

Regulation of Phosphate Homeostasis by MicroRNA in *Arabidopsis*^W

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In this study, we reveal a mechanism by which plants regulate inorganic phosphate (Pi) homeostasis to adapt to environmental changes in Pi availability. This mechanism involves the suppression of a ubiquitin-conjugating E2 enzyme by a specific microRNA, miR399. Upon Pi starvation, the miR399 is upregulated and its target gene, a ubiquitin-conjugating E2 enzyme, is downregulated in *Arabidopsis thaliana*. Accumulation of the E2 transcript is suppressed in transgenic *Arabidopsis* overexpressing miR399. Transgenic plants accumulated five to six times the normal Pi level in shoots and displayed Pi toxicity symptoms that were phenocopied by a loss-of-function E2 mutant. Pi toxicity was caused by increased Pi uptake and by translocation of Pi from roots to shoots and retention of Pi in the shoots. Moreover, unlike wild-type plants, in which Pi in old leaves was readily retranslocated to other developing young tissues, remobilization of Pi in miR399-overexpressing plants was impaired. These results provide evidence that miRNA controls Pi homeostasis by regulating the expression of a component of the proteolysis machinery in plants.

INTRODUCTION

Phosphorous (P) is one of the mineral nutrients essential for plant growth, development, and reproduction. Not only is it a major component of fundamental macromolecules, such as nucleic acids and phospholipids, but it also plays an important role in energy transfer and the regulation of enzyme reactions and metabolic pathways (Bieleski and Ferguson, 1983; Theodorou and Plaxton, 1993). Despite the importance of P in agricultural production, most of the P in the soil is unavailable for plant uptake because of adsorption, precipitation, or conversion to organic forms (Marschner, 1995; Holford, 1997; Raghothama, 1999).

To overcome problems with P availability, plants have evolved a series of adaptive responses to maintain P homeostasis. These responses include conservation and remobilization of internal P and enhanced acquisition of external P (Raghothama, 1999; Poirier and Bucher, 2002), which involve rapid and distinct changes in gene expression. Although many P-responsive genes have been reported in large-scale expression profiling (Hammond et al., 2003; Wasaki et al., 2003; Wu et al., 2003; Misson et al., 2005), the molecular mechanisms regulating these P starvation-responsive genes remain to be determined. PHOS-

PHATE STARVATION RESPONSE1, a MYB transcription factor, is the only well-characterized regulator involved in the upregulation of a specific group of P-responsive genes through a GNATATNC *cis* element (Rubio et al., 2001; Franco-Zorrilla et al., 2004).

Inorganic phosphate (Pi) is the predominant form of P directly absorbed by plant roots and a major transported form of P within the plants (Marschner, 1995; Schachtman et al., 1998). Stable cytoplasmic Pi concentrations and systemic distribution of Pi should be tightly regulated despite large fluctuations in the external environment (Schachtman et al., 1998). The maintenance of Pi homeostasis in plants involves Pi uptake by roots and subsequent allocation among different tissues and into different compartments in the cell.

To date, however, little is known about the regulatory systems supervising such complex processes (Poirier and Bucher, 2002; Ticconi and Abel, 2004). Two *Arabidopsis thaliana* mutants, *pho1* and *pho2*, which are defective in Pi homeostasis, have been identified (Poirier et al., 1991; Delhaize and Randall, 1995). Pi concentration in the leaves of *pho1* was strongly reduced, whereas Pi concentration in the root was similar to that in wild-type plants (Poirier et al., 1991), which suggests that the *pho1* mutant was impaired in a protein involved in the loading of Pi to the xylem in the root. The *PHO1* gene was later identified to be a membrane protein located in the stellar cells of the root, which is consistent with a role in loading Pi into the xylem (Hamburger et al., 2002). However, *pho2* accumulated up to threefold more Pi in the shoots but had a similar or lower Pi concentration in the roots compared with the wild type (Delhaize and Randall, 1995). *pho2* could be impaired in the phloem transport of Pi between shoots and roots or defective in regulating leaf Pi concentration (Dong et al., 1998). In addition, a shoot-specific low-affinity Pi

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transporter, PHT2;1, was hypothesized to play a role in Pi loading of shoot (Daram et al., 1999). Further analysis indicated the localization of PHT2;1 in the chloroplast and suggested that PHT2;1 may also be involved in the Pi allocation between shoots and roots and the translocation of Pi within leaves, by the characterization of a *pht2;1* null mutant (Versaw and Harrison, 2002).

MicroRNAs (miRNAs) represent a class of noncoding small RNAs that generally function as posttranscriptional negative regulators through base pairing to nearly complementary sequences in the target mRNAs (Lee et al., 1993; Reinhart et al., 2000, 2002; Carrington and Ambros, 2003; Bartel, 2004). Most plant miRNAs regulate their targets by directing mRNA cleavage in the coding regions (Bartel and Bartel, 2003; Carrington and Ambros, 2003; Bartel, 2004; Dugas and Bartel, 2004). Several plant miRNAs were demonstrated to be critical in regulating leaf or flower development by targeting to the relevant transcription factors (Reinhart et al., 2002; Bartel and Bartel, 2003). Other than transcription factors, miRNAs may target a wide range of transcripts (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Adai et al., 2005; Axtell and Bartel, 2005). Their expression has been shown to be regulated by development in a tissue-specific manner or in response to a range of environmental stresses (Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Adai et al., 2005; Axtell and Bartel, 2005; Lu et al., 2005a, 2005b; Sunkar et al., 2005). The diverse expression patterns and large varieties of potential target genes of miRNAs suggest that miRNAs can regulate various developmental and physiological processes and might play a direct role in cell-to-cell signaling in plants (Dugas and Bartel, 2004; He and Hannon, 2004; Yoo et al., 2004; Kidner and Martienssen, 2005).

