OsMYB2P-1, an R2R3 MYB Transcription Factor, Is Involved in the Regulation of Phosphate-Starvation Responses and Root Architecture in Rice[1][C][W][OA]

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An R2R3 MYB transcription factor, *OsMYB2P-1*, was identified from microarray data by monitoring the expression profile of rice (*Oryza sativa* ssp. japonica) seedlings exposed to phosphate (Pi)-deficient medium. Expression of *OsMYB2P-1* was induced by Pi starvation. *OsMYB2P-1* was localized in the nuclei and exhibited transcriptional activation activity. Overexpression of *OsMYB2P-1* in Arabidopsis (*Arabidopsis thaliana*) and rice enhanced tolerance to Pi starvation, while suppression of *OsMYB2P-1* by RNA interference in rice rendered the transgenic rice more sensitive to Pi deficiency. Furthermore, primary roots of *OsMYB2P-1*-overexpressing plants were shorter than those in wild-type plants under Pi-sufficient conditions, while primary roots and adventitious roots of *OsMYB2P-1*-overexpressing plants were longer than those of wild-type plants under Pi-deficient conditions. These results suggest that *OsMYB2P-1* may also be associated with the regulation of root system architecture. Overexpression of *OsMYB2P-1* led to greater expression of Pi-responsive genes such as *Oryza sativa* UDP-sulfoquinovose synthase, *OsIPS1*, *OsPAP10*, *OsmiR399a*, and *OsmiR399j*. In contrast, overexpression of *OsMYB2P-1* suppressed the expression of OsPHO2 under both Pi-sufficient and Pi-deficient conditions. Moreover, expression of *OsPT2*, which encodes a low-affinity Pi transporter, was up-regulated in *OsMYB2P-1*-overexpressing plants under Pi-sufficient conditions, whereas expression of the high-affinity Pi transporters *OsPT6*, *OsPT8*, and *OsPT10* was up-regulated by overexpression of *OsMYB2P-1* under Pi-deficient conditions, suggesting that *OsMYB2P-1* may act as a Pi-dependent regulator in controlling the expression of Pi transporters. These findings demonstrate that OsMYB2P-1 is a novel R2R3 MYB transcriptional factor associated with Pi starvation signaling in rice.

Phosphorus (P), as an essential macronutrient for plant growth and development, is a constituent of key molecules such as ATP, nucleic acids, and phospholipids (Rubio et al., 2001; Cheng et al., 2011). Although the overall P content in soil is high, P is one of the limiting factors for plant growth due to its rapid immobilization by soil organic and inorganic components in many natural and agricultural ecosystems (for review, see Richardson et al., 2009; Rouached et al., 2010; Hinsinger et al., 2011). To cope with the low availability of soil phosphate (Pi), which is a major form of P used by plants, plants have evolved numerous strategies to optimize Pi acquisition from soil solution and its distribution to different organs and subcellular compartments (Raghothama, 1999; Vance et al., 2003). There are profound changes in root architecture by stimulating the growth of lateral roots and root hairs to maximize root surface area for Pi uptake under Pi-deficient conditions (Williamson et al., 2001; López-Bucio et al., 2003). Exudation of organic acids and phosphatases as well as acidification of rhizosphere have been widely observed to solubilize Pi bound to the soil particles and to release inorganic P from organic sources (Jones, 1998; Richardson et al., 2009). In addition, Pi-starved plants can modulate multiple metabolic processes to reprioritize the utilization of internal Pi and maximize the acquisition of external Pi, leading to adaptation to low-Pi environments (Vance et al., 2003; Wasaki et al., 2003). Upon exposure of plants to Pi-deficient medium, numerous genes are activated, which in turn orchestrates changes in molecular, cellular, and physiological processes, thus allowing plants to effectively adapt to the low-Pi environment (Hammond et al., 2003; Smith et al., 2010; Yang and Finnegan, 2010; Chen et al., 2011; Hammond and White, 2011). Furthermore, microRNAs as posttranscriptional regulators have also been reported to play a role in the response of plants to Pi deficiency (Fujii et al., 2005; Chiou et al., 2006; Vance, 2010). In Arabidopsis (*Arabidopsis thaliana*), a major transcriptional regulatory system that involves *PHR1*, *SIZ1*, *miR399*, and *PHO2* in response to Pi deficiency has been identified (Bari et al., 2006; Schachtman and Shin, 2007). *PHR1*, a MYB transcription factor (TF), is a key

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regulator of Pi starvation signaling cascades and sumoylated by SIZ1, which is a small plant ubiquitin-like modifier E3 ligase (Miura et al., 2005). *miR399*, the target gene of PHR1, is specifically induced by Pi starvation (Fujii et al., 2005) and negatively regulates *PHO2* expression at the transcriptional level (Chiou et al., 2006). Mutations in *PHO2* and overexpression of both *PHR1* and *miR399* in Arabidopsis result in excessive Pi accumulation in shoots and the activation of Pi starvation-induced gene expression (Rubio et al., 2001; Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Nilsson et al., 2007).

The *PHR1-miR399-PHO2* pathway is a central component of the Pi starvation response, but other pathways may also be required for the Pi starvation response (Yi et al., 2005; Wang et al., 2009). Available evidence indicates that some Pi-responsive TFs do not participate in the *PHR1-miR399-PHO2* pathway. These include *OsPTF1* (Yi et al., 2005) in rice (*Oryza sativa*) and *MYB62* (Devaiah et al., 2009), *WRKY75* (Devaiah et al., 2007a), *ZAT6* (Devaiah et al., 2007b), and *BHLH32* (Chen et al., 2007) in Arabidopsis. These findings suggest that TFs play a crucial role in controlling the expression of downstream genes as well as the regulation of cross talk among different signaling pathways.

Rice is one of the most important food crops in Asia (Cantrell and Reeves, 2002). Rice productivity is limited by low Pi availability in cultivated areas worldwide (Raghothama, 1999). To improve rice yield under Pi-deficient conditions, it is essential to decipher the molecular mechanisms by which rice plants respond and adapt to low-Pi stress. Recent studies show that the *PHR1-miR399-PHO2* signaling pathway is also operating in rice plants in response to Pi deficiency. For instance, Zhou et al. (2008) found that *OsPHR2*, the homolog of *AtPHR1*, is a key regulator for Pi starvation signaling in rice. *OsSPX1* is associated with Pi homeostasis, and the function of *OsPHR2* is suppressed by *OsSPX1* (Wang et al., 2009; Liu et al., 2010). However, little is known about the molecular mechanisms underlying the signaling pathways for sensing and responding to Pi deficiency in rice. Therefore, the identification of novel genes that are involved in sensing and responding to Pi deficiency, and unraveling their regulatory networks, are of critical importance for our understanding how plants tolerate Pi deficiency in soil. Here, we report the identification and functional characterization of a nucleus-localized R2R3-type MYB TF, *OsMYB2P-1* (for *MYB2* phosphate-responsive gene 1) in rice. Our results demonstrate that overexpression of *OsMYB2P-1* in Arabidopsis and rice conferred the greater tolerance of transgenic plants to low-Pi stress. We further show that overexpression *OsMYB2P-1* activated the expression of *OsPT6*, *OsPT8*, and *OsPT10* under low-Pi stress, whereas it induced the expression of *OsPT2* under Pi-sufficient conditions, leading to excess accumulation of Pi in shoots. These findings shed important light on the mechanism by which rice plants regulate Pi uptake and translocation under Pi-deficient conditions.

RESULTS

Identification of Pi-Responsive MYB TFs from Rice

Expression profiles of 1-week-old rice seedlings exposed to Pi-deficient solution for 6, 24, 48, or 72 h were monitored by a microarray that contains approximately 60,000 rice clones. The probes were prepared from RNAs isolated from rice seedlings exposed to Pi-deficient solution for 6, 24, 48, or 72 h and nontreated controls. For hybridization, two biological replicates were used to extract RNAs from different batches of plants. The Pi-inducible and Pi-repressive genes were taken as the expression ratio (treatment relative to control) greater than and less than 2-fold, respectively (Supplemental Tables S1–S4). Among the Pi starvation-responsive genes, an EST (accession no. Os.9514.1.S1_at [Affymetrix GeneChip]) encoding a putative R2R3 MYB TF was identified and functionally characterized. In our microarray hybridization, the transcript level of Os.9514.1.S1_at was increased by 2.8-, 6.3-, 6.1-, and 4.4-fold after Pi starvation for 6, 24, 48, and 72 h, respectively (Fig. 1A; Supplemental Tables S1–S4). The expression of Os.9514.1.S1_at in the microarrary analysis was confirmed by real-time PCR. A comparable change in the expression of Os.9514.1.S1_at in response to Pi starvation was observed by real-time PCR (Fig. 1B). Thus, these results validate the Pi-regulated expression patterns from the microarray analysis (Fig. 1A).

Structural Features, Phylogenetic Tree, and Subcellular Localization of OsMYB2P-1

To investigate the function of Os.9514.1.S1_at, we amplified its full-length cDNA by reverse transcription (RT)-PCR from rice seedlings exposed to Pi-depleted medium for 6 h. The full-length cDNA contained an open reading frame of 427 amino acids with a calculated molecular mass of 46.5 kD. Homological analysis revealed that the gene shared the greatest sequence similarity with the R2R3-type MYB TFs. Bioinformatics analysis demonstrated that the protein contained two

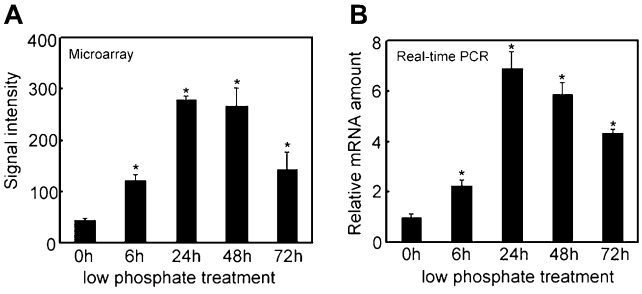

Figure 1. Isolation of Pi deprivation-inducible MYB TF from microarray hybridization. A, Signal intensity of Os.9514.1.S1_at in microarray hybridization. B, Real-time PCR to validate Os.9514.1.S1_at microarray results presented in A. Expression was normalized to that of *Actin*. Data are means ± SD ($n = 3$). Asterisks indicate significant differences at $P < 0.05$ compared with the control by Student's *t* test.

