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A miRNA Involved in Phosphate-Starvation Response in *Arabidopsis*

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Summary

Although microRNAs (miRNAs) have been documented to regulate development in plants and animals [1, 2], the function of miRNAs in physiology is unclear. miR399 has multiple target sites in the 5′ untranslated region (UTR) of a gene encoding a putative ubiquitin-conjugating enzyme (UBC) in *Arabidopsis thaliana* [3]. We report here that miR399 was highly induced, whereas the target UBC mRNA was reduced by low-phosphate (Pi) stress. In transgenic plants with constitutive expression of miR399, UBC mRNA accumulation was suppressed even under high Pi. The expression of transgene UBC mRNA with 5′ UTR miR399 target sites, but not the one without 5′ UTR, was reduced under low-Pi condition. Furthermore, transgenic *Arabidopsis* plants with constitutive expression of miR399 accumulated more Pi than the wild-type, and transgenic plants expressing the UBC mRNA without 5′ UTR (miRNA-deregulated) showed less inhibition of primary root growth and less induction of a Pi transporter gene by low-Pi stress than those of wild-type plants. We conclude that miR399 downregulates UBC mRNA accumulation by targeting the 5′ UTR, and this regulation is important for plant responses to Pi starvation. The results suggest that miRNAs have functional roles for plants to cope with fluctuations in mineral-nutrient availability in the soil.

Results and Discussion

miR399 Is Induced by Low Pi

miRNAs are endogenous noncoding small RNAs that are produced from pre-miRNA transcripts that form fold-back structures [1, 2, 4]. miRNAs silence genes that have complementary or partially complementary sequences to the miRNAs by causing mRNA cleavage or translational repression [1, 2, 4]. miR399 was identified in *Arabidopsis* and rice *Oryza sativa* [3]. There are six loci in *Arabidopsis* and 11 loci in rice for this family. The target in *Arabidopsis* is a gene encoding a putative ubiquitin-conjugating enzyme (UBC; At2g33770), which has five complementary sequences to miR399 in its 5′ UTR [3, 5]. The location of miRNA target sites in 5′ UTR is unusual, and the functional significance of this has not been explored in plants or animals.

Mineral nutrients are of fundamental importance to plants and indirectly to all living organisms that are dependent on plants. Of the 16 essential elements for plants, with the exception of C and O, all are provided by the soil through plant roots. During their life cycle, plants experience frequent fluctuations in soil nutrient status. Various factors such as water content and pH alter the availability of nutrients in the soil. Plants must alter their architecture and metabolism to adapt to varying nutrient conditions. It is possible that miRNAs are involved in not only programmed development but also the adjustment to fluctuations in nutrient availability. Recently, it was shown that miR395 is induced by sulfate starvation and that its predicted target gene, ATP sulfurylase, is reduced in low sulfate conditions [6]. The functional role of miR395 in sulfate metabolism remains to be determined, and the involvement of miRNAs in the regulation of critical plant mineral nutrients such as nitrogen, phosphate, and potassium is unknown.

For a number of miRNAs that have low expression under normal growth conditions [3], we tested their expression under various abiotic stress conditions including nutrient deficiencies, drought, salt, and cold stress. We did not find any substantial regulation of miR399 by salt, drought, or cold stress (data not shown). To investigate the regulation of miR399 by nutrient deficiency stress, we cultured plants in Murashige-Skoog (MS) liquid medium lacking Pi, K, or N for 4 days. Northern-blot analysis with oligonucleotides complementary to miR399f as a probe showed that the expression of miR399 was induced by low Pi, but not by low K or low N (Figure 1A). As a control, we show that the expression of miR172 was not induced (Figure 1A). Northern analysis with RNA extracted separately from shoots and roots of seedlings grown on high- or low-Pi agar plates indicated that the induction of miR399 by low Pi occurred in both shoots and roots (Figure 1B). When plants cultured in high Pi were transferred to low-Pi culture medium or to high-Pi culture medium as a control, the accumulation of miR399 was detected at 24 hr and 48 hr after the plants were transferred to low Pi, whereas little induction was detected at 12 hr (Figure 1C). No induction was seen after the transfer to the control high-Pi culture medium. These results show that miR399 is inducted by low Pi.

