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Role of AtMYB2 in Pi starvation

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Regulation of miR399f transcription by AtMYB2 affects phosphate-starvation responses in Arabidopsis

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ABSTRACT

Although a role for microRNA399 (miR399) in plant responses to phosphate (Pi) starvation has been indicated, the regulatory mechanism underlying miR399 gene expression is not clear. Here we report that AtMYB2 functions as a direct transcriptional activator for miR399 in Arabidopsis Pi starvation signaling. Compared to untransformed control plants, transgenic plants constitutively overexpressing AtMYB2 showed increased miR399f expression and tissue Pi contents under high Pi growth, and exhibited elevated expression of a subset of Pi starvation-induced (PSI) genes. Pi-starvation-induced root architectural changes were more exaggerated in AtMYB2 overexpressing transgenic plants compared to wild type. AtMYB2 directly binds to a MYB-binding site in the miR399f promoter in vitro, as well as in vivo, and stimulates miR399f promoter activity in Arabidopsis protoplasts. Transcription of AtMYB2 itself is induced in response to Pi deficiency, and the tissue expression pattern of miR399f and AtMYB2 are similar. Both genes are expressed mainly in vascular tissues of cotyledons and in roots. Our results suggest that AtMYB2 regulates plant responses to Pi-starvation by regulating the expression of the miR399 gene.
INTRODUCTION

Phosphorus (P) is an essential component of all organisms as it is found, among other compounds, in nucleic acids, ATP, and membrane phospholipids. It is an essential nutrient for plants. Phosphorus can be acquired by plants only as inorganic phosphate (Pi). Therefore most of the P content of soils is unavailable for plant growth and development (Bieleski, 1973; Hinsinger, 2001). To overcome the problem of Pi limitation, plants have developed a variety of adaptive responses that conserve internal P while activating mechanisms that enhance accessibility and uptake of external P. The accompanying gene expression changes produce changes in root architecture, enhanced Pi uptake activity, secretion of organic acids and secretion of phosphatases (Raghothama, 1999; Poirier and Bucher, 2002; Yuan and Liu, 2008; Péret et al., 2011). The synchronization of Pi availability with plant growth and development is orchestrated by several phytohormones, including ABA, ethylene, auxin and cytokinin (Hillwig et al., 2008; Devaiah et al., 2009; Lei et al., 2011).

A few transcription factors have been characterized that appear to regulate subsets of the response to Pi stress, either positively or negatively. PHR1 is a MYB transcription factor that initiates up-regulation of Pi starvation-responsive genes in plants and unicellular algae (Rubio et al., 2001). WRKY75, a WRKY transcription factor family member, has been identified as a key regulator of Pi acquisition and root architecture in response to Pi starvation (Devaiah et al., 2007a). MYB62, a R2R3-type MYB transcription factor, connects Pi homeostasis and gibberellic acid (GA) signaling during Pi starvation (Devaiah et al., 2009). ZAT6, a C2H2-type zinc finger transcription factor, regulates Pi homeostasis and exerts some control over root development (Devaiah et al., 2007b). The BHLH32 transcription factor is a negative regulator of several Pi starvation responses (Chen et al., 2007). PTF1, of Oryza sativa and Zea mays, encodes a bHLH transcription factor that is involved in Pi signaling (Yi et al.,
2005; Li et al., 2011). These transcription factors function in crosstalk between Pi starvation signaling and signaling by phytohormones, or photosynthates, to govern physiological responses to Pi limitation (Rouached et al., 2010).

MicroRNAs (miRNAs) are endogenous non-coding RNAs, 21 to 24 nt in length, that contribute to the regulation of gene expression. They have emerged as master regulators in plant development, and they orchestrate adaptive responses to stresses owing to posttranscriptional regulation of gene expression (Bonnet et al., 2006; Mallory and Vaucheret, 2006; Sunkar et al., 2012). Recently, the regulation of phosphate, copper, and sulfate homeostasis in plants has been found to involve miRNAs (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005; Chiou et al., 2006; Yamasaki et al., 2007; Liang et al., 2010; Kuo and Chiou, 2011). Pi deprivation induces the expression of several miRNAs in Arabidopsis, including miR156, miR399, miR778, miR827, and miR2111 (Fujii et al., 2005; Hsieh et al., 2009; Pant et al., 2009). Of these, miR2111 upregulates the expression of At3g27150, which encodes a Kelch repeat containing F-box protein (Hsieh et al., 2009). miR827 mediates cross-talk between Pi and nitrogen limitation signaling based on the regulation of anthocyanin synthesis. It also down-regulates the expression of At1g02860 that encodes a ubiquitin E3 ligase (Hsieh et al., 2009; Pant et al., 2009). Irrespective, the precise role of these Pi limitation-induced miRNAs in the regulation of Pi homeostasis remains unknown (Doerner, 2008; Kuo and Chiou, 2011).