MYB repeats that are most similar to R2 and R3 motif at its N terminus. Therefore, this gene is an R2R3-type MYB TF and was designated *OsMYB2P-1*. Interestingly, it also contained a novel DUF3651 conserved domain at its C terminus (Fig. 2A; Supplemental Fig. S1). A search of PROSITE (http://www.expasy.org/prosite) revealed that the OsMYB2P-1 protein contained five potential domains of the sumoylation target (Supplemental Fig. S1).

AtPHR1, CrPSR1, OsPHR1, and OsPHR2 belong to MYB-CC subgroup I, which have been shown to be involved in responses to Pi starvation (Wykoff et al., 1999; Rubio et al., 2001; Zhou et al., 2008). In a recent report, Zhang et al. (2012) classified rice MYB proteins related to abiotic stress to the C17, C20, and C25 subgroups. We constructed a phylogenetic tree based on amino acid sequences of rice MYB proteins involved in abiotic stress, MYB-CC subgroup I and myb62 (a Pi-induced MYB gene from Arabidopsis), using the DNAMAN program. According to the phylogenetic tree, the MYB62 protein had the highest similarity with EEC69869 from rice. OsMYB2P-1 was not grouped with any MYB proteins that are associated with abiotic stress; rather, it formed a separate branch with an Arabidopsis R2R3-MYB protein with unknown function (Fig. 2B).

To determine its subcellular localization, *OsMYB2P-1* was fused in frame to the 5′ terminus of the *GFP* reporter gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. The recombinant constructs of the *OsMYB2P-1-GFP* fusion gene and *GFP* alone were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment. As shown in Figure 2C, the OsMYB2P-1-GFP fusion protein accumulated mainly in the nucleus, whereas GFP alone was present throughout the whole cell, suggesting that OsMYB2P-1 is a nucleus-localized protein. This result also is consistent with its predicted function as a TF (Fig. 2C).

A yeast GAL4 system was used to determine the transcription activity of OsMYB2P-1. The full-length cDNA of *OsMYB2P-1* was fused to the GAL4 DNA-binding domain of the pGBKT7 vector, and the fusion plasmid pBD-*OsMYB2P-1* was transformed into the yeast strain AH109. Figure 2D shows yeast growth on screened synthetic dextrose medium lacking tryptophan and adenine (SD/-Trp/-Ade), synthetic dextrose medium lacking tryptophan, adenine, and histidine (SD/-Trp/-Ade/-His), as well as galactosidase. Strong blue signals reflecting a healthy growth of yeast on both media were detected in the transformants containing the

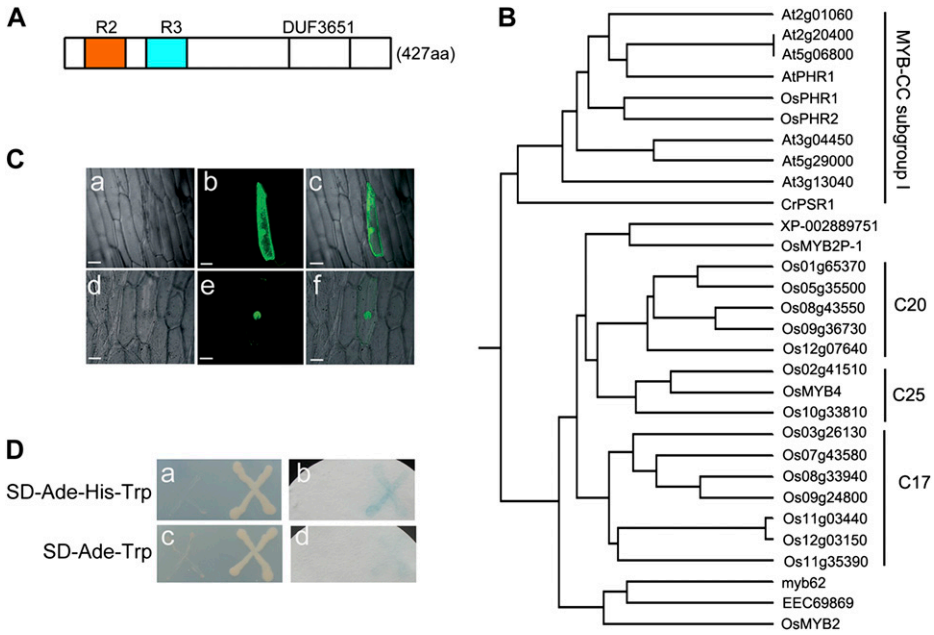

Figure 2. Homology, localization, and transcriptional activation of OsMYB2P-1. A, Scheme showing the structures of OsMYB2P-1 proteins. aa, Amino acids. B, Phylogenetic tree of Myb proteins. The tree was constructed with the DNAMAN tree program with amino acid sequences of OsMYB2P-1 and other members of the Myb family isolated from Arabidopsis, rice, and *Chlamydomonas*. The full-length amino acid sequences were downloaded from The Institute for Genomic Research (http://www.tigr.org) and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). C, Localization of OsMYB2P-1-GFP protein. Individual panels show GFP alone (b) or OsMYB2P-1-GFP (e) in onion epidermal cells, corresponding bright-field images (a and d), and merged images (c and f) of a and b and of d and e, respectively. GFP and OsMYB2P-1-GFP fusion was driven by the control of the CaMV 35S promoter. Onion epidermal peels were bombarded with DNA-coated gold particles, and GFP expression was visualized 24 h later. Bars = 50 μm. D, Transcription activation analysis of OsMYB2P-1 protein. Individual panels show that the transformants with pBD (left) and pBD-OsMYB2P-1 (right) grow on SD/-Trp/-Ade and SD/-Trp/-Ade/-His medium (a and c) and the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside activation detection of transformed yeast thalli on SD/-Trp/-Ade and SD/-Trp/-Ade/-His plates with solid SD medium shown in a and c (b and d).

full-length cDNA of *OsMYB2P-1* compared with the control yeast transformed with empty vector, suggesting that the OsMYB2P-1 protein is involved in transcriptional activation of genes.

Expression Patterns of *OsMYB2P-1*

The tissue-specific expression of *OsMYB2P-1* in rice was studied by real-time RT-PCR. *OsMYB2P-1* was expressed in all organs examined, with the expression being highest in stems and lowest in leaves (Fig. 3A). The expression patterns of *OsMYB2P-1* under Pi-sufficient and Pi-deficient conditions were evaluated by real-time RT-PCR using RNA samples extracted from roots, stems, and leaves. Induction of *OsMYB2P-1* transcripts by Pi starvation was observed mainly in roots and stems up to 7 d (Fig. 3B). The increases in *OsMYB2P-1* transcripts in leaves, stems, and roots were detected after 6 h of Pi starvation, peaked at 24 h of Pi starvation, and declined gradually thereafter. This observation is consistent with our microarray results (Fig. 1A). To determine whether the up-regulation of the *OsMYB2P-1* gene was specific to Pi starvation, the responsiveness of *OsMYB2P-1* expression to deprivation of other mineral nutrients, including nitrogen (N), potassium (K), and iron (Fe), was also investigated. Similar to Pi deprivation, the expression of *OsMYB2P-1* in both roots and stems was markedly enhanced by deprivation of Fe (Fig. 3C). In contrast, the expression of *OsMYB2P-1* in roots, stems, and leaves was not responsive to deprivation of N and K (Fig. 3D; Supplemental Fig. S2). Taken together, these results suggest that the expression of *OsMYB2P-1* is induced by deficiency of Pi and Fe, implying that it may play a regulatory role in response and adaptation to these mineral stresses.

Molecular Characterization of *OsMYB2P-1*-Overexpressed and RNA Interference Knockdown Transgenic Lines

To investigate the function of *OsMYB2P-1* in plants, we overexpressed *OsMYB2P-1* in Arabidopsis and rice under the control of a CaMV 35S promoter and a ubiquitin promoter of maize (*Zea mays*), respectively. In addition, we suppressed the expression of *OsMYB2P-1* in rice under the control of a ubiquitin promoter of maize. Transgenic lines of *OsMYB2P-1* in rice were confirmed by hygromycin selection and Southern blotting. Southern blotting was performed by using the DNA digested with *Hin*dIII or *Eco*RI and the *GUS* gene as a probe. Three overexpressed lines and the two RNA interference (RNAi) lines were randomly selected, and different hybridized patterns to the *GUS* probe were observed. In wild-type rice plants, no signals were detected under the same conditions (Fig. 4A). Therefore, the three overexpressed transgenic lines and the two RNAi transgenic lines are likely to be independent. Furthermore, real-time PCR analysis showed that expression of *OsMYB2P-1* was markedly increased in the three independent overexpressing lines but decreased in the two RNAi transgenic lines (Fig. 4B).