miR399 Target, a UBC mRNA, Is Suppressed under Low Pi

We analyzed the expression of miR399 target, a putative UBC, under low Pi. Northern-blot analysis showed that the accumulation of UBC mRNA was reduced under low-Pi condition (Figure 1D). The largest and strongest band (approximately 4.5 kb) corresponds to the full-length UBC mRNA. A very faint, lower band was also detected. The size of the lower band (approximately 3.3 kb) is similar to the UBC mRNA without 5′ UTR (see Figure 2D). The reduction of the UBC mRNA level was detected 24 hr after the transfer to low-Pi medium. At 48 hr after the transfer to low Pi, UBC mRNA could hardly

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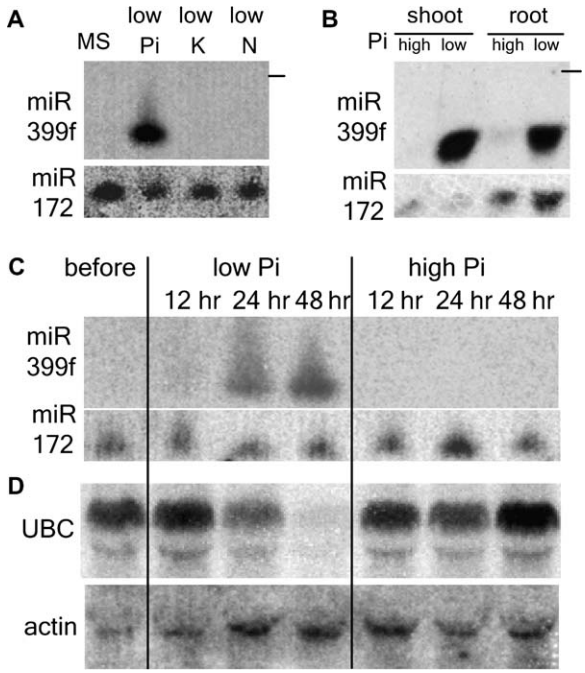

Figure 1. Northern-Blot Analysis of miR399 and UBC mRNA under Low Pi

(A) Northern-blot analysis of miR399 under low-nutrient stress. Fifty micrograms of total RNA from *Arabidopsis* seedlings treated with nutrient-rich MS media, low-Pi, low-potassium (K), or low-nitrogen (N) media for 4 days was subsequently probed with oligonucleotides complementary to miR399f and unrelated miR172 as control. The marker on the right indicates the position of 24 nt control RNA.

(B) Northern-blot analysis of miR399 in shoots and roots under low Pi. Twenty micrograms of total RNA from shoots or roots of *Arabidopsis* grown on high- or low-Pi plates for 14 days was probed with oligonucleotides complementary to miR399f and the unrelated miR172. The marker on the right indicates the position of 24 nt control RNA.

(C and D) Northern-blot analysis of miR399 (C) and UBC mRNA (D) at 12 hr, 24 hr, and 48 hr after transfer to high- or low-Pi media. Fifty (C) or twenty (D) micrograms of total RNA was probed with oligonucleotides complementary to miR399f and unrelated miR172 (C) or with UBC and actin2 cDNA (D), respectively.

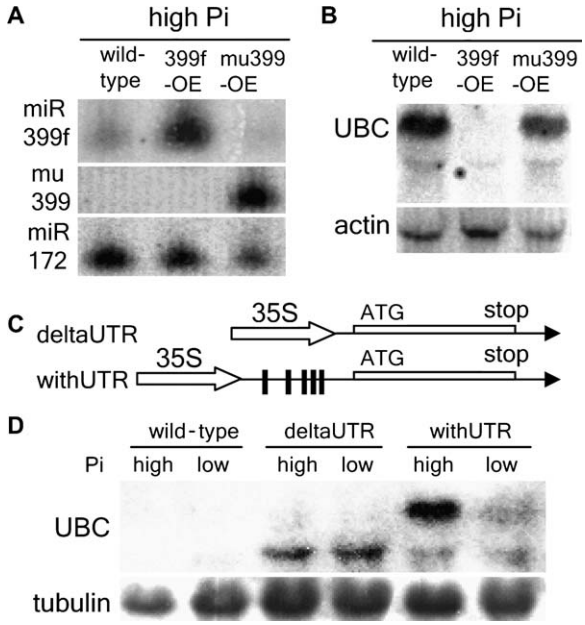

Figure 2. Northern-Blot Analysis of miR399 and UBC mRNA in Wild-Type, miR399f-, or mu399-Overexpression Plants, and in deltaUTR-UBC- or withUTR-UBC-Overexpression Plants