In contrast, the mode of action for miR399 during plant responses to Pi starvation is well characterized. Expression of miR399 is strongly induced upon Pi starvation, especially in vascular tissues of the shoot. Mature miR399 is then translocated to roots and binds to 5’-UTR of PHO2 (UBC24, encodes an ubiquitin-conjugating E2 enzyme) transcripts, leading to the degradation of PHO2 mRNA. The resulting decrease of PHO2 protein level activates the
expression of phosphate transporter genes, such as *Pht1;8* and *Pht1;9*, thereby facilitating Pi uptake and transport to the shoot (Fujii et al., 2005; Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Pant et al., 2009). Thus, induction of *miR399* gene expression by Pi limitation plays an important role as the trigger in the restoration of Pi homeostasis, by promoting Pi acquisition in roots and Pi allocation to shoots. Missing are mechanistic details of the regulation of *miR399* gene expression in response to Pi starvation. In fact, information on the transcriptional regulation of *miRNA* genes is generally scarce, although much is known about genomic organization of miRNA genes, molecular mechanisms of miRNA biogenesis and miRNA functions in animals and plants (Jones-Rhoades et al., 2006).

We demonstrate here that positive regulation of *miR399* gene expression in response to Pi starvation is mediated at least in part by the transcription factor AtMYB2. AtMYB2, a transcription factor that is known to function in abiotic stress signaling in *Arabidopsis* (Urao et al., 1996; Abe et al., 1997; Abe et al., 2003; Yoo et al., 2005), directly binds to a MYB-binding site located in the *miR399f* promoter. This enhances *miR399f* promoter activity. AtMYB2 is co-expressed with *miR399f* in vascular tissue, and its transcript level is increased by Pi deprivation like that of *miR399f*. Constitutive over-expression of *AtMYB2* in *Arabidopsis* activates the transcription of *miR399f* and increases a subset of PHOSPHATE STARVATION INDUCED (*PSI*) gene expression, Pi uptake, and promotes changes in root architecture. The results uncover a missing link between Pi starvation and *miR399* transcription that also connects abiotic stress signaling to growth responses and Pi acquisition in the plant.
RESULTS

AtMYB2 expression, like mi399f, is induced by phosphate deficiency

Induction of miR399f gene expression in response to Pi deficit is the earliest known step in the signaling pathway leading from the sensing of Pi deficiency to changes in root architecture and restoration of Pi homeostasis in Arabidopsis (Fujii et al., 2005; Hsieh et al., 2009). To uncover mechanisms involved in controlling miR399f gene expression we performed an in silico analysis of its presumptive promoter region using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Figure 1). Several cis-acting regulatory elements typically associated with biotic and abiotic stress responses, such as defense-, jasmonic acid-, and light-signaling, were identified in this region. Also found were two canonical binding sites for AtMYB2, a drought-inducible transcription activator of the dehydration-responsive gene RD22 that also participates in ABA- and salt-stress signaling (Urao et al., 1996; Abe et al., 1997; Abe et al., 2003; Yoo et al., 2005). To test whether AtMYB2 plays a role in miR399f-mediated Pi starvation signaling, we compared the expression of AtMYB2 and the miR399f precursor transcript in wild type seedlings after transfer from normal growth medium to high Pi (1.25 mM KH₂PO₄), low Pi (0.0125 mM KH₂PO₄), or Pi deficiency (0 mM KH₂PO₄) media by quantitative real-time PCR (qRT-PCR). The temporal expression pattern of miR399f precursor was similar to that of AtMYB2 at all three Pi levels (Figure 2). Significant increases in the steady-state levels of AtMYB2 and miR399f transcripts were observed in shoots and roots after 5 d and 7 d exposure to low Pi or Pi deficiency. However, no increase in AtMYB2 or miR399f transcript abundance was observed after exposure to high Pi. These results suggested that AtMYB2 may be involved in miR399f-mediated Pi deficiency signaling in Arabidopsis.
miR399f and AtMYB2 are expressed in the same plant tissues

miR399 is expressed mainly in the vascular tissues of cotyledons, leaves, and roots. The expression in these tissues is strongly enhanced by Pi starvation (Aung et al., 2006). In order to investigate whether AtMYB2 is expressed in the same plant organs and tissues, we performed histochemical analysis of GUS expression in tissues of PromiR399f:GUS and ProAtMYB2:GUS transgenic plants grown in high Pi, low Pi and Pi deficiency medium (Figure 3). As reported earlier (Aung et al., 2006), weak expression of the miR399f promoter was detected by GUS staining in the vascular tissues of cotyledons and leaves, but not in roots of seedlings grown in high Pi (Figures 3, A-D). Strong miR399f promoter activity was observed in vascular tissues of cotyledons, rosette leaves, primary and lateral roots of seedlings grown under Pi deficit, but no activity was evident in root tips (Figures 3, E-L). Under high Pi, low Pi and Pi deficiency conditions, GUS activity was weaker in tissues of ProAtMYB2:GUS transgenic seedlings than in the corresponding tissues of PromiR399f:GUS seedlings. Consequently, only weak GUS staining was observed in vascular tissues of cotyledons in ProAtMYB2:GUS seedlings in high Pi medium and no GUS stain was observed in rosette leaves, primary and lateral roots (Figures 3, M-P). Clear induction of AtMYB2 promoter activity was observed in response to Pi limitation in cotyledons (Figures 3, Q and U) and lateral roots (Figures 3, T and X). Overall, the GUS reporter expression patterns in tissues of ProAtMYB2:GUS and PromiR399f:GUS seedlings were essentially similar, indicating that AtMYB2 and miR399f are expressed in the same plant tissues, particularly under Pi limitation. These results suggest direct regulation of miR399f expression by AtMYB2.

