them up on a floating board system. Hydroponic solution was replaced every 5 days. The solution for Pi-sufficient and starvation treatment contained 200 μM and 5 μM of $NH_4H_2PO_4$, respectively. For growing Arabidopsis plants in sand, seeds were germinated on solid medium with 500 μM (Pi-sufficient), or 10 μM (Pi-starvation) of $NH_4H_2PO_4$ for 14 days, followed by transplanting to silica sand. Arabidopsis plants were further grown in silica sand for 14 days with nutrient solution, which contained 500 μM (Pi-sufficient), or 10 μM (Pi-starvation) of $NH_4H_2PO_4$, supplied to the roots. Images of root system architecture were captured with a Nikon D7200 (Nikon) digital camera. Acquired images were processed and analyzed using ImageJ software, as described previously [88].

Shoot and root tissues were collected from hydroponically cultured Arabidopsis plants and were used to measure biomass and Pi concentration, as described previously [34,87]. Briefly, collected shoot and root tissues were frozen in liquid nitrogen after measurement of fresh weight and then homogenized using a bead beater. Ground samples were digested with 5M H_2SO_4 and then Pi concentrations were determined using a continuous flow analyzer (Skalar), following the manufacturer's instructions.

Four-week-old Arabidopsis plants were used for the split-root assays, as described previously [12,89], with modifications. Briefly, Arabidopsis roots were divided into two parts and then immersed in two separate compartments, which contained either the same nutrient solution with 200 μM Pi (C200) or 0 μM Pi (C0), or a different nutrient solution with 200 μM Pi (SP200) and 0 μM Pi (SP0), for 7 days [12,89].

4.3. Phylogenetic analysis

The amino acid sequences of AtMYBs were obtained from the Arabidopsis Information Resource Database (https://www.arabidopsis.org/) and aligned using MEGA 11 software. The phylogenetic tree was constructed using a neighbor-Joining (NJ) method with 1,000 bootstrap replicates.

4.4. β-Glucuronidase (GUS) histochemical analysis

The 2,916 bp upstream region, which was from the ATG start codon of *AtMYB44*, was amplified from genomic DNA of Arabidopsis WT (Col-0), using the primer set listed in Table S1. The fragment was then inserted into the TOPO-D vector (Invitrogen), followed by LR clonase reaction (Invitrogen), to subclone the *AtMYB44* promoter into pBGWFS7. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 to generate transgenic Arabidopsis plants. The GUS staining of transgenic plants, carrying a *GUS* reporter gene under the control of the *AtMYB44* native promoter, was

conducted, as described previously [84]. Briefly, 12-day-old transgenic plants were immersed in 50 mM sodium phosphate buffer (pH 7.0) with 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide sodium salt and 10 mM EDTA (pH 8.0) and incubated at 37 °C. The chlorophyll was eliminated using 70% ethanol. T3 plants collected from three independent transgenic lines were used for GUS assays and imaged, using a THUNDER stereomicroscope (Leica) and Zeiss Axioskop 2 Plus microscope.

4.5. Micrografting

Arabidopsis micrografting was performed, as described previously [76,90]. Briefly, hypocotyls of 5-day-old Arabidopsis seedlings, grown on 1/2 × MS medium with 0.8% agar, were cut on nylon membranes using a surgical blade. The scion and stock were placed together to connect the shoot and root. Grafted Arabidopsis plants were grown on 1/2 × MS medium with 1.5% agar for 5 days. Successfully grafted plants were transferred to 1/2 × MS medium with 0.8% agar for further studies.

4.6. RNA extraction and qRT-PCR

Total RNA was extracted from Arabidopsis leaves and roots, using the TRIzol® Reagent (Invitrogen, Life Technologies), following the manufacturer's instructions. Total RNA of 1 µg was used for cDNA synthesis with the SuperScript IV first-strand synthesis system (Invitrogen). The qRT-PCR analysis was conducted, as described previously [91]. Briefly, the qRT-PCR was performed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher), using the QuantStudio™ 6 Flex Real-Time PCR Systems (Life Technologies), to detect *AtMYB44*, *AtMYB70*, *AtMYB73*, *AtMYB77*, *PHT1;2* and *PHT1;4*, with the primer sets listed in Table S1. *AtActin* was used as a reference gene for normalization of transcript levels. Transcript levels and ratios were calculated using the $2^{-\Delta Ct}$ or the $2^{-\Delta\Delta Ct}$ method, respectively. Statistical analyses were performed with the Student's t-test and Tukey's HSD (honestly significant difference) test. At least three biological and three technical replicates were used for qRT-PCR analyses.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Phylogenetic analysis of the *A. thaliana* MYB family; Figure S2: AtMYB44 is involved in primary and lateral root development; Figure S3: AtMYB44 serves as a potential negative regulator in shoot and root development; Figure S4: Mobility of *AtMYB44* mRNA Table S1: List of PCR primers used in this study; Table S2: Potential AtMYB44-mediated gene regulation in Arabidopsis.

Author Contributions: TO: JC and BH conceived the project. TO, JC and BH designed the experiments. TO, JC, YZ and CQ conducted the experiments; BH and LK provided technical advice. All authors contributed to perform the data analysis and interpretation. JC and TO prepared the draft and BH revised the manuscript. All authors read and approved this article.

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Data Availability Statement: The data in this study are available within the article.

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