(A and B) Northern-blot analysis of miR399 and UBC mRNA in wild-type, miR399f-, or mu399-overexpression plants. Fifty (A) or twenty (B) micrograms of total RNA from wild-type seedlings and seedlings expressing miR399f precursor (399f-OE, line #15) or mu399 precursor (mu399-OE) under the 35S promoter was probed with oligonucleotides complementary to miR399f, mu399, and unrelated miR172 (A) or with UBC and actin2 cDNAs (B).

(C) Diagram of UBC expression constructs, deltaUTR and withUTR. Black boxes indicate target sites of miR399.

(D) Northern-blot analysis of UBC in seedlings expressing deltaUTR or withUTR UBC. Twenty micrograms of total RNA from wild-type seedlings and seedlings expressing deltaUTR (line #1) or withUTR (line #4) UBC treated with high- or low-Pi media was probed with UBC and β-tubulin cDNA.

be detected. In contrast, at 48 hr or any other time after transfer to high-Pi medium, strong UBC mRNA expression could be detected (Figure 1D). The time course of UBC mRNA reduction in response to low Pi largely coincided with the time course of miR399 induction. These results show that low Pi suppresses the accumulation of UBC mRNA and suggest that the suppression was mediated by miR399.

Constitutive Expression of miR399 Suppresses the Accumulation of UBC mRNA Even under High Pi

To further investigate the relationship between miR399 accumulation and suppression of UBC mRNA under low Pi, we generated transgenic plants expressing miR399f constitutively. Five hundred base pairs genomic DNA including the stem-loop part of the MIR399f gene was cloned. As a negative control, we constructed a mutated miR399f gene (mu399) (UGCCAACGGC GAUUUGCCCAG; the underlined three nucleotides were changed from A, A, and G, respectively) with site-directed mutagenesis. Because these nucleotides are originally mismatched in the foldback structure of miR399f precursor, it is expected that the mutations would not affect the foldback structure and the altered gene would yield a mature mutated miRNA. Both the wild-type and mutated precursors were expressed under the strong constitutive cauliflower mosaic virus 35S promoter (herein referred to as the 35S promoter) in *Arabidopsis*. Transgenic lines were selected with hygromycin resistance, and the expression of miR399f and mu399 were confirmed by northern blot analysis with oligonucleotide probes complementary to miR399f and mu399, respectively (Figure 2A, and Figure S1A in the Supplemental Data available with this article online). In transgenic plants expressing miR399f constitutively, the UBC mRNA was not detected even under high-Pi condition, whereas the UBC mRNA was detected in the wild-type and the transgenic plants expressing mu399 constitutively (Figure 2B and Figure S1B). These results further support that miR399 suppresses UBC mRNA accumulation.

UBC mRNA Regulation by miR399 Requires the 5′ UTR

To investigate whether the regulation of UBC mRNA accumulation by miR399 occurs via the 5′ UTR of UBC,