Constitutive overexpression of AtMYB2 promotes miR399f expression and increases
tissue Pi content

Next, we generated transgenic CaMV35S:AtMYB2 plants and selected three lines (AtMYB2 OE) that showed constitutive high-, middle-, and low-level overexpression of AtMYB2 under normal growth conditions (Figure 4A). RNA gel blot analysis showed that miR399f mRNA abundance was significantly higher in the AtMYB2 OE transgenic plants compared to wild type plants grown under identical conditions (Figure 4C). The level of miR399f accumulation was correlated positively with the level of AtMYB2 expression (Figures 4, A and C). Thus, overexpression of AtMYB2 leads to a proportional increase miR399f expression, even in the presence of sufficient Pi.

Constitutive expression of miR399 leads to degradation of the UBC24 (PHO2) transcript and elevated Pi accumulation in Arabidopsis even under high Pi (Fujii et al., 2005; Chiou et al., 2006). Accordingly, AtMYB2 OE plants grown under high Pi accumulated lower levels of UBC24 transcript than wild type plants (Figure 4B). A negative correlation was observed between mRNA levels of UBC24 and AtMYB2 or miR399f (Figures 4, A, B and C). The Pi content in shoots of all three AtMYB2 OE lines was significantly higher than that in wild type plants (Figure 4D), as predicted from their elevated miR399f expression levels and reduced UBC2 expression levels on the basis of earlier reports (Fujii et al., 2005; Chiou et al., 2006). Elevated Pi accumulation was also observed in roots of the two AtMYB2 OE lines that expressed the highest levels of mi399f transcript. Moreover, it has been reported that elevated Pi accumulation in pho2 mutant and miR399 OE Arabidopsis transgenic plant induced Pi toxicity-mediated chlorosis symptoms on their leaves (Fujii et al., 2005; Aung et al., 2006). To test whether AtMYB2 OE plants also exhibit chlorosis symptoms, we grew wild type and AtMYB2 OE plants on Pi-sufficient MS medium for three weeks under constant light condition. We observed development of typical chlorosis symptoms on the leaves of AtMYB2
OE plants and also less chlorophyll content in AtMYB2 OE plants compared to wild type plants, supporting higher Pi content in AtMYB2 OE plants than wild type (Figure S1, A and B). These results suggest that overexpression of AtMYB2 affects Pi homeostasis in Arabidopsis by activating miR399f-mediated phosphate starvation signaling.

Pi starvation-induced root architectural changes are exaggerated in AtMYB2 overexpression transgenic plants

Under Pi limitation, root architecture is altered. Lateral root growth is promoted (increased lateral root number and length), while primary root length is reduced due to reduced cell elongation (Desnos, 2008; Osmont et al., 2007). Based on our results that overexpression of AtMYB2 activated miR399 accumulation and miR399-mediated Pi starvation signaling, we hypothesized that at least some Pi limitation-induced root architecture changes should be exaggerated in the AtMYB2 OE lines. Accordingly, we investigated root morphology in wild type plants, transgenic plants expressing empty vector and AtMYB2 OE transgenic plants 7 d after transfer from normal growth media to high and low Pi media and to Pi-deficient media (Figure 5). Compared to plants grown in high Pi medium, primary root lengths of wild type and vector control seedlings grown in low Pi and Pi deficiency media was lower by 20-30 % (Figures 5, A and B). However, low Pi and Pi deficiency conditions resulted in a dramatic reduction (60-70 %) of primary root length of AtMYB2 OE seedlings relative to growth in high Pi. Primary root lengths of AtMYB2 OE seedlings were about 20 % less than those of wild type and vector control seedlings even in high Pi (Figures 5, A and B). Similarly, AtMYB2 OE plants developed 5-fold more hairs near the tip of the primary root than wild type plants even under normal Pi conditions (Figures 5, C and D). The Pi limitation-induced reduction of primary root length and the increase in root
hair density was exaggerated in *AtMYB2* OE lines as expected. However, overexpression of *AtMYB2* did not exaggerate the effect of Pi limitation on lateral root development. The lateral root numbers of wild type, vector control seedlings and *AtMYB2* OE plants all increased by 20% to 30% in response to low Pi and Pi deficiency, respectively (Figure 5E). Thus overexpression of *AtMYB2* exaggerates some, not all, of Pi limitation-induced root architectural changes.

Taken together, the results suggested that overexpression of *AtMYB2* leads to a constitutive Pi starvation-induced reprogramming of root development, such as suppression of primary root growth and activation of root hair development, under Pi sufficient condition. Also, *AtMYB2* OE plants become more sensitive to Pi limitation than wild type plants. These results led us to hypothesize that the inactivation of *AtMYB2* should lead to the inhibition of Pi limitation responses of roots, reduced expression of *miR399f* under Pi limitation, and reduced Pi content under Pi sufficiency or excess.

**Inactivation of AtMYB2 does not affect Pi starvation responses**

To verify the above hypothesis, we obtained an *atmyb2* mutant (SALK_045455) that contains a T-DNA insertion in the third exon of *AtMYB2* (Figure S2A). This insertion mutant was designated as *atmyb2*-3. RT-PCR analysis of *AtMYB2* expression showed that the *atmyb2*-3 mutant did not produce any detectable *AtMYB2* transcript (Figure S2B). In low Pi and Pi deficiency media, inhibition of primary root growth and *miR399f* transcript levels in wild type and *atmyb2*-3 plants were comparable (Figures S2, C and D). Furthermore, there was no difference in the Pi contents of roots and shoots of wild type and *atmyb2*-3 plants grown in high Pi medium (Figure S2E). As the inactivation of *AtMYB2* did not produce the expected phenotypes, we concluded that there is redundancy of the function that *AtMYB2*
fulfills in Pi starvation signaling.