To examine phenotypes of the transgenic lines, T2 and T3 progeny of the *OsMYB2P-1*-overexpressed lines, the *OsMYB2P-1* RNAi transgenic lines, and wild-type plants were grown in a greenhouse under identical conditions. When grown in Pi-sufficient medium, primary roots in the overexpressed rice plants were shorter than those in wild-type plants (Fig. 4, C and D). By contrast, when grown in Pi-deficient medium, the *OsMYB2P-1*-overexpressed lines had greater tiller numbers than wild-type plants (Fig. 4, E and F). In addition to rice, the expression of *OsMYB2P-1* in transgenic Arabidopsis

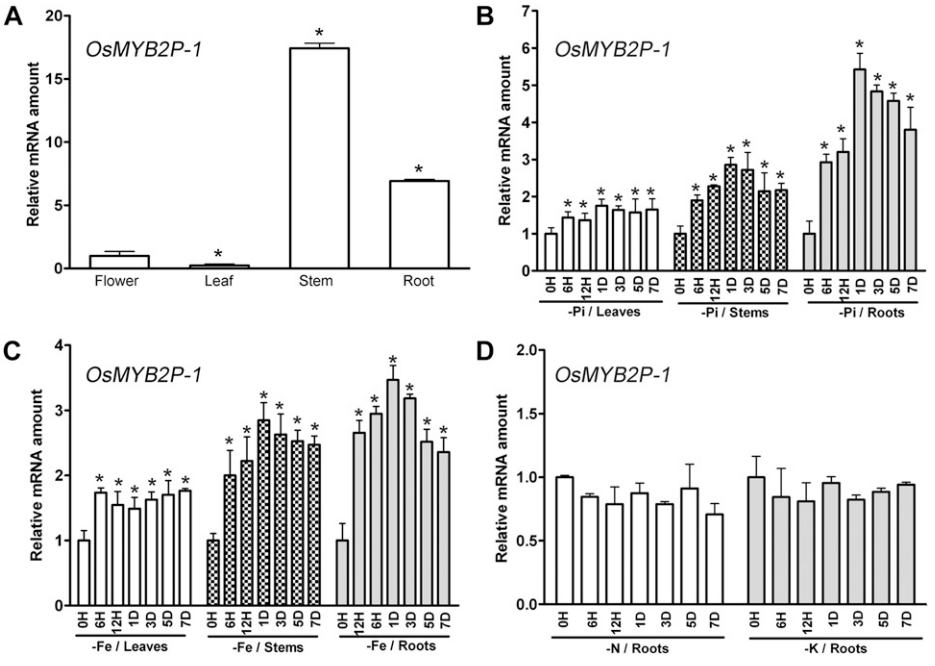

Figure 3. Expression patterns of *OsMYB2P-1* in different organs, and effects of the deprivation of Pi, N, K, and Fe on the expression of *OsMYB2P-1*. A, *OsMYB2P-1* expression in different tissues. B, Time course of *OsMYB2P-1* expression in response to Pi deprivation. C and D, Response of *OsMYB2P-1* to Fe, N, and K deprivation. *Actin* was used as an internal control. Expression was normalized to that of *Actin*. Data are means ± SD ($n = 3$). Asterisks indicate significant differences at $P < 0.05$ compared with the control by Student's *t* test.

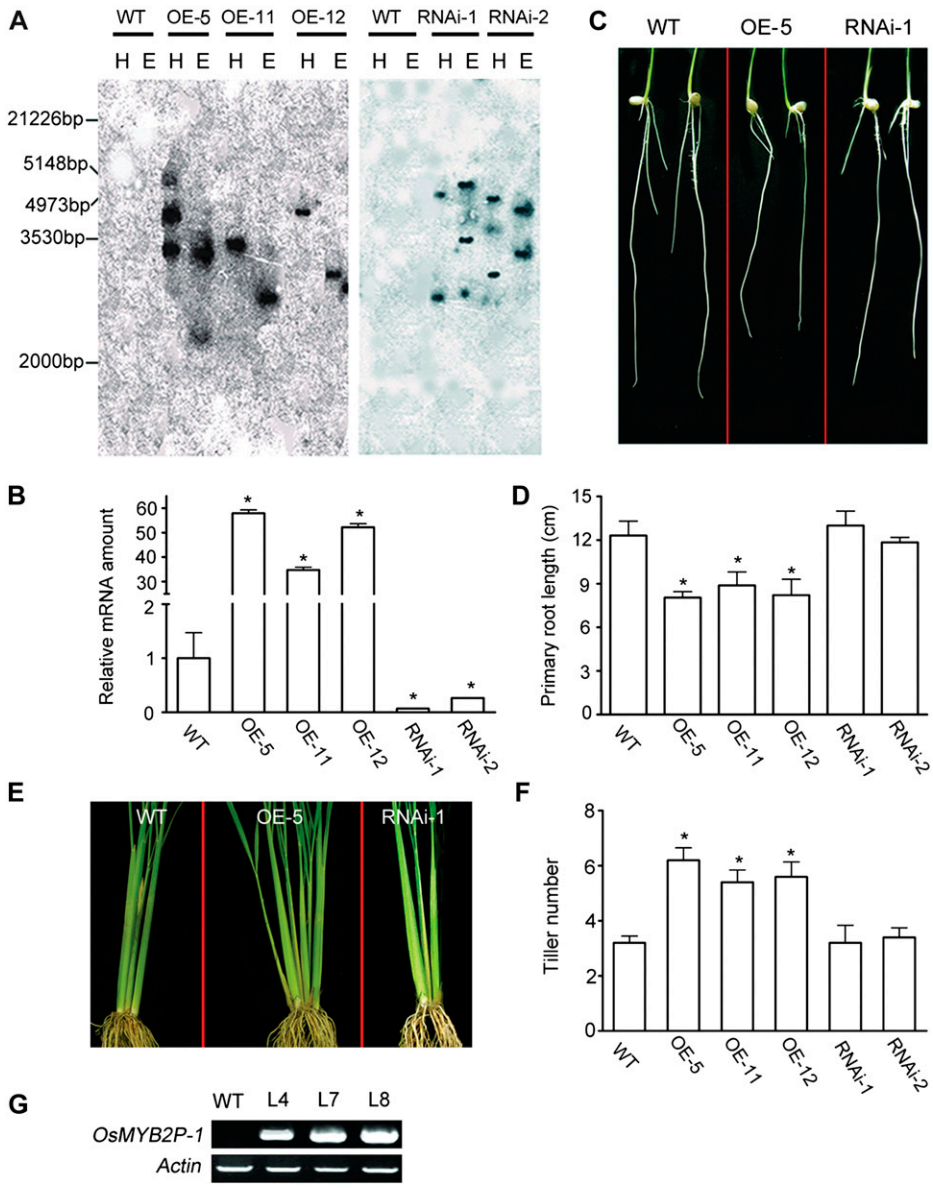

Figure 4. Molecular characterization and phenotypes of *OsMYB2P-1* transgenic plants. A, Southern-blot assay for rice transgenic plants. Genomic DNA isolated from wild-type (WT) and transformed plants was digested with *Eco*RI (E) or *Hin*dIII (H). The blot was hybridized with the open reading frame of the *GUS* gene labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP as described in "Materials and Methods." B, Expression of independent transgenic rice by real-time PCR analysis. Expression was normalized to that of *Actin*. The transcript level from the wild-type was set to 1. Data are means ± SD (n = 3). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's t test. C, Primary root length in transgenic rice. Wild-type, *OsMYB2P-1*-overexpressed, and *OsMYB2P-1* RNAi lines are shown after 1 week of germination at 28°C. D, Quantitative analysis of the plant primary root length as shown in C. Data are means ± SD ($n \geq 30$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's t test. E, Comparison of tiller numbers between *OsMYB2P-1*-overexpressed and wild-type rice plants. Wild-type, *OsMYB2P-1*-overexpressed, and *OsMYB2P-1* RNAi lines are shown after 2 months of growth under the Pi-deficient condition. F, Quantitative analysis of the tiller number as shown in E. Data are means ± SD ($n \geq 25$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's t test. G, Examination of transgenic plant lines of Arabidopsis by semiquantitative RT-PCR.

was examined by semiquantitative RT-PCR. *OsMYB2P-1* was highly expressed at the transcriptional level in transgenic Arabidopsis, while no *OsMYB2P-1* was detected in wild-type Arabidopsis plants (Fig. 4G).

Response of Rice and Arabidopsis Lines Expressing *OsMYB2P-1* to Pi Starvation

To functionally characterize the role of *OsMYB2P-1* in response and adaptation to Pi starvation, T2 progeny of 1-week-old transgenic lines and wild-type seedlings were exposed to hydroponic solution containing a high level of Pi (HP; 0.323 mM Pi, approximately 10 mg L^{-1} Pi) and a low level of Pi (LP; 0.016 mM Pi, approximately 0.5 mg L^{-1} Pi) for 30 d. The three *OsMYB2P-1*-overexpressing lines showed retarded growth compared with wild-type plants, as shown by the lower biomass and shorter plant height of the transgenic plants than the wild-type plants when grown in HP solution (Fig. 5A; Table I). Moreover, Pi concentrations in shoots and roots of the *OsMYB2P-1*-overexpressing lines grown in the HP medium were higher than in the wild type (Fig. 5B). In contrast, the *OsMYB2P-1*-overexpressing lines showed better growth than wild-type plants when grown in the LP medium (Fig. 5A; Table I). There was a significant difference in the biomass of root and shoot between wild-type and *OsMYB2P-1*-overexpressing plants under both HP and LP conditions (Table I). Pi concentrations in roots of the transgenic plants overexpressing *OsMYB2P-1* were significantly higher than those of wild-type plants when grown in HP medium. There were sharp reductions in Pi concentrations in shoots and roots of both wild-type and transgenic plants when they were incubated in LP medium (Fig. 5C). In contrast to plants grown in HP medium, the *OsMYB2P-1*-overexpressing plants had