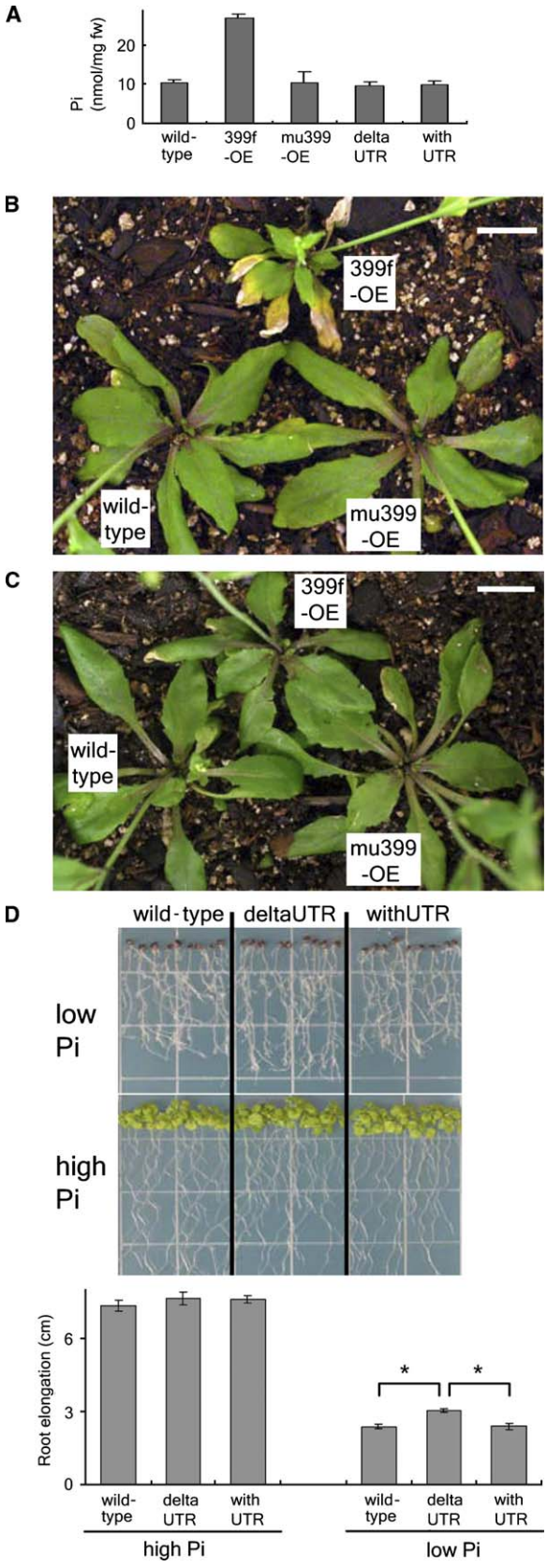

Figure 3. Analysis of Transgenic Plants

(A) Pi concentration (nmol/mg fresh weight) in shoots of the wild-type and transgenic plants overexpressing miR399 (399f-OE, line #15), mu399 (mu399-OE), deltaUTR UBC (line #1), or withUTR UBC (line #4) under the 35S promoter (mean ± standard error [SE], n = 5 each). Plants were grown in soil for 3 weeks.

(B and C) Necrosis in the margins of leaves of transgenic plants expressing miR399f constitutively (399f-OE, line #15). The wild-type and transgenic plants overexpressing mu399 (mu399-OE) are shown as controls. Plants were grown in soil and placed under a fan with continuous light (B) or placed in a growth chamber (C) for 4 weeks. The scale bar represents 1 cm.

(D) Root-elongation assay under low and high Pi. Four-day-old wild-type seedlings and seedlings expressing deltaUTR UBC (line #1) or withUTR UBC (line #4) were transferred to high- or low-Pi plates. Photographs were taken 12 days after the transfer. The grid scale of the plate is 13.5 mm. The bar graph indicates the elongation of primary roots during day 2–12 after transfer to the high- or low-Pi plates (mean ± SE, n = 18 each). Asterisks indicate significant differences in one-way ANOVA followed by Turkey's multiple-comparison tests.

a region that contains five target sites of miR399, we constructed two types of UBC cDNAs for transgenic expression. One contains only the ORF of UBC (deltaUTR) behind the 35S promoter, whereas the other contains the 5′ UTR and ORF of UBC (withUTR) behind the 35S promoter (Figure 2C). These constructs were introduced into *Arabidopsis*, and transgenic lines expressing the transgenes were selected and evaluated. In all of the transgenic lines, no morphological or developmental alterations were observed under normal conditions. The transgenic lines were treated with low or high Pi for 4 days, and RNA was collected for northern analysis. The amount of withUTR-UBC mRNA was substantially reduced in low-Pi medium, whereas the amount of deltaUTR-UBC mRNA was not changed by low Pi (Figure 2D and Figure S1C). After longer time exposure, bands corresponding to the endogenous UBC mRNA were detected in the lanes of the wild-type and transgenic lines expressing deltaUTR UBC treated with high Pi (data not shown). These results indicate that the 5′ UTR is important for the suppression of UBC mRNA under low Pi and that the deltaUTR UBC behaves as a miRNA-deregulated form of UBC.