**Overexpression of AtMYB2 affects the expression of PSI genes**

In addition to changes in the root architecture, Pi starvation also induces the expression of PSI genes such as the Pi transporters *AtPT1* (*Pht1;1*) (Shin et al., 2004) and *AtPT2* (*Pht1;4*) (Shin et al., 2004), an acid phosphatase (*AtPS2*) (Baldwin et al., 2001), a glycerol-3-phosphate permease (*AtPS3*) (Ramaiah et al. 2011), a S-like ribonuclease (*AtRNS1*) (Bariola et al., 1999), and a noncoding transcript (*AtIPS1*) (Franco-Zorrilla et al., 2007). To test whether overexpression of *AtMYB2* also affects the expression of PSI genes, the mRNA levels of several PSI genes were analyzed in *AtMYB2* OE plants after transfer from normal growth medium to high Pi, low Pi and Pi deficiency medium. qRT-PCR analyses showed that the expression of all PSI genes tested (*AtPT1*, *AtPT2*, *AtPS2*, *AtPS3*, *AtIPS1*, and *AtRNS1*) was highly induced by Pi limitation in wild type plants (Figure 6). In *AtMYB2* OE plants, abundance of transcripts of the phosphate transporters *AtPT1* and *AtPT2* was higher than that in wild type plants under high Pi and low Pi limitation conditions, but was not evident under Pi deficiency because the expression level of the wild type was equally high (Figures 6, A and B). These results provide some explanation of the higher Pi content of *AtMYB2* OE plants compared to the wild type under high Pi growth (Figure 4D). However, it is possible that other PSI genes for which we did not analyze the expression patterns in this study, may also play important roles in enhanced Pi uptake of *AtMYB2* OE plants. Expression of the acid phosphatase *AtPS2* was comparable in wild type and *AtMYB2* OE plants grown under high Pi, but was induced to a greater extent in *AtMYB2* OE plants compared to the wild type under low Pi and Pi deficiency (Figure 6C). In contrast, abundance of *AtRNS1* and *AtIPS1* mRNA was comparable in wild type and *AtMYB2* OE plants grown under high Pi. Although these
transcripts were induced by Pi limitation in the \textit{AtMYB2} OE plants, the magnitude of induction was less than that in wild type plants grown under the same conditions (Figures 6, E and F). These data indicate that \textit{AtMYB2} is involved in the regulation of a subset of \textit{PSI} gene expression.

\textbf{\textit{AtMYB2} directly binds to the \textit{miR399f} promoter and activates \textit{miR399f} expression}

Two putative MYB binding sites (MBS, 5'-TAACTG-3’) that have opposite orientation were found by \textit{in silico} analysis of the putative regulatory region of \textit{miR399f} (Figures 1B and 7A). To examine whether the AtMYB2 protein binds to one or both of these MBSs, we performed electrophoretic mobility shift assays (EMSA) with $^{32}$P-labeled oligonucleotides corresponding to promoter fragments containing the MBS-1 or MBS-2 motifs (140 bp and 149 bp, respectively) and recombinant GST-AtMYB2 or GST proteins. A GST-AtMYB2-specific mobility-retarded band indicating binding to AtMYB2 was observed with the MBS-2 oligonucleotide (Figure 7A). The intensity of this band was enhanced by increasing the amount of GST-AtMYB2 protein in the binding reaction. No mobility-retarded bands were observed with MBS-1 oligonucleotide indicating absence of binding.

Next, a chromatin immunoprecipitation (ChIP) assay was performed using total protein extracts of wild type and \textit{CaMV35S:FLAG-AtMYB2} transgenic plants. After immunoprecipitation with an antiserum against the FLAG tag, the relative contents of \textit{miR399f} promoter fragments P1 to P4, (Figure 7B) in the immunoprecipitates was estimated by qRT-PCR (Figure 7C). The amplicons P1 and P2, which surrounded and included the MBS-2 region, respectively, were significantly enriched by qRT-PCR. No enrichment of the P3 amplicon that includes MBS-1, or the P4 amplicon, was observed in the \textit{CaMV35S:FLAG-AtMYB2} extracts. Together, the results from EMSA and ChIP assays indicate that AtMYB2
directly binds to the MBS-2 region in the miR399f promoter in vitro and in vivo.

Next, we verified that AtMYB2 was a nuclear-localized protein. As shown in Figure S3, the GFP signal in Arabidopsis protoplasts transiently transformed with CaMV35S:AtMYB2-sGFP was exclusively localized in the nucleus. We then tested whether AtMYB2 can transactivate reporter gene expression from the miR399f promoter. Co-transformation of atmyb2-3 protoplasts with PromiR399f:GUS reporter and CaMV35S:AtMYB2-sGFP or CaMV35S:sGFP (negative control) as effector constructs showed that AtMYB2-sGFP greatly increases miR399f promoter activity compared to sGFP alone (Figure 8). The results indicate that AtMYB2 can function as a transcriptional activator for the miR399f gene in vivo.
DISCUSSION

Our results show that \textit{AtMYB2} binds to the \textit{miR399f} promoter leading to the activation of \textit{miR399f} expression. \textit{AtMYB2} and \textit{miR399f} are expressed in the same tissues, particularly under Pi limitation, are also induced by Pi limitation, and they activate the same subset of \textit{PSI} genes. Thus, we infer that \textit{AtMYB2} functions as a transcription factor regulating \textit{miR399f}-mediated signaling in the establishment of Pi homeostasis under Pi limitation. We subsequently were able to support this role for \textit{AtMYB2} in the plant response to Pi limitation on the basis of the phenotypes of transgenic \textit{AtMYB2} overexpression lines. As there were no differences with respect to phenotype in the response to Pi starvation between wild type and null \textit{atmyb2-3} plants (Figure S2), we conclude further that \textit{AtMYB2} is functionally redundant.