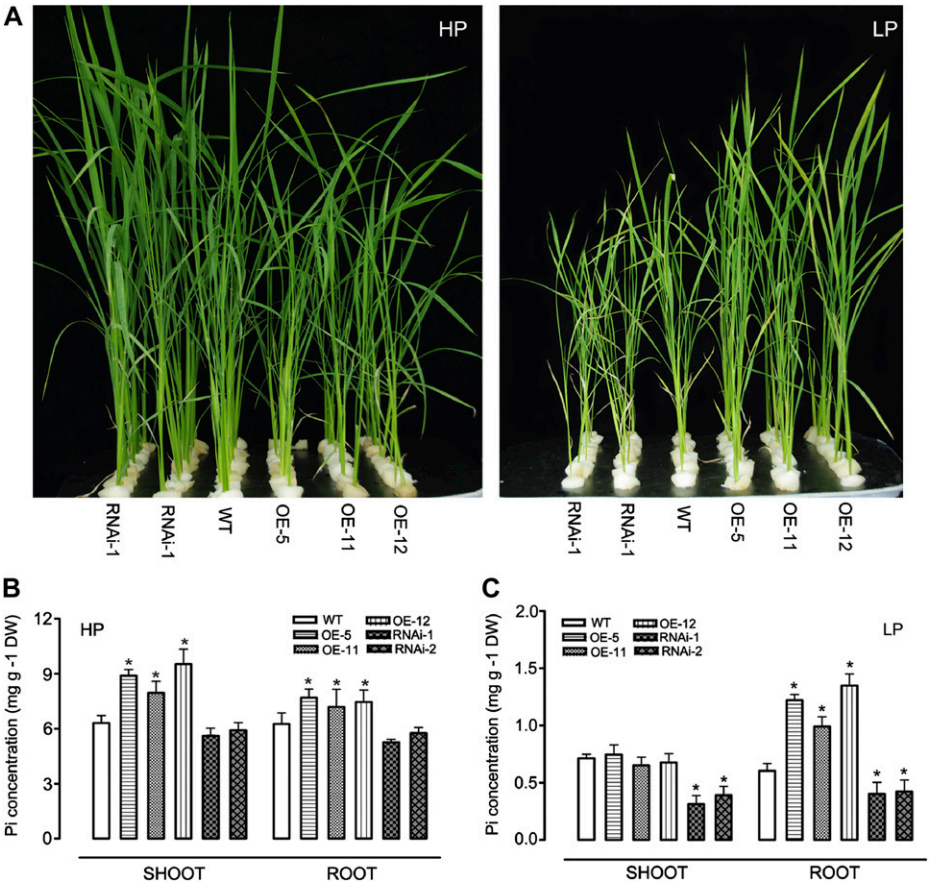

Figure 5. Effects of *OsMYB2P-1* expression on tolerance to Pi deficiency. A, Phenotypes of wild-type (WT), *OsMYB2P-1*-overexpressed, and *OsMYB2P-1* RNAi plants grown in the greenhouse for 30 d under Pi-sufficient or Pi-deficient conditions. Plants were pregerminated in water for 7 d and grown hydroponically for 30 d in medium containing 0.323 or 0.0161 mM Pi. B and C, Pi contents in roots and shoots of wild-type, *OsMYB2P-1*-overexpressed, and *OsMYB2P-1* RNAi knockdown plants grown in the greenhouse for 30 d under Pi-sufficient or Pi-deficient conditions. Data are means of three replicates with errors bars indicating SD. Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. DW, Dry weight.

higher Pi concentrations in roots than wild-type plants under LP conditions, while no differences in Pi concentrations in shoots between wild-type and *OsMYB2P-1*-overexpressing plants were observed under LP conditions (Fig. 5C).

To test whether knockdown of *OsMYB2P-1* expression can alter the sensitivity of rice plants to Pi deficiency, two independent RNAi transgenic lines of *OsMYB2P-1* (RNAi-1 and RNAi-2) were also used to study the response to Pi deficiency. No evident differences in phenotypes and Pi concentrations between the wild type and the two *OsMYB2P-1* RNAi knockdown lines were observed when grown in HP medium. However, the two RNAi lines exhibited greater

Table I. *Plant height, dry shoot biomass, and dry root biomass of wild-type and transgenic plants*

Plants were pregerminated in water for 7 d and grown hydroponically for 30 d in HP or LP medium, and then plants were sampled for the measurements. The values are means ± SD of three independent experiments, with 10 seedlings being used in each experiment. Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test.

Genotype	Shoot Biomass	Root Biomass	Plant Height
	g dry weight		*cm*
HP (0.323 mM Pi)			
Wild type	1.664 ± 0.056	0.259 ± 0.023	54.657 ± 1.050
OE-5	0.849 ± 0.075*	0.127 ± 0.018*	43.100 ± 0.641*
OE-11	0.935 ± 0.015*	0.130 ± 0.012*	46.329 ± 0.312*
OE-12	0.692 ± 0.026*	0.109 ± 0.006*	45.357 ± 0.940*
RNAi-1	1.508 ± 0.044	0.212 ± 0.003	53.652 ± 0.867
RNAi-2	1.601 ± 0.061	0.231 ± 0.008	52.613 ± 2.012
LP (0.0161 mM Pi)			
Wild type	0.692 ± 0.021	0.198 ± 0.033	43.171 ± 0.615
OE-5	0.797 ± 0.010*	0.223 ± 0.009*	46.029 ± 2.040*
OE-11	0.733 ± 0.046*	0.210 ± 0.025*	45.329 ± 1.177*
OE-12	0.861 ± 0.012*	0.261 ± 0.011*	47.300 ± 0.344*
RNAi-1	0.422 ± 0.031*	0.131 ± 0.015*	40.057 ± 0.822*
RNAi-2	0.384 ± 0.028*	0.112 ± 0.027*	41.163 ± 0.907*

growth inhibition than the wild-type plants when grown in LP medium (Fig. 5A; Table I). For instance, shoot biomass of the *OsMYB2P-1* RNAi transgenic rice was about 40% less than that of wild-type plants after exposure to Pi-deficient solution for 30 d. Pi concentrations in shoots and roots of *OsMYB2P-1* RNAi knockdown lines were lower than those of wild-type plants under Pi-deficient conditions (Fig. 5, B and C), while Pi concentrations in both shoots and roots in the two RNAi lines were comparable to those of wild-type plants when grown in HP medium (Fig. 5, B and C). Thus, these results suggest that interference of *OsMYB2P-1* renders rice seedlings more sensitive to Pi deficiency.

To further assess the role of *OsMYB2P-1* in response to Pi deficiency, T3 progeny of three homozygous *OsMYB2P-1*-overexpressed transgenic Arabidopsis lines (L4, L7, and L8) and wild-type plants were also used (Fig. 6A). Overexpression of *OsMYB2P-1* enhanced the accumulation of Pi in both shoots and roots of Arabidopsis seedlings when grown in HP solution (Fig. 6B). Pi concentrations in shoots of the *OsMYB2P-1*-overexpressed Arabidopsis lines did not differ from their wild-type counterparts when grown in LP solution (Fig. 6B), while Pi concentrations in roots of the *OsMYB2P-1*-overexpressed transgenic Arabidopsis lines were significantly higher than those in roots of wild-type plants grown in LP medium (Fig. 6C). Compared with wild-type plants, the biomass of shoots and roots in the transgenic plants was reduced when grown in HP medium, while the transgenic Arabidopsis seedlings had greater biomass than wild-type plants when grown in LP medium (Fig. 6, D and E). Enhanced accumulation of anthocyanin in aerial parts of plants is a common phenomenon for plants suffering from low-Pi stress. When grown on Murashige and Skoog medium supplemented with sufficient Pi, little anthocyanin was detected in both wild-type and transgenic lines (Fig. 6, A and F). A marked increase in the accumulation of anthocyanin was observed in both wild-type and transgenic lines upon exposure to LP medium, and the Pi deficiency-induced increase in anthocyanin was significantly higher in the wild type than in the three transgenic lines (Fig. 6F). Therefore, the higher biomass and lower anthocyanin content in the transgenic plants than in the wild-type plants under Pi-deficient conditions imply that the overexpression of *OsMYB2P-1* in Arabidopsis also renders Arabidopsis more tolerant to Pi deficiency.

OsMYB2P-1 Altered Root System Architecture

The root architecture system is sensitive to Pi status in growth medium (López-Bucio et al., 2003; Jain et al., 2007). To test whether the improved performance of transgenic rice plants grown in Pi-deficient medium is related to changes in the root architecture system, 1-week-old wild-type and transgenic lines with over-

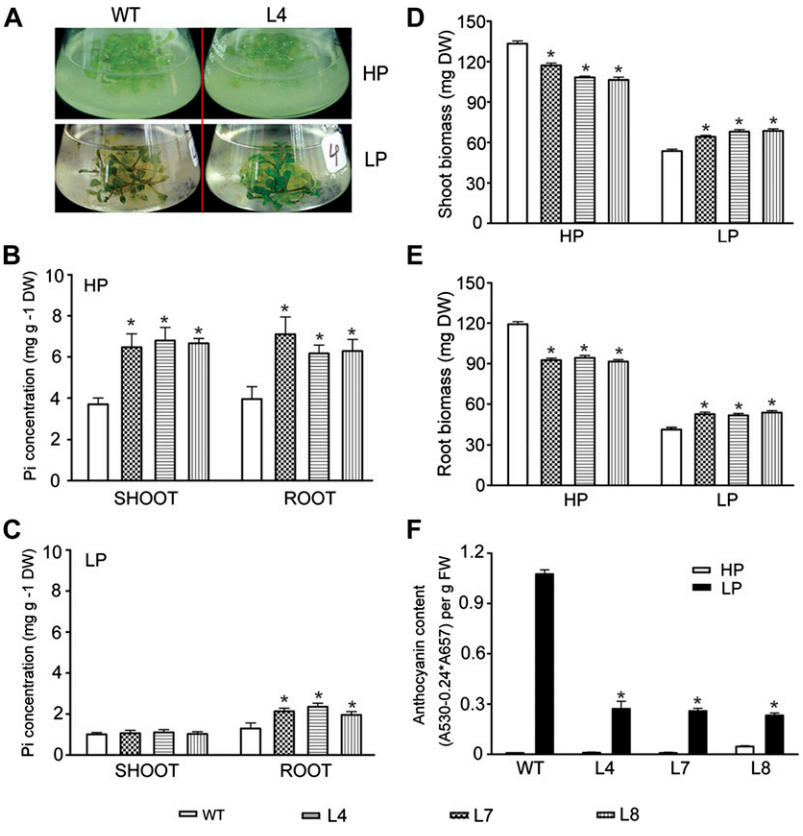

Figure 6. Stress response of *OsMYB2P-1*-overexpressing Arabidopsis. A, Phenotypes of wild-type (WT) and transgenic Arabidopsis plants grown in HP and LP medium. Four-day-old wild-type and *OsMYB2P-1*-overexpressing plants were transferred to LP (10 μM) and HP (1 mM) conditions and grown for 3 weeks in hydroponic medium. B and C, Pi concentrations in roots and shoots for *OsMYB2P-1*-overexpressing Arabidopsis and wild-type plants. Data are means ± SD. Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. D and E, Biomass in wild-type and transgenic plants. Dry weight (DW) for shoots and roots of *OsMYB2P-1*-overexpressing Arabidopsis and wild-type plants was determined after growth in HP or LP medium for 3 weeks. Data are means ± SD ($n \geq 30$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. F, Total anthocyanin content in wild-type and *OsMYB2P-1*-overexpressing (L4, L7, and L8) plants grown hydroponically in HP and LP medium. Data are means ± SD ($n \geq 30$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. FW, Fresh weight.

expressing and RNAi lines of *OsMYB2P-1* grown in hydroponic solution containing high and low Pi for 14 d were used to compare the primary root length and total length of the three longest adventitious roots. In HP medium, primary and adventitious roots in *OsMYB2P-1*-overexpressed plants were shorter than those in wild-type plants, while no significant differences in root architecture were observed between the wild type and *OsMYB2P-1* RNAi knockdown lines (Fig. 7). However, both primary and adventitious roots in the *OsMYB2P-1*-overexpressed plants were significantly longer than those in wild-type plants under LP conditions (Fig. 7).