Constitutive Expression of miR399 Induces the Accumulation of Pi

Phosphorus is one of the most limiting macronutrients in natural and agricultural soils. Despite the high amount of total phosphorus in the soil, phosphorus is not freely available for assimilation by roots because of the formation of insoluble compounds and low mobility of orthophosphate, which is the preferential form for uptake [7]. To examine whether miR399 affects plant Pi homeostasis, we measured Pi content in shoots of transgenic plants growing in soil for 3 weeks. Pi content in transgenic plants expressing miR399f constitutively was more than two times as high as that in the wild-type or in the transgenic plants expressing mu399 constitutively (Figure 3A and Figure S2A). No substantial difference was detected between the wild-type and deltaUTR-UBC- or withUTR-UBC-overexpression lines (Figure 3A). The Pi high-accumulator mutant *pho2* of *Arabidopsis* has been reported to show symptoms of Pi toxicity, which is dependent on transpiration rate [8]. We created a high-transpiration condition by placing the plants under a fan and with continuous light. Under this high-transpiration condition for 4 weeks, the margins of

leaves of transgenic plants expressing miR399f constitutively became necrotic, and old leaves senesced earlier than those in wild-type plants. This is a typical symptom of Pi toxicity [8]. Wild-type plants and transgenic plants expressing mu399 constitutively (Figure 3B and Figure S2B) or expressing deltaUTR or withUTR UBC did not show such a symptom (data not shown). The symptom was not seen in any of the plants when they were not grown under the high-transpiration condition (i.e., when they were grown in our regular growth-chamber conditions) (Figure 3C and data not shown). These results suggest that miR399 overexpression enhances Pi accumulation, which can result in Pi toxicity.

miRNA-Deregulated UBC Alters Pi Starvation Response in Plants

Plants show several responses, including the accumulation of anthocyanins, attenuation of primary-root elongation, and the proliferation of lateral roots and root hairs, to Pi-deficient conditions [9]. Pi starvation also induces the expression of some coding and noncoding RNAs, including RNAs for phosphate transporters AtPT1 (Pht1;1) and AtPT2 (Pht1;4) and for AtIPS1, RNS1, and AT4 RNAs [10].

We examined the Pi-starvation response of UBC-overexpression plants. Four-day-old seedlings grown on MS (high-Pi) agar plates were transferred to 1/20 MS agar plates with high or low Pi. During the first 2 days after transfer to low-Pi plates, the elongation of primary roots was not significantly different among the wild-type and plants expressing deltaUTR or withUTR UBC (1.1 ± 0.1 cm, 1.1 ± 0.1 cm, and 1.2 ± 0.1 cm, respectively, n = 18 each). During the next 10 days, the elongation of primary roots of plants expressing deltaUTR UBC (3.0 ± 0.3 cm) was larger than that of the wild-type or plants expressing withUTR UBC transferred to the low-Pi plates (2.4 ± 0.4 and 2.4 ± 0.6 cm, respectively, n = 18 each, $p < 0.05$ in one-way ANOVA followed by Turkey's multiple-comparison tests), whereas significant difference was not detected among the elongation of primary roots of the wild-type and plants expressing deltaUTR or withUTR UBC transferred to high-Pi plates (7.3 ± 1.0, 7.6 ± 1.1, and 7.6 ± 0.7 cm, respectively, n = 18 each) (Figure 3D and Figure S2C). Little difference in lateral-root density was detected among these lines under low Pi (5.2 ± 1.0, 5.0 ± 0.9 and 5.2 ± 1.1/cm, respectively, n = 18 each). These results indicate that miRNA-deregulated UBC alters one of the plant phenotypic responses to Pi starvation. We also tested the miR399f-overexpression transgenic plants under the low-Pi conditions but did not detect any significant phenotypic differences with the wild-type or mu399 transgenic plants (data not shown).