Our discovery of \textit{AtMYB2} as a transcription factor activating \textit{miR399f} expression began with an \textit{in silico} analysis for \textit{cis}-acting elements in the 1384 bp region upstream of the primary transcript of \textit{miR399f} gene (pri-\textit{miR399f}; \textit{At2g34208}). It has been reported that a TATA box-like sequence is located within 50 nt upstream of the majority of the pri-miRNA transcripts of \textit{Arabidopsis miRNA} genes (Xie et al., 2005). A TATA box-like sequence, TATAATTA, was mapped at 45 nt upstream of \textit{miR399f} precursor (Figure 1A), indicating that \textit{miR399f} is a typical RNA polymerase II-transcribed independent transcription unit. In addition to the TATA box-like motif and MYB binding sites, we found several canonical \textit{cis}-regulatory elements in the \textit{miR399f} promoter. A \textit{GNATATNC} element was located at -84 bp on the \textit{miR399f} promoter (Figure 1B). This sequence is known to bind \textit{PHR1}, a MYB transcription factor that causes up-regulation of Pi-responsive genes (Rubio et al., 2001). The presence of a \textit{PHR1}-binding motif in the \textit{miR399f} promoter raises the possibility that the MYB-family transcription factor \textit{PHR1} compensates at least partly for \textit{AtMYB2} function. More experiments are needed in order to both verify the role, if any, of \textit{PHR1} in the
regulation of miR399f expression, and to investigate how much the two promoter binding factors overlap or diverge in the activating capacity.

The miR399 family in Arabidopsis consists of six members, miR399a to miR399f, all of which are induced by Pi starvation and function in Pi homeostasis by regulating the expression of UBC24 (Fujii et al., 2005; Aung et al., 2006; Chiou et al., 2006; Doerner, 2008; Pant et al., 2008; Hsieh et al., 2009; Kuo and Chiou, 2011). We found that the putative promoter regions of miR399a, miR399b, and miR399c also contain GNATATNC elements (data not shown). Soybean miRNA genes responsible for Pi starvation-signaling contain several types of Pi-responsive cis-elements in their promoters, including the PHR1-binding site (Zeng et al., 2010). This suggests that induction of several miRNA399 family members, including miRNA399f, in response to Pi limitation could be mediated in part by PHR1. Defense-, hormone-, light-, and water stress-responsive cis-regulatory elements were detected in the miR399f promoter (Figure 1B). This is consistent with a previous report indicating that the cis-acting elements involved in hormone and abiotic stress responses are overrepresented in miRNA promoters when compared to promoters of protein-coding genes (Megraw et al., 2006). It would be interesting to ascertain whether miR399f and other members of this family constitute a hub that is important for co-ordinating environmental cues with nutrient acquisition to modulate plant growth. It must be noted that AtMYB2, established by us as a transcriptional activator of miR399f, also functions in hormonal and abiotic stress signaling in Arabidopsis (Urao et al., 1996; Abe et al., 1997; Abe et al., 2003; Yoo et al., 2005; Guo and Gan, 2011)

Of the two MBS motifs in the AtMYB2 promoter, only MBS-2, the more distal element in the pri-miR399f sequence, was identified as a functional binding site for the AtMYB2 transcription factor in vitro and in vivo (Figure 7). This suggests that both the core element
sequences and the flanking sequences are important for efficient binding of AtMYB2 on the miR399f promoter. MBS motifs are found in the promoters of many miR399 family members of Arabidopsis and rice (data not shown). From an analysis of 1.5 kb upstream sequences of miR399a, miR399b, miR399c, miR399d, and miR399e, we found that miR399b and miR399c have MBS motifs in their promoter regions (Table S2). However, in contrast to miR399f, transcript levels of miR399b and miR399c genes in AtMYB2 OE plants were comparable to those in wild type plants (Figure S4). These in silico promoter analyses and subsequent gene expression analyses suggest that the transcriptional regulation by AtMYB2 affects specifically the miRNA399f gene among the miR399 family members. The upstream region of other Pi-responsive miRNA genes, including miR156a, miR156e, and miR2111a, also contain MBS motifs (Table S2). Additional experimental data will be required to ascertain whether AtMYB2 is also involved in the regulation of miR156a, miR156e, and miR2111a expression in the response to Pi starvation, or whether this group of miRNAs diversifies into additional functions. Further analyses of promoters of the miRNA genes that regulate low Pi responses should illuminate other mechanisms and the signaling crosstalk that govern their expression.
MATERIALS AND METHODS