In addition to rice, the effect of Pi deficiency on the root system architecture of wild-type and *OsMYB2P-1*-overexpressing Arabidopsis was also examined. Wild-type and transgenic Arabidopsis seedlings were grown on vertically oriented agar plates containing sufficient Pi (1 mM) and deficient Pi (10 μM). There were no significant differences between the wild type and the three transgenic lines under Pi-sufficient conditions in terms of primary root length and lateral root density. However, the lateral root density in the *OsMYB2P-1*-overexpressing Arabidopsis seedlings became significantly higher than in wild-type plants grown in Pi-deficient medium (Supplemental Fig. S3). Although the primary root growth was inhibited in both the wild-type and transgenic plants under Pi-deficient conditions, the primary root length was slightly longer in *OsMYB2P-1*-overexpressing Arabidopsis than in wild-type plants (Supplemental Fig. S3). Taken together, these results are suggestive that *OsMYB2P-1* is likely to play a role in the regulation of Pi-dependent root architecture, which in turn may facilitate Pi acquisition under Pi-deficient conditions.

OsMYB2P-1 Regulates the Expression of Pi-Responsive Genes

To elucidate the molecular mechanism by which OsMYB2P-1 regulates the Pi starvation response, the expression of several Pi starvation-inducible genes was monitored by real-time PCR. The Pi starvation-inducible genes, including *Oryza sativa* UDP-sulfoquinovose synthase (*OsSQD*), *OsPAP10*, *OsIPS1*, *OsmiR399a*, and *OsmiR399j*, were markedly induced in both wild-type and *OsMYB2P-1*-overexpressing transgenic plants when grown in Pi-deficient medium (Fig. 8). This observation is consistent with previous studies (Essigmann et al., 1998; Yu et al., 2002; Hou et al., 2005; Wang et al., 2006). However, the expression of *OsSQD*, *OsPAP10*, *OsIPS1*, *OsmiR399a*, and *OsmiR399j* in roots of *OsMYB2P-1*-overexpressed plants was significantly higher than in wild-type plants under both Pi-sufficient and Pi-deficient conditions (Fig. 8). In addition, these genes were also activated by *OsMYB2P-1* in shoots of *OsMYB2P-1*-overexpressing plants under both Pi-sufficient and Pi-deficient conditions (Supplemental Fig. S4). In contrast to *OsMYB2P-1*-overexpressing transgenic plants, the expression of *OsSQD*, *OsPAP10*, *OsIPS1*, *OsmiR399a*, and *OsmiR399j* in roots of RNAi *OsMYB2P-1* transgenic plants was significantly reduced compared with wild-type plants under both Pi-sufficient and Pi-deficient conditions (Fig. 8). *OsPHO2*, which acts as a target gene of *miR399*, plays an important role in Pi starvation signaling (Hu et al., 2011). In our study, the expression of *OsPHO2* was suppressed in the *OsMYB2P-1*-overexpressing plants compared with the wild type under both Pi-sufficient and Pi-deficient conditions (Fig. 8), while the expression of *OsPHO2* in the RNAi *OsMYB2P-1* transgenic plants was enhanced compared with wild-type plants under both Pi-deficient and Pi-sufficient conditions (Fig. 8; Supplemental Fig. S4).

There are 13 putative genes encoding high-affinity Pi transporters in rice (Paszkowski et al., 2002). The effect of *OsMYB2P-1* on the expression of these genes was analyzed. The expression of *OsPT11* and *OsPT13* was not detected in both wild-type and transgenic plants under both Pi-sufficient and Pi-deficient conditions. These observations are consistent with previous reports that *OsPT11* and *OsPT13* are exclusively induced in roots by inoculation with arbuscular mycorrhiza fungi (Paszkowski et al., 2002; Glassop et al., 2005). Among the 11 genes, disruption of *OsMYB2P-1* expression in

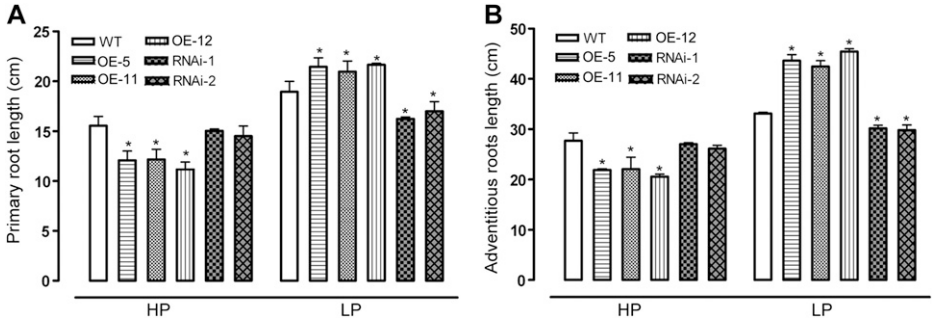

Figure 7. Effects of Pi availability in the medium on root architecture in wild-type (WT) and transgenic rice. Quantitative analysis is shown for the length of primary roots (A) and the length of the three longest adventitious roots (B) of wild-type and *OsMYB2P-1*-overexpressed and *OsMYB2P-1* RNAi rice seedlings after growth in Pi-sufficient or Pi-deficient medium for 14 d. Error bars indicate SD. Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test.

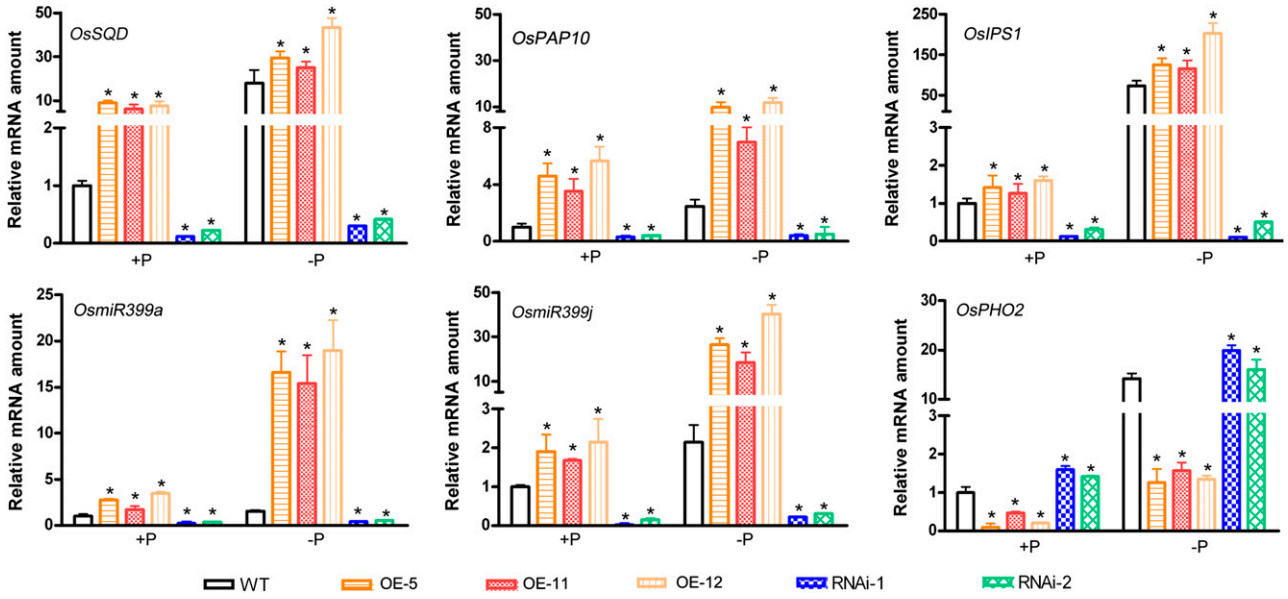

Figure 8. Expression of Pi starvation-induced genes in wild-type (WT) and *OsMYB2P-1* transgenic plants. Total RNA samples were extracted from roots of seedlings grown in normal nutrient solution for 7 d, followed by treatment with HP or LP medium for 14 d. Expression was normalized to that of *Actin*. Data are means ± SD ($n = 3$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. [See online article for color version of this figure.]

transgenic rice plants alters the expression of *OsPT2*, *OsPT6*, *OsPT8*, and *OsPT10* under either Pi-sufficient or Pi-deficient conditions (Fig. 9), while expression of the remaining genes in the wild type was comparable to that in the transgenic rice plants under both Pi-sufficient and Pi-deficient conditions (data not shown). For instance, *OsPT2* was significantly up-regulated in roots and shoots of *OsMYB2P-1*-overexpressing plants compared with wild-type plants under Pi-sufficient, but not Pi-deficient, conditions, whereas no significant difference ;in the expression of *OsPT6*, *OsPT8*, and *OsPT10* was observed among the RNAi *OsMYB2P-1*, *OsMYB2P-1*-overexpressing transgenic, and wild-type plants under Pi-sufficient conditions (Fig. 9; Supplemental Fig. S5). Suppression of *OsMYB2P-1* reduced the expression of *OsPT6*, *OsPT8*, and *OsPT10* under Pi-deficient conditions (Fig. 9).

DISCUSSION

The response of higher plants to Pi starvation is a highly regulated event that involves the activation of numerous genes, leading to changes in many physiological and morphological processes (Franco-Zorrilla et al., 2004; Yang and Finnegan, 2010). However, information about the TFs involved in this complex process is limited. In this study, we identified a novel gene, *OsMYB2P-1*, belonging to a MYB family TF, and characterized its role in response and adaptation to Pi deficiency by overexpressing/interfering with *OsMYB2P-1* in rice and Arabidopsis. Our results demonstrate that *OsMYB2P-1* was rapidly induced by Pi deprivation and that overexpression of *OsMYB2P-1* in rice allowed the transgenic rice plants to have a greater root system and to maintain a relatively higher Pi concentration in roots under Pi-deficient conditions due to enhanced up-regulation of Pi transporter genes and Pi-responsive genes of *OsSQD*, *OsIPS1*, *OsMiR399*, thus rendering rice plants more tolerant to Pi deficiency.