In addition, we analyzed the expression of genes induced by Pi starvation in plants overexpressing miR399f, withUTR UBC, or deltaUTR UBC. AtPT1, AtPT2, AtIPS1, RNS1, and AT4 were used as probes. Plants were cultured in high- or low-Pi liquid media for 4 days. In plants overexpressing miR399f, AtPT1 mRNA accumulation was slightly higher than that in the wild-type under high-Pi condition (Figure 4A and Figure S3A). On the other hand, AtPT1 mRNA accumulation in plants expressing the miR399-deregulated deltaUTR UBC was less than that in the wild-type under low-Pi condition (Figure 4A). This

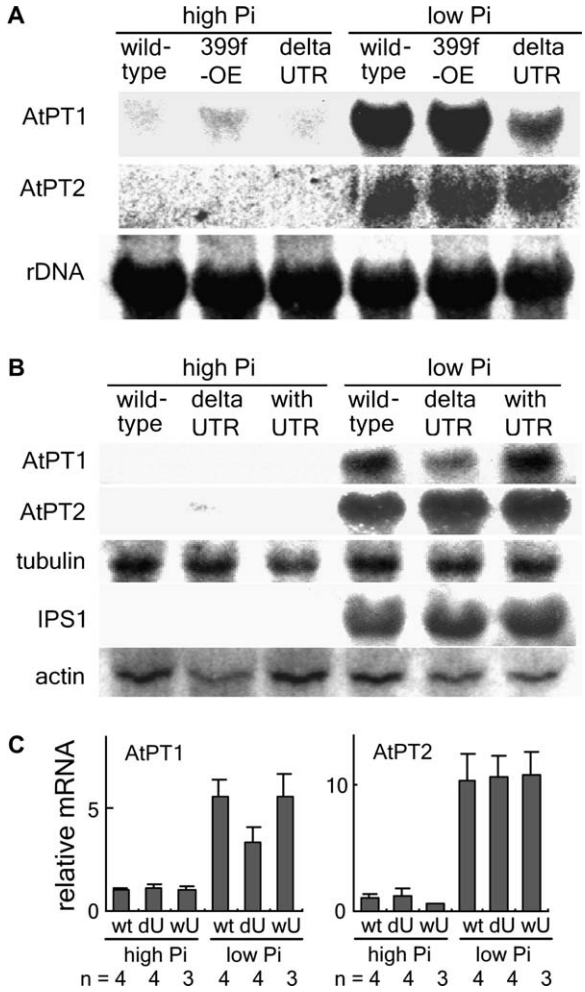

Figure 4. Expression of Pi-Starvation-Responsive Genes in Plants Expressing miR399f, deltaUTR UBC, or withUTR UBC

(A and B) Northern-blot analysis of Pi-starvation-responsive genes.
(A) Twenty micrograms of total RNA from wild-type seedlings and seedlings expressing miR399f (399f-OE line #15) or deltaUTR UBC (line #1) treated with high- or low-Pi media was sequentially probed with AtPT1, AtPT2, and ribosomal DNA (rDNA).
(B) Twenty micrograms of total RNA from wild-type seedlings and seedlings expressing deltaUTR UBC (line #1) or withUTR UBC (line #4) treated with high- or low-Pi media was probed with AtPT1, AtPT2, and β-tubulin or IPS1 and actin2.
(C) Amount of AtPT1 and AtPT2 mRNA relative to tubulin7 determined by quantitative real-time PCR (mean ± SE). Total RNA from wild-type seedlings (wt) and seedlings expressing deltaUTR UBC (dU, line #1) or withUTR UBC (wU, line #4) treated with high- or low-Pi media for 4 days was used for reverse transcription followed by quantitative PCR. Experiments were performed with indicated biological replicates (n) and four technical replicates.