Plant Materials and Stress Treatments

All *Arabidopsis thaliana* lines were in the Col-0 background. The *atmyb2-3* mutant (SALK_045455) was obtained from *Arabidopsis* Biological Resource Center (ABRC; Ohio State University; http://www.arabidopsis.org/). Transgenic lines were generated by *Agrobacterium tumefaciens* mediated transformation using the floral dip method as described (Clough and Bent., 1998). Homozygous lines were generated by back crossing and were used in the experiments. Genotype of the transformants was verified by PCR. Seeds were germinated and grown on Murashige and Skoog (MS) medium containing 1 % sucrose, and 0.7 % (or 1.2 %) agar, pH 5.7. For testing the effect of Pi limitation, 5- or 7-day old seedlings were transferred to growth medium containing 1 % sucrose, 1/20X micronutrients (Miura et al., 2005) and 1.25 mM KH₂PO₄ (high Pi; this is equivalent to the Pi content of 1 X MS), 0.0125 mM KH₂PO₄ (low Pi) or 0 mM KH₂PO₄ (Pi deficiency) for indicated times (Miura et al., 2005). Plants were grown in a growth chamber at 22 °C under a 16 h light/8 h dark cycle.

Quantitative Real-time PCR (qRT-PCR) Analysis

Total RNA was isolated using an RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction, and treated with DNase I (Promega, Madison, WI, USA) to remove the genomic DNA contamination. Total RNA (2 µg) was used for first strand cDNA synthesis using a cDNA synthesis Kit (Invitrogen, Carlsbad, CA, USA), and subjected to qRT-PCR analysis. The primers used in qRT-PCR analysis are described in Supplementary Table 1. The SsoFast™ EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) was used for the
PCR reactions. PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. The relative expression levels of all the samples were automatically calculated and analyzed three times using CFX Manager software (Bio-Rad, Hercules, CA, USA).

**Northern Blot Analysis of microRNA**

Northern blot analysis of microRNA was performed essentially as described (Xie et al., 2005).

Total RNA was extracted from seedlings using Plant RNA reagent (Invitrogen, Carlsbad, CA, USA) following the supplier’s instructions. Briefly, total RNA (20 µg) was resolved on a 15 % polyacrylamide gel containing 7 M urea and transferred to Amersham Hybrid-N membrane (GE Healthcare, Buckinghamshire, UK). The probe complementary to miR399f (5’-UGCCAAAGGAGAUUUGCCGG-3’) was 5’-end labeled with γ³²P-ATP using Optikinase (USB, cleveland, OH, USA). Blots were pre-hybridized for at least 1 h and hybridized for 24 h in PerfectHyb Plus Hybridization Buffer (Sigma, St. Louis, MO, USA) at 37 °C. Post-hybridization, blots were washed successively at 42°C with 2 X SSC and 0.1 % SDS for 15 min, 0.5 X SSC and 0.1 % SDS for 15 min, and 0.1 X SSC and 0.1 % SDS for 15 min.

**Pi Measurement**

Total inorganic Pi contents were analyzed as described previously (Fujii et al., 2005).
Expression and Purification of Recombinant GST-AtMYB2 Protein

AtMYB2 cDNA was inserted as a BamHI/SalI fragment into the same sites of pGEX-2T (Amersham Biosciences) to create an in-frame GST-fusion. The primers used in cDNA cloning are described in Table S1. The construct was verified by sequencing. pGEX-2T::AtMYB2 introduced into Escherichia coli strain BL21 (Merck KGaA, Darmstadt, Germany). For protein expression, cells were induced for 3 h at 30°C with 0.5 mM IPTG. Induced cells were harvested, suspended in 1 X GST Bind/Wash buffer (4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.3), incubated on ice for 20 min and lysed by sonication. After centrifugation at 12,000 rpm at 4°C for 30 min, the supernatant was added to 0.5 ml Glutathione-Agarose 4B (PEPTRON, Daejeon, South Korea) that had been equilibrated with 1 X GST Bind/Wash buffer. The slurry was mixed gently by shaking at room temperature for 30 min. The resin was then collected and washed 2 or 3 times with 10 ml of 1 X GST Bind/Wash Buffer. GST-AtMYB2 was eluted in 1 ml of 1 X GST elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione).

Electrophoretic Mobility Shift Assay (EMSA)

To generate the $^{32}$P-labeled DNA probes, oligonucleotides spanning the MYB binding sites on the miR399f promoter, MBS-1 (140 bp) and MBS-2 (149 bp), were annealed and the 5’ overhangs were filled in using the Klenow fragment of DNA polymerase (Takara, Tokyo, Japan), dCTP, dGTP, dTTP and $\alpha^{32}$P-dATP. The DNA binding reaction was allowed to proceed at 25°C for 20 min in binding buffer (20 mM HEPES pH 7.9, 0.5 mM dithiothreitol, 0.1 mM EDTA), 50 mM KCl, 15 % glycerol, 1 µg of poly (dI-dC) and various concentrations...
of purified bacterially expressed AtMYB2 protein. The reaction was started by adding $^{32}$P-labeled DNA probe (40,000 cpm) and allowed to proceed at 25°C for 30 min. The reaction mixture was then subjected to electrophoresis on an 8% polyacrylamide gel in 0.5 X TBE buffer at 80 V for 3 h. The gel was dried, mounted for autoradiography with intensifying screens, and exposed at -70°C.