OsMYB2P-1 Encodes a Novel Pi-Responsive R2R3 MYB TF

Transcriptional factors such as WRKY (Devaiah et al., 2007a; Chen et al., 2009), zinc finger (Devaiah et al., 2007b), bHLH (Yi et al., 2005), and MYB (Rubio et al., 2001; Zhou et al., 2008; Devaiah et al., 2009) have been reported to regulate Pi starvation responses either positively or negatively in the literature. The MYB protein family is one of the largest TF families in plants and has been shown to be involved in numerous physiological processes (Jin and Martin, 1999; Ito et al., 2001; Stracke et al., 2001; Zhang et al., 2012). In the rice genome, there are 183 MYB-encoding genes (Yanhui et al., 2006), and the emerging evidence indicates the important roles played by MYB proteins in response to abiotic stress in general (Golldack et al., 2011; Yang et al., 2012) and nutrient deficiency in particular (Nilsson et al., 2010). The involvement of MYB in the regulation of the response to Pi deficiency in plants has been reported. These include four R2R3-MYB TFs, *PHR1*, *myb62*, *OsPHR1*, and *OsPHR2* (Nilsson et al.,

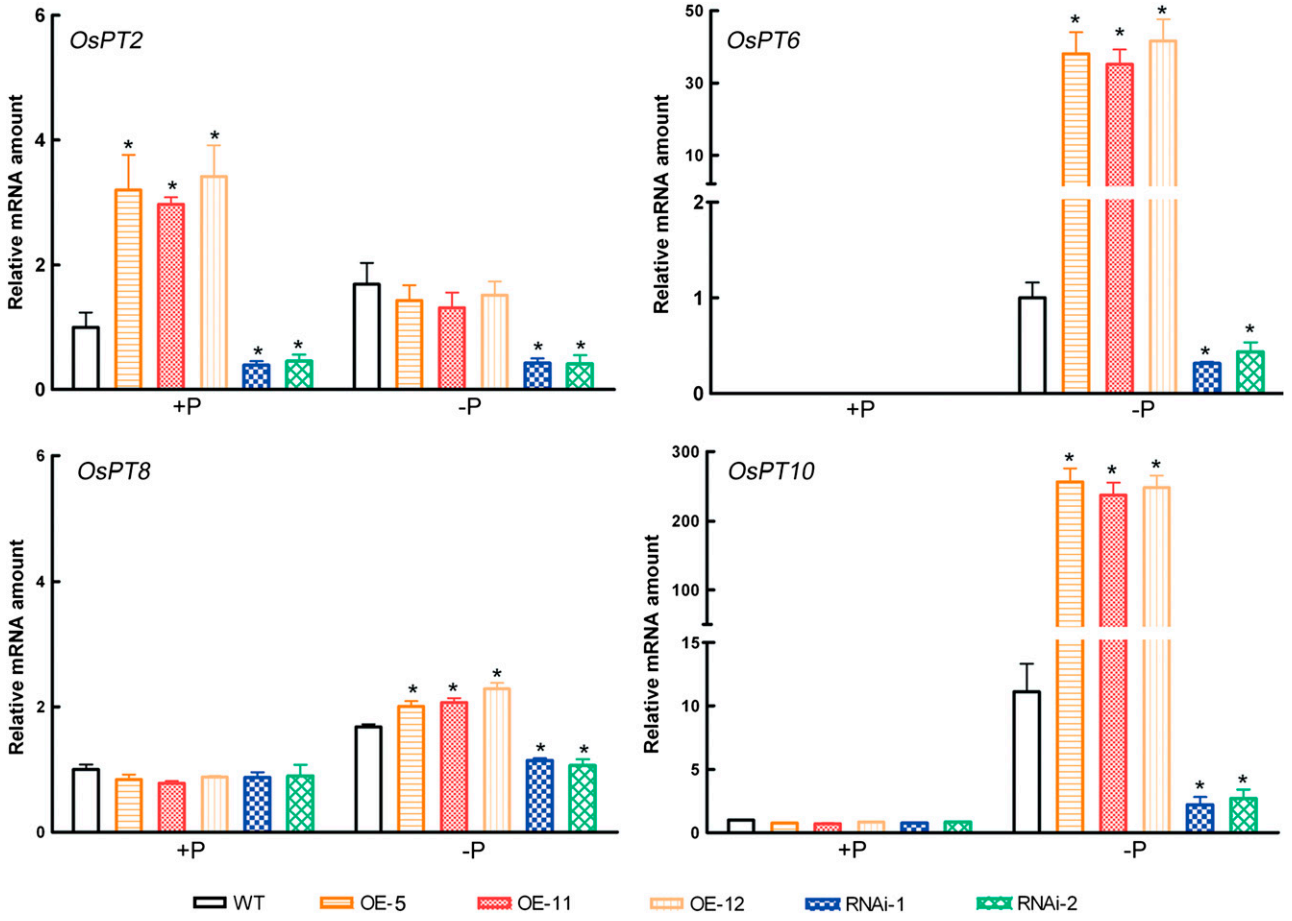

Figure 9. Expression of Pi transporter genes in wild-type (WT) and *OsMYB2P-1* transgenic plants. Total RNA samples were extracted from roots of seedlings grown in normal nutrient solution for 7 d, followed by treatment with HP or LP medium for 14 d. Expression was normalized to that of *Actin*. Data are means ± SD ($n = 3$). Asterisks indicate significant differences at $P < 0.05$ compared with the wild type by Student's *t* test. [See online article for color version of this figure.]

2007; Zhou et al., 2008; Devaiah et al., 2009). *OsPHR1* and *OsPHR2*, the homologs to *PHR1* that belong to a MYB-CC family, may play essential roles in Pi deficiency in rice by possibly interacting with the *PHR-miR399-PHO2* pathway (Zhou et al., 2008). *myb62* is involved in the regulation of the Pi starvation response by targeting the GA pathway (Devaiah et al., 2009). Our results showed that *OsMYB2P-1* was mainly expressed in stems and roots and that its expression was induced differentially among leaves, stems, and roots by deprivation of Pi and Fe in the growth medium (Fig. 3, B and C). This expression pattern is unique compared with other reported MYB TFs. For instance, expression of *AtPHR1*, *OsPHR1*, and *OsPHR2* exhibits constitutive patterns in all tissues, with higher expression levels in roots and leaves (Rubio et al., 2001; Zhou et al., 2008). *myb62* is induced specifically in the leaves during Pi deprivation (Devaiah et al., 2009). In addition, it has been shown that Pi deprivation can lead to increases in Fe contents (Zheng et al., 2009). In our study, we found that overexpression of *OsMYB2P-1* enhanced the accumulation of Fe in both roots and shoots regardless of Pi status in the growth medium (Supplemental Fig. S6).

The OsMYB2P-1 protein is close to Os01g65370, Os05g3550, and OsMYB4 (Fig. 2B). OsMYB4 has been shown to be involved in cold stress in rice (Vannini et al., 2004). In our study, we found that expression of *OsMYB2P-1* was sensitive to cold, salt, and osmotic stress (Supplemental Fig. S7). It has been proposed that Pi starvation and cold stress might share common regulatory cascades and that P may participate in the acclimatization to cold stress, as some cold-responsive genes are also regulated by Pi deficiency (Hammond et al., 2003). Therefore, *OsMYB2P-1* is likely to play a role in sensing and transducing signals associated with Pi starvation and cold stress. To the best our knowledge, this is the first report showing that *OsMYB2P-1* is a member of the R2R3 MYB TFs involved in the regulation of plants in response to deficiencies in Pi and Fe as well as cold stress. Further work on the role of *OsMYB2P-1* in Fe deficiency and cold stress is under way in our laboratory.

Overexpression of *OsMYB2P-1* Confers Tolerance to Low-Pi Stress in Arabidopsis and Rice

The improvement of tolerance to low-Pi stress by expressing Pi deficiency-induced genes such as *OsPTF1* and *PHR* has been reported in the literature (Yi et al., 2005; Nilsson et al., 2007). The expression of *OsMYB2P-1* was rapidly induced upon deprivation of Pi in the medium (Fig. 3B). To characterize the function of *OsMYB2P-1*, we overexpressed and suppressed *OsMYB2P-1* in rice and Arabidopsis and compared the performance of the transgenic plants with wild-type plants. Our results showed that overexpression of *OsMYB2P-1* substantially improved the performance of rice and Arabidopsis plants grown under Pi-deficient conditions, as indicated by the increased shoot and root biomass (Figs. 5 and 6). In addition, overexpression of *OsMYB2P-1* in rice led to increases in the number of tillers under Pi-deficient, but not under Pi-sufficient, conditions (Fig. 4, E and F). The number of rice tillers is an important indicator for Pi nutrient status in plants, such that the number of tillers is positively correlated with the tolerance to low-Pi stress (IRRI, 1996). Furthermore, the enhanced tolerance of *OsMYB2P-1*-overexpressed rice to low-Pi stress coincides with the up-regulation of low-Pi-responsive genes, including *OsPAP10* and *OsSQD* (Fig. 8). *OsPAP10* encodes an acid phosphatases that is activated by Pi starvation in rice (Zhou et al., 2008). *OsSQD* is involved in sulfolipid biosynthesis (Yu et al., 2002; Zhou et al., 2008). Production of acid phosphatases and activation of scavenging systems are adaptive mechanisms to maximize Pi availability for plants under low-Pi conditions (Abel et al., 2002; Richardson et al., 2009). Thus, the enhanced tolerance of *OsMYB2P-1* transgenic plants to low-Pi stress might depend in part on changes in the expression of those genes.