reduction was not observed in plants expressing withUTR UBC (Figure 4B). No substantial differences were detected in the RNA blot in the induction of AtPT2, IPS1, RNS1, or AT4 mRNAs (Figure 4B and data not shown) among these plants. Quantitative real-time PCR (polymerase chain reaction) experiments showed that the amounts of AtPT1 mRNA in plants expressing the miR399-deregulated deltaUTR UBC and withUTR UBC under low-Pi condition were 60.5 ± 13.4% and 101.1 ± 18.8% of that in the wild-type, respectively (Figure 4C and Figure S3B). The

amounts of AtPT2 mRNA in plants expressing the miR399-deregulated deltaUTR UBC and withUTR UBC under low-Pi condition was 103.3 ± 13.8% and 103.3 ± 19.3% of that in the wild-type, respectively (Figure 4C and Figure S3B). These results suggest that miRNA regulation of UBC is also involved in the regulation of AtPT1 mRNA expression by Pi availability.

Pi, K, and N are three essential plant macronutrients that are often deficient in soils. Consequently, crop plants are almost always supplemented with one or more of these elements in the form of fertilizers, in order to achieve high yields. We show in this study that miRNA is involved in Pi nutrition. Our results suggest that low Pi induces miR399, which then downregulates UBC mRNA accumulation. The downregulation of UBC appears important for the attenuation of primary-root elongation and for the induction of the phosphate transporter gene AtPT1. The attenuation of primary-root elongation, proliferation of lateral roots and root hairs, and the induction of high-affinity phosphate transporters all contribute to the acquisition of Pi and help maintain cellular Pi homeostasis under low-Pi stress. Our observation that miR399 overexpression increased Pi accumulation suggests that Pi uptake and/or root-shoot translocation is enhanced by the presence of miR399. Because the expression of AtPT1 mRNA in miR399-overexpression lines was only marginally higher than that in the control plants, the expression or activity of some unidentified transporter(s) may be enhanced by miR399 overexpression. Interestingly, miR399-regulation of UBC seems to have only a very slight effect or no effect on the proliferation of lateral roots and root hairs under low Pi because the expression of the miRNA-deregulated form of UBC had little impact on these phenotypes.

Because the miR399 target gene UBC encodes a putative ubiquitin-conjugating enzyme, our results suggest that posttranslational regulation of some regulator(s) of low-Pi response pathways by ubiquitin or ubiquitin-like molecules is important for the prevention of Pi-starvation response under high-Pi conditions. Recently, a SUMO (small ubiquitin-like modifier) E3 ligase was reported to be involved in the regulation of Pi-starvation responses, suggesting that a certain regulator (or regulators) of Pi nutrition is sumoylated [11]. It is possible that the UBC functions together with the E3 ligase in the sumoylation pathway. Alternatively, the UBC may be involved in the ubiquitination pathways for ethylene [12] or auxin [13] signaling because these two hormones control plant root architecture, and plant sensitivity to them has been shown to be modulated by Pi starvation [14, 15].

It is a very unique feature that the miR399 target sites are located in the 5′ UTR of UBC. The results in Figure 2D showed that the 5′ UTR is necessary for the reduction of UBC mRNA by low Pi. This is consistent with results from 5′ RACE assays, in which the cleavage products corresponding to the target sites were found [5]. Thus, mRNAs containing target sites in the 5′ UTR can be efficiently cleaved by miRNA-RISC complex. This cleavage must somehow trigger the degradation of the resulting mRNA with a truncated 5′ UTR. The biochemical features of the cleaved products or of the cleavage reaction that trigger mRNA degradation are unknown. The degradation of UBC mRNA cleavage products could potentially also be regulated by low-Pi stress because the band corresponding to the putative UBC mRNA with a truncated 5′ UTR was not increased under low Pi despite enhanced mRNA cleavage (Figure 2D).

Conclusions

The regulatory role of miRNAs in development is now well documented [1, 2, 4]. Our results here demonstrate that a miRNA is involved in the regulation of plant responses to Pi nutrient deficiency. Plants have evolved elaborate mechanisms to cope with frequent fluctuations in nutrient availability. It would be of interest to determine whether plant responses to other nutritional deficiencies (e.g., low K or N) may also be regulated by some of the known miRNAs or by yet-to-be-discovered miRNAs. In this regard, it is tempting to speculate that miRNAs may have general roles in the regulation of physiological homeostasis of higher organisms, in addition to their important functions in development.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/22/2038/DC1/.

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