**Chromatin Immunoprecipitation (ChIP) Assay**

The Gateway system was used to generate a CaMV35S: FLAG-AtMYB2 construct in the pGWB12 vector. This construct expresses FLAG-tagged full-length AtMYB2 protein. The construct was introduced into wild type Arabidopsis plants through Agrobacterium-mediated (A. tumefaciens strain GV3101) transformation. ChIP assays were performed as described by Saleh et al., (2008) using leaf tissue (100 mg) from 3-week-old plants. Monoclonal anti-FLAG M2 (Sigma, St. Louis, MO, USA) was used for immunoprecipitation. The amount of immunoprecipitated DNA was quantified by qRT-PCR. The primers used in the ChIP assay are listed in Table S1.

**Measurement of Promoter Activity**

Transcriptional activity of the miR399f promoter was analyzed in Arabidopsis protoplasts as described by Zhu et al. (2008). The reporter construct was PromiR399f:GUS and the effector constructs were CaMV35S:AtMYB2-sGFP and CaMV35S:sGFP. Plasmids carrying the reporter and an effector gene construct, along with an internal control plasmid carrying a CaMV35:LUC gene construct, were introduced into protoplasts prepared from leaves of 20-
day-old *atmyb2-3* plants by PEG-mediated transformation as described in Baek et al. (2004). Fluorescence was measured using a SpectraMax GEMINI XPS spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) and using the SoftMax Pro 5 software. GUS activity was normalized to luciferase activity to eliminate experimental variation between samples.

**Histochemical Analysis of GUS Activity**

Plants expressing the *PromiR399f:GUS* or *ProAtMYB2:GUS* transgenes in wild type background were used for histological analysis. Seedlings of transgenic plants grown in various levels of Pi were incubated at 30°C for 6 h in the dark, in staining buffer (0.5 M Tris, pH 7.0, 10% Triton X-100) containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide). Chlorophyll was removed with an ethanol series consisting of 20 %, 35 %, 50 %, and 70 % ethanol washes at room temperature for 30 min each.
Supplemental Data

The following supporting information is available for the article online:

**Supplemental Figure S1.** Chlorosis phenotype in *AtMYB2* OE plants.

**Supplemental Figure S2.** Responses of *atmyb2-3* to Pi starvation.

**Supplemental Figure S3.** Subcellular localization of AtMYB2.

**Supplemental Figure S4.** Expression patterns of *miR399b* and *miR399c* in *AtMYB2* OE plants.

**Supplemental Table S1.** List of primers used in this study.

**Supplemental Table S2.** MBS elements in Pi-responsive microRNA promoter.
ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center (ABRC; Ohio State University) for providing mutant seeds.
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domain of ATMYB2, a drought-inducible Arabidopsis Myb-related protein. Plant J 10: 1145-1148


FIGURE LEGENDS

Figure 1. Putative cis-acting regulatory elements in the miR399f promoter. A, Genomic organization of miR399f (At2g34208) flanking regions. Location of the TATA-like sequence (TATAATTA) of miR399f gene is indicated. B, Putative cis-acting regulatory sequences on the miR399 promoter. 1,384 bp upstream of the transcription start site were analyzed using PlantCARE. Selected matrix score for all cis-acting elements was ≥ 5. MBS; MYB2 binding site, LTR; low temperature response, ARE; anaerobic response element, ACE; ACGT-containing element, MRE; MYB recognition element, AE-box; activating element-box.

Figure 2. Expression of AtMYB2 and miR399f precursor is induced in response to phosphate deficit. A to D, Wild-type plants were grown on MS medium for 7 days, transferred to high Pi, low Pi or Pi deficiency growth medium, and allowed to grow further for 0, 2, 5, and 7 days. Transcript levels were measured by qRT-PCR in total RNA extracted from shoots and roots at indicated time points. Transcript levels of AtMYB2 (A and B) and miR399f precursor (C and D), normalized to the transcript level of TUBULIN2, are shown. Bars represent mean ± standard deviation of three biological replicates with two technical replicates each.

Figure 3. Spatial expression patterns of miR399f and AtMYB2. Seeds of PromiR399f:GUS and ProAtMYB2:GUS transgenic lines, that express the GUS reporter from the miR399f and AtMYB2 promoters, respectively, were grown as described in Figure 5. Tissues were stained 7 d after transfer to high Pi, low Pi and Pi deficiency media. Blue color indicates GUS activity. (A to L) Tissues of PromiR399f:GUS transgenic plants. (M to X) Tissues of ProAtMYB2:GUS transgenic plants. Scale bar, 0.5 mm. Arrow indicates lateral roots.

Figure 4. Overexpression of AtMYB2 induces miR399f expression and Pi accumulation. Wild type (WT) and three independent lines of CaMV35S:AtMYB2 (AtMYB2 OE) were grown in MS medium. Ten day-old seedlings were analysed. A and B, Shown are the expression levels of AtMYB2 (A) and UBC24 (B), normalized to the level of TUBULIN2. Transcript levels were analyzed in total RNA extracted from the seedlings by qRT-PCR. Bars represent mean ±
standard deviation for three biological replicates with two technical replicates each. C, Northern blot analysis of miR399f expression in total RNA. Ethidium bromide stained 5S rRNA bands are shown as loading controls. D, Inorganic Pi concentrations were measured in the roots and shoots. Bars represent mean ± standard deviation for two biological replicates. Asterisks represent significant differences from the WT (p-value ≤ 0.05 from a Student’s t-test).