OsMYB2P-1 Regulates Root Development

P is an important signal to regulate root system architecture. In Arabidopsis, low Pi availability can increase lateral root density and length and reduce primary root growth, due to the reduction in cell elongation (Williamson et al., 2001; López-Bucio et al., 2002). Unlike Arabidopsis, alterations in both primary and adventitious root elongation are typical phenomena in response to Pi deprivation in rice (Wissuwa, 2003; Yi et al., 2005). In this study, we found that the root system architecture of *OsMYB2P-1* transgenic plants was significantly altered compared with wild-type plants under Pi-deficient conditions (Fig. 7). There was a significant increase in primary and adventitious root lengths of the *OsMYB2P-1*-overexpressing plants grown in Pi-deficient medium compared with wild-type plants, whereas the reduced expression of *OsMYB2P-1* led to suppression of the growth rate of primary root and adventitious roots (Fig. 7). Similarly, Arabidopsis plants overexpressing *OsMYB2P-1* exhibited longer primary roots and more lateral roots than wild-type plants (Supplemental Fig. S3). These results suggest that *OsMYB2P-1* plays a regulatory role in the modulation of root architecture in response to Pi deficiency. The greater root systems of *OsMYB2P-1*-overexpressing rice plants grown in Pi-deficient medium would allow these plants to exploit more soils and increase in root surface area for Pi uptake, thus conferring on them more efficient acquisition of Pi under Pi-deficient conditions. *WRKY75*, a member of the WRKY TFs, is up-regulated during Pi deprivation and negatively regulates lateral root and root hair growth that is independent of Pi status in plants (Devaiah et al., 2007a). *ZAT6*, a Cys-2/His-2 zinc finger TF, negatively regulates primary root growth, while it increases lateral root growth (Devaiah et al., 2007b). The regulation of root development by *ZAT6* is independent of the Pi status of plants (Devaiah et al., 2007b). Interestingly, under Pi-sufficient conditions, overexpression of *OsMYB2P-1* led to changes in root system architecture, suggesting that *OsMYB2P-1* regulates root system architecture by different pathways under Pi-sufficient and Pi-deficient conditions. A similar phenotype has also been observed in *MYB62*-overexpressing plants. For instance, the root system architecture of *MYB62*-overexpressing plants is significantly altered as compared with wild-type plants under both Pi-sufficient and Pi-deficient conditions, although the precise mechanisms remain to be elucidated (Devaiah et al., 2009). Therefore, *OsMYB2P-1* may be involved in the regulation of root development under both Pi-sufficient and Pi-deficient conditions. The observation that overexpression of *OsMYB2P-1* in the transgenic plants led to a significant growth inhibition (Table I) is in line with this proposition.

Overexpression of *OsMYB2P-1* Leads to Pi Accumulation in Shoots under Pi-Sufficient Conditions

Alterations in the expression of several genes have been suggested to account for Pi accumulation in shoots. These include *OsPHR2*, *OsSPX1*, *LTN1* (*OsPHO2*), *OsPT2*, and *miR399* (Zhou et al., 2008; Ai et al., 2009; Wang et al., 2009; Liu et al., 2010; Hu et al., 2011). Overexpression of *miR399* shows a similar phenotype to the *pho2* mutant, leading to enhanced Pi accumulation in shoots under Pi-sufficient conditions (Franco-Zorrilla et al., 2007). Acquisition and transport of Pi are mediated by Pi transporters (Harrison et al., 2002; Misson et al., 2004). Up-regulation of the expression of Pi transporters to maximize Pi uptake and transport under Pi starvation has been widely observed (Liu et al., 1998; Karthikeyan et al., 2002). Recent studies reported that overexpansion of *OsPT2* leads to the overaccumulation of Pi in shoots of rice plants under Pi-sufficient conditions (Ai et al., 2009). Our results demonstrated that overexpression of *OsMYB2P-1* increased Pi content in shoots under Pi-sufficient conditions (Figs. 5 and 6). The transcription of *OsPHO2* was repressed in the *OsMYB2P-1*-overexpressing lines, whereas *OsmiR399a*, *OsmiR399j*, and *OsPT2* were up-regulated in the *OsMYB2P-1*-overexpressed rice plants under Pi-

sufficient conditions (Figs. 8 and 9). Therefore, the changes in the expression of those genes may account for the enhanced accumulation of Pi in shoots of *OsMYB2P-1*-overexpressing rice plants.

OsMYB2P-1 Acts as a Pi-Dependent Regulator in Controlling the Expression of Low- and High-Affinity Pi Transporters

A number of genes encoding Pi transporters have been identified, and these transporters have been classified into four families, *Pht1* to *Pht4* (Rausch and Bucher, 2002; Rae et al., 2003). Among the known Pi transporters, members belonging to the *Pht1* family, which presumably encodes high-affinity Pi transporters, have been intensively studied (Paszkowski, 2006; Bucher, 2007). For instance, in Arabidopsis, *AtPht1;1* and *Atpht1;4* are responsible for Pi acquisition from both low- and high-Pi environments (Shin et al., 2004). Two of the nine *Pht1* Pi transporters have been functionally characterized so far (Misson et al., 2004; Shin et al., 2004; Catarecha et al., 2007). In rice, 13 putative high-affinity Pi transporter genes belonging to the *Pht1* family (*OsPT1*–*OsPT13*) have been identified (Paszkowski, 2006). However, their roles in the acquisition and translocation of Pi remain largely unknown, except for *OsPT2*, *OsPT6*, and *OsPT8* (Ai et al., 2009; Jia et al., 2011). In this study, we found that, among the 13 Pi transporters examined in rice, *OsPT2* was up-regulated in shoots and roots of *OsMYB2P-1*-overexpressing plants under Pi-sufficient conditions. Recent studies showed that *OsPT2*, unlike other *Pht1* members, is a low-affinity Pi transporter that appears to mediate Pi translocation (Ai et al., 2009). However, we found that the expression of *OsPT2* in *OsMYB2P-1*-overexpressing plants was not affected under Pi-deficient conditions. Several high-affinity Pi transporters, such as *OsPT6*, *OsPT8*, and *OsPT10*, may account for the Pi uptake in roots of *OsMYB2P-1*-overexpressing plants under Pi-deficient conditions, as evidenced by the greater up-regulation of these genes in *OsMYB2P-1*-overexpressing plants than in wild-type plants (Fig. 9). *OsPT6* differs from *OsPT2* in terms of their kinetics in Pi uptake and translocation (Ai et al., 2009). *OsPT8* is a high-affinity Pi transporter involved in Pi homeostasis, such that overexpression of *OsPT8* results in excessive accumulation of Pi in roots (Jia et al., 2011). Therefore, it is likely that *OsMYB2P-1* may act as a Pi-dependent regulator in controlling the expression of a low-affinity Pi transporter and a high-affinity Pi transporter.

In summary, we characterized a novel R2R3-type MYB protein that was localized at the nucleus in rice and induced by deficiencies of Pi and Fe. The OsMYB2P-1 protein was involved in the mediation of tolerance of plants to low Pi stress, such that overexpressing *OsMYB2P-1* in rice and Arabidopsis rendered the transgenic plants more tolerant to low-Pi stress. Further studies to unravel the molecular networks by which OsMYB2P-1 regulates responses to Pi deficiency are warranted.

MATERIALS AND METHODS

Microarray Analysis

Rice (*Oryza sativa* ssp. Japonica) seedlings exposed to low-Pi solution for varying periods (6, 24, 48, and 72 h) were used to extract RNA for microarray studies. Total RNA was isolated from rice plants in two independent experiments with the RNA extraction kit (Trizol reagent; Invitrogen). For analysis of the Affymetrix GeneChip, 8 μg of total RNA was used for making biotin-labeled complementary RNA (cRNA) target. All the processes for cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning were conducted according to the GeneChip Standard Protocol (Eukaryotic Target Preparation; Affymetrix). Information on the GeneChip Rice Genome Array (MAS 5.0) was accessed from the Affymetrix Web site: http://www.affymetrix.com/products/arrays/specific/rice.affx. GCOS software (Affymetrix GeneChip Operating Software) was used for data collection and normalization. The overall intensity of all arrays was equivalent, and each probe set is assigned with "Present", "Absent", "Marginal," and a *P* value from the algorithm in GCOS.

Plant Material and Growth Conditions

Growth of Rice Plants

Japonica rice cv Zhonghua 10 was used in physiological experiments and rice transformation throughout this study. Rice seeds were surface sterilized for 5 min with ethanol (75%, v/v) and for 10 min with commercially diluted (1:3, v/v) NaClO, followed by thorough rinses with sterile water. Seed germination was conducted in the dark at 28°C for 72 h. Then, the 7-d-old seedlings were transferred to nutrient solution containing 1.425 mM NH_4NO_3, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.168 mM Na_2SiO_3, 0.125 mM Fe-EDTA, 0.019 mM H_3BO_3, 0.009 mM $MnCl_2$, 0.155 mM $CuSO_4$, 0.152 mM $ZnSO_4$, and 0.075 mM Na_2MoO_4, pH 5.5, supplemented with 0.323 mM NaH_2PO_4 (HP; approximately 10 mg L^{-1} Pi) or 0.016 mM NaH_2PO_4 (LP; approximately 0.5 mg L^{-1} Pi). The hydroponic experiments were carried out in a growth room with a 16-h-light (30°C)/8-h-dark (22°C) photoperiod, and the relative humidity was controlled at approximately 70%. The solution was refreshed every 3 d (Wang et al., 2009).

The optimal time and concentrations used for the low-Pi stress were determined following protocols described by Liu et al. (2010). The concentrations of Pi deficiency were set to be 0.016 mM throughout this study. One-week-old wild-type and transgenic rice plants were exposed to the low-Pi solution (0.5 μL L^{-1}, 0.016 mM) for 14 or 30 d. For analyses of root system architecture and RT-PCR, rice seedlings grown in the low-Pi (0.016 mM Pi) solution for 14 d were used. Pi concentration, shoot biomass, and root biomass were measured after 30 d of Pi starvation. The tiller number was observed using pot experiments after Pi starvation for 60 d.

Growth of Arabidopsis

The response of wild-type and transgenic Arabidopsis (*Arabidopsis thaliana*) plants to low-Pi stress was examined as described by Jiang et al. (2007). Briefly, 4-d-old seedlings were transferred to LP medium (10 μM KH_2PO_4) and HP medium (1 mM KH_2PO_4) for 3 weeks. The LP and HP media were supplemented with 2.0 mM NH_4NO_3, 1.9 mM KNO_3, 0.3 mM $CaCl_2.2H_2O$, 0.15 mM $MgSO_4.7H_2O$, 5 μM KI, 100 μM HBO_3, 100 μM $MnSO_4.H_2O$, 30 μM $ZnSO_4.7H_2O$, 1 μM $Na_2MoO_4.2H_2O$, 0.1 μM $CuSO_4.5H_2O$, 0.1 μM $CoCl_2.6H_2O$, 100 μM $FeSO_4.7H_2O$, 100 μM $Na_2EDTA.2H_2O$, and 1% Suc. To maintain identical concentrations of K between Pi-sufficient and Pi-deficient media, 0.99 mM KCl was also added in the Pi-deficient medium. Plants were maintained at 65 μmol m^{-2} s^{-1} photosynthetically active radiation and placed in vertical orientation in controlled-environment chambers (22°C, 16 h of light).

Plasmid Construction

For *OsMYB2P-1* RNAi, a fragment of 459 bp was amplified from *OsMYB2P-1* with two primers, 5′-GGGGTACCACTAGTATGAATTCACTCTGGTTC-3′ (*Kpn*I and *Spe*I sites underlined) and 5′-CGGGATCCGAGCTCCCAAGAGGCACATATCCC-3′ (*Bam*HI and *Sac*I sites underlined), containing two restriction enzymes at their 5′ ends. The plasmid was constructed as described previously (Wang et al., 2004). The hairpin structure consisting of an antisense *OsMYB2P-1* fragment, a rice intron, and an *OsMYB2P-1* sense fragment was inserted

between the maize (*Zea mays*) ubiquitin promoter and the nopaline synthase terminator of vector pTCK303 (Supplemental Fig. S8A).

For *OsMYB2P-1* overexpression, the full-length cDNA of *OsMYB2P-1* was amplified using two primers, 5′-CGCGGATCCATGGGGAGGCATTCTTGCTGC-3′ (*Bam*HI site underlined) and 5′-CGGGGTACCCTAGATATGTTCAAAAGACAAGG-3′ (*Kpn*I site underlined), by RT-PCR with Pyrobest DNA Polymerase (TaKaRa), ligated into pGEM-T Easy vector (Promega), and sequenced. The digestion product of *OsMYB2P-1* from pT Easy-*OsMYB2P-1* was directionally cloned into the *Kpn*I-*Bam*HI sites of a pUN1301 vector to create the pUN1301-*OsMYB2P-1* construct. *OsMYB2P-1* was driven by a ubiquitin promoter in the construct (Supplemental Fig. S8B). These constructs were electroporated into *Agrobacterium tumefaciens* EHA105.

Generation of Transgenic Rice Plants

Plant was performed as described previously (Ge et al., 2004). Briefly, rice embryonic calli were induced on scutella from germinated seeds and transformed with strain EHA105 of *A. tumefaciens* containing the desired binary vector. Transgenic plants were selected in half-strength Murashige and Skoog medium containing 75 mg L^{-1} hygromycin (Sigma). Hygromycin-resistant plants from calli, defined as transgenic plants of the T0 generation, were transplanted into soil and grown in a greenhouse at 28°C. T2 and T3 seeds were harvested and used for subsequent experiments.

Transformation of OsMYB2P-1 in Arabidopsis

The digestion product *OsMYB2P-1* from pT-*OsMYB2P-1* was directionally cloned into the *Kpn*I-*Bam*HI sites of an SN1301 vector to create SN1301-*OsMYB2P-1* (Supplemental Fig. S8C). *OsMYB2P-1* was driven by a CaMV 35S promoter in the construct. The construct was electroporated into *A. tumefaciens* C58. Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998). Arabidopsis ecotype Columbia was used for the transformation.

Transactivation Assay Using the Yeast GAL4 System

The cDNA fragments of *OsMYB2P-1* were generated by PCR amplification, cloned into *Eco*RI and *Bam*HI sites, and fused in frame to the GAL4 DNA-binding domain in the pGBKT7 vector. A transactivation assay was performed as described (Choi et al., 2004). The *OsMYB2P-1*-pGBKT7 constructs were transformed into AH109 cells by the lithium acetate-mediated method (Gietz et al., 1992), and the transformants were selected on synthetic dextrose medium lacking tryptophan (SD/-Trp) at 28°C for 2 d. Yeast transformants from SD/-Trp were then transferred and streaked onto solid SD/-Trp/-Ade or SD/-Trp/-Ade/-His to score the growth response after 3 d. For the β-galactosidase assay, the transformants were blotted on Whatman filter paper, and the cells imprinted on the filter were lysed by freezing in liquid nitrogen, then thawed at room temperature. The filter was then incubated in 2.5 mL of Z buffer containing 0.8 mg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside supplemented with 16.1 g L^{-1} $Na_2HPO_4{\cdot}7H_2O$, 5.5 g L^{-1} $NaH_2PO_4{\cdot}H_2O$, 0.7 g L^{-1} KCl, and 0.246 g L^{-1} $MgSO_4{\cdot}7H_2O$ at 30°C. The color reaction was monitored.

Semiquantitative RT-PCR and Quantitative Real-Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). Three biological replicates, each comprising five individual plants, were used for semiquantitative RT-PCR and quantitative real-time PCR. To confirm the reliability of *OsMYB2P-1* in microarray hybridization, semiquantitative RT-PCR using the One Step RNA PCR Kit (avian myeloblastosis virus; TaKaRa) with gene-specific primers for *OsMYB2P-1* and *Actin* was performed (Supplemental Table S5). Total RNA was isolated from materials collected for microarray hybridization. One microgram of total RNA was used as a template in one reaction. The same amplification reaction was conducted with a rice *Actin* gene used as a template RNA loading control. RT-PCR was repeated three times.

For real-time PCR, 2 μg of total RNA was treated with DNase I (Promega) and then transcribed in a total volume of 20 μL with 1 μg of oligo$(dT)_{18}$, 10 mM deoxynucleotide triphosphate, and 200 units of SuperScript II reverse transcriptase (Invitrogen). The cDNA samples were diluted to 2 and 8 ng μL^{-1}. Triplicate quantitative assays were performed on 1 μL of each cDNA dilution with the SYBR Green Master Mix or TaqMan reagents (TaKaRa) and an ABI 7900 sequence detection system according to the manufacturer's protocol (Applied Biosystems). The relative quantification method (Delta-Delta cycle threshold) was used to evaluate quantitative variation between the replicates examined. The PCR signals were normalized to those of *Actin* or *rice polyubiquitin1* (*RubQ1*). All the primers used for quantitative RT-PCR are listed in Supplemental Tables S5 and S6.

Localization of OsMYB2P-1-GFP Fusion Proteins

The localization assay was performed as described by Wang et al. (2004). The whole coding sequence of *OsMYB2P-1* was amplified with two primers, 5′-GCTCTAGAATGGGGAGGCATTCTTGCTGC-3′ (*Xba*I site underlined) and 5′-CGGGGTACCGATATGTTCAAAAGACAAGG-3′ (*Kpn*I site underlined). The PCR product was subcloned into the pBI221 vector to generate *pBI221-OsMYB2P-1-GFP*, containing an *OsMYB2P-1-GFP* fusion construct under the control of the CaMV 35S promoter. The construct was confirmed by sequencing and used for transient transformation of onion (*Allium cepa*) epidermis via a gene gun (Bio-Rad). Transformed onion cells were observed with a confocal microscope (Nikon).

DNA Gel-Blot Analysis

DNA gel-blot analysis was performed as described by Wang et al. (2004). Genomic DNA isolated from 3-week-old Arabidopsis seedlings was digested with *Eco*RI or *Hin*dIII, fractioned electrophoretically on a 0.8% (w/v) agarose gel, and blotted onto a nylon membrane (Amersham Pharmacia Biotech). The membrane was prehybridized at 65°C for 2 h and hybridized in the same solution containing [α-^{32}P]ATP- and CTP-labeled *GUS* for 20 h at 65°C. After hybridization, the membrane was washed once with 2× SSC plus 0.1% SDS at 65°C for 20 min and then twice with 1× SSC plus 0.1% SDS at 37°C for 30 min. The membrane was exposed to x-ray film (Eastman-Kodak) at –70°C for 3 to 7 d.

Determination of Pi and Anthocyanin

The dry root and shoot samples were separated and digested with concentrated nitric acid and hydrogen peroxide, and total Pi and Fe were determined by using inductively coupled plasma mass spectrometry following the protocols described by Song et al. (2011). About 100 mg (fresh weight) of seedlings was collected, and anthocyanin content was measured as described by Devaiah et al. (2009).

Raw microarray data have been submitted to the National Center for Biotechnology Information with the accession number GSE35984.

Supplemental Data

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

Supplemental Figure S1. Deduced amino acid sequence of OsMYB2P-1.

Supplemental Figure S2. Response of *OsMYB2P-1* to N and K deprivation in leaves and stems.

Supplemental Figure S3. Effects of Pi availability in the medium on root architecture in wild-type and transgenic Arabidopsis.

Supplemental Figure S4. Expression of Pi starvation-induced genes in wild-type and *OsMYB2P-1* transgenic plants.

Supplemental Figure S5. Expression of Pi transporter genes in wild-type and *OsMYB2P-1* transgenic plants.

Supplemental Figure S6. Fe contents in wild-type and *OsMYB2P-1* overexpression transgenic rice under Pi-sufficient conditions (A) and Pi-deficient conditions (B).

Supplemental Figure S7. *OsMYB2P-1* expression response to cold, salt, and polyethylene glycol stress.

Supplemental Figure S8. Plasmid construction for plant transformation.

Supplemental Table S1. List of genes that up- or down-regulated in rice seedling after Pi starvation for 6 h.

Supplemental Table S2. List of genes that up- or down-regulated in rice seedling after Pi starvation for 24 h.

Supplemental Table S3. List of genes that up- or down-regulated in rice seedling after Pi starvation for 48 h.

Supplemental Table S4. List of genes that up- or down-regulated in rice seedling after Pi starvation for 72 h.

Supplemental Table S5. Primers used in semiquantitative and real-time RT-PCR.

Supplemental Table S6. Sequences of forward and reverse primers and 6-carboxyfluorescein 5′ end-labeled probes designed for the 3′-untranslated region of the rice Pi transporter genes and the *RubQ1* gene for quantitative RT-PCR.

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