**Figure 5.** *AtMYB2* overexpression enhances Pi deficiency responses in root development and also affects root hair development. A, Seeds of untransformed (WT), empty vector (VC) transformants and three independent lines of *AtMYB2* OE transformants were grown on MS agar medium for 5 days, then transferred to nutrient medium containing 1.25 mM (high Pi), 0.0125 mM (low Pi) or 0 mM (Pi deficiency) KH₂PO₄. Seedlings were photographed 7 d after transfer. B, Quantification of primary root lengths of seedlings depicted above. Bars represent means ± standard error of three replicates with 16 seedlings per replicate. Asterisks represent significant differences from the values of each line under high Pi condition (p-value ≤ 0.05 from a Student’s t-test). C, Root hair development at tips of the primary root of seedlings grown in MS medium for 7 d. Bar indicates 1 mm. D, Quantification of root hair densities at the primary root tip of plants shown in (D). Root density is the number of root hairs along 5 mm of each root above the tip. Bars represent means ± standard error of three replicates with 16 seedlings per replicate. E, Quantification of lateral root numbers per plant of seedlings depicted above. Bars represent means ± standard error of three replicates with 16 seedlings per replicate. Asterisks represent significant differences from the values of each line under high Pi condition (p-value ≤ 0.05 from a Student’s t-test).

**Figure 6.** Expression patterns of Pi starvation-induced genes in *AtMYB2* OE plants. Seeds of untransformed (WT), and three independent lines of *AtMYB2* OE transformants were grown on MS agar medium for 7 d, then transferred to the high Pi, low Pi or Pi deficiency media described in Figure 4. Transcript levels of *AtPT1* (A), *AtPT2* (B), *AtPS2* (C), *AtPS3* (D), *AtIPS1* (E), and *AtRNS1* (F) were analyzed by qRT-PCR in total RNA extracted from the seedlings 7 d after transfer. *TUBULIN2* transcript level was used for normalization. Bars
represent mean ± standard deviation of three biological replicates with two technical replicates each. Asterisks represent significant differences from the WT ($p$-value $\leq 0.05$ from a Student’s $t$-test).

**Figure 7.** AtMYB2 binds to MBS-2 on the *miR399f* promoter region. A, (Top) Schematic representation of predicted Myb binding sites (MBS-1 and MBS-2) in the *miR399f* promoter. (Bottom) EMSA of the binding of recombinant AtMYB2 protein to oligonucleotides spanning the MBS-2 and MBS-1 regions. The autoradiogram shows resolved binding reactions of $^32$P-labeled DNA probes (MBS-2 and MBS-1) without protein (Free) or with indicated amounts of AtMYB2-GST (AtMYB2) or GST (negative control). B, Schematic drawing of the *miR399f* locus and locations of the ChIP assay amplicons (P1, P2, P3 and P4). C, ChIP assay for *miR399f* chromatin regions associated with AtMYB2. The ChIP assay was performed on total protein extracts of MS-grown three week-old seedlings of untransformed (WT) and *CaMV35S:FLAG-AtMYB2* transformed *Arabidopsis*. Fold enrichment is the ratio of *CaMV35S:FLAG-AtMYB2* to WT signal. Bars represent the mean ± standard deviation for three technical replicates.

**Figure 8.** AtMYB2 enhances the *miR399f* promoter activity. Shown (top) is a schematic representation of the effector and reporter constructs used in the transient expression assay of *miR399f* promoter activity. Each effector construct was introduced into *atmyb2-3* protoplasts along with the reporter construct and an internal control *CaMV35S:LUC* construct by PEG-mediated transformation. GUS reporter activity in each sample was obtained after normalization to LUC activity. Fold induction is the ratio of the GUS activity of *CaMV35S:AtMYB2-sGFP* transformed protoplasts (AtMYB2) relative to the GUS activity of *CaMV35S:sGFP* transformed protoplasts (VC). Bars represent mean ± standard deviation of three technical replicates.
Figure 1.

A

B

Putative cis-acting elements in miR399f promoter

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a This is the species in which the cis-acting element sequence was first described.
b Positions are relative to the miR399f precursor start site.
c (+) and (-) indicate sense or antisense DNA strands.
Figure 2.
Figure 5.

A

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<td>![image]</td>
</tr>
<tr>
<td>Pi deficiency</td>
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B

- **WT**
- **VC**
- **AtMYB2 OE #4**
- **AtMYB2 OE #5**
- **AtMYB2 OE #6**

Primary root length (mm)

High Pi  | Low Pi  | Pi deficiency
---|---|---
60 | 50 | 40

C

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D

Root hair density

High Pi  | Low Pi  | Pi deficiency
---|---|---
0 | 10 | 20

E

- **WT**
- **VC**
- **AtMYB2 OE #4**
- **AtMYB2 OE #5**
- **AtMYB2 OE #6**

Lateral root number

High Pi  | Low Pi  | Pi deficiency
---|---|---
0 | 2 | 4

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Figure 7.

A

5' -833 CAGTTA TAACTG -574 MBS-2 MBS-1 miR399f precursor (At2g34208) 3'

B

At2g34204 MBS-2 MBS-1 miR399f precursor (At2g34208)

P1 P2 P3 P4 Amplicon 100bp

C

Enrichment fold

MBS-2 MBS-1 : Probes

Free GST AtMYB2 Free GST AtMYB2

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WT  35S:FLAG-AtMYB2

Amplicon

P1 P2 P3 P4
Figure 8.

Effectors:
- CaMV 35S
- sGFP
- CaMV 35S
- AtMYB2
- sGFP

Reporter:
- PromiR399f
- GUS

Fold induction graph:
- VC
- AtMYB2