We were interested in understanding how Pi starvation-responsive genes are regulated and how plants coordinate Pi acquisition and allocation to maintain Pi homeostasis during Pi starvation. The correlation between the upregulation of miR395 and the suppression of corresponding target genes (ATP sulfurylases) during sulfate starvation suggests the regulation of nutrient starvation responses by miRNAs (Jones-Rhoades and Bartel, 2004). Here, we demonstrated that miR399 controls Pi homeostasis by regulating the expression of a ubiquitin-conjugating E2 enzyme in *Arabidopsis*.

RESULTS

miR399 Is Upregulated by Pi Starvation

To investigate whether miRNA is involved in regulating Pi starvation-responsive genes, we examined the expression of predicted miRNA target genes with Pi starvation. Analyses of the *Arabidopsis* Affymetrix ATH1 GeneChip revealed that the expression of a predicted target gene of miR399, At2g33770 (Sunkar and Zhu, 2004), was downregulated fivefold in Pi-starved roots. miR399 was predicted to target multiple sites on the 5' untranslated region (UTR) of At2g33770 mRNA encoding a ubiquitin-conjugating E2 enzyme, a component in the ubiquitin-dependent protein degradation pathway (Sunkar and Zhu, 2004) (Figure 1A). This E2 enzyme is designated as AtUBC24 (Kraft et al., 2005). To determine whether this decrease in E2 mRNA level during

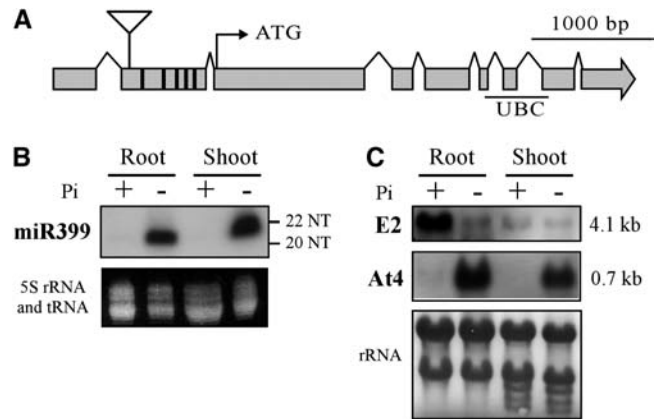


Figure 1. Gene Organization and Expression of At2g33770 Encoding a Ubiquitin-Conjugating E2 Enzyme.

(A) Exons and five putative miR399 target sites within the second exon are shown as gray boxes and black bars, respectively. The translation initiation site and the ubiquitin-conjugating conserved domain (UBC) are indicated. The triangle indicates the T-DNA insertion site in the SAIL_47_E01 line.

(B) and (C) RNA gel blot analyses of miR399 (B) and E2 (C) transcripts in seedlings grown in high-Pi (+Pi) or low-Pi (-Pi) medium. *At4*, a Pi starvation-induced gene (Burleigh and Harrison, 1999), was used as a positive control. 5S rRNA and tRNA and 25S and 18S rRNA staining are shown as loading controls. NT, nucleotides.

Pi starvation was a direct result of negative regulation by miR399, we revealed that highly induced miR399 expression in response to Pi starvation in *Arabidopsis* seedlings grown in low Pi medium ($10 \mu\text{M KH}_2\text{PO}_4$) was undetectable under high-Pi conditions ($1 \text{ mM KH}_2\text{PO}_4$) (Figure 1B). The accumulation of miR399 was accompanied by a decreased level of E2 mRNA in Pi-starved roots (Figure 1C), which suggests that E2 mRNA was cleaved by the targeting of miR399. Significantly, this cleavage is directly linked to the Pi status of the plant.

During Pi starvation, downregulation of E2 occurred primarily in roots, with only a slight reduction in E2 mRNA level in shoots (Figure 1C). Even though miR399 accumulated to approximately an equal amount in both roots and shoots, the suppression of E2 mRNA accumulation in roots was much greater than that in shoots.

Although miR399s were originally cloned from an abiotic stress-treated small RNA library (Sunkar and Zhu, 2004), later analyses showed that they did not respond to these stresses (see Supplemental Figure 1 online). Expression of miR399 in Pi-starved tissues but not in untreated tissues agrees with early observations of no miR399 expression in tissues grown in normal nutrient media (Jones-Rhoades and Bartel, 2004).

Several miRNAs have been shown to be evolutionarily conserved among different plant species (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Axtell and Bartel, 2005; Sunkar et al., 2005). Genes encoding miR399 homologs have been predicted in rice (*Oryza sativa*) and other plant species (Sunkar and Zhu, 2004; Xie et al., 2005). We examined whether the upregulation of miR399 by Pi starvation also exists in plant species other than *Arabidopsis*. Like *Arabidopsis*, the expression

of miR399 homologs was strongly detected in Pi-starved roots, stems, and leaves of tomato (*Lycopersicon esculentum*) plants (Figure 2A). However, the upregulation of miR399 homologs was barely detected in the shoots of rice (Figure 2B). Whether this observation resulted from differences in the regulation of miR399 or responses to Pi deficiency between dicots and monocots remains to be studied. Nevertheless, these results suggest that this miR399-based regulatory system may have been conserved across a broad spectrum of angiosperms.

Overexpression of miR399 Suppresses the Accumulation of the E2 Transcript

To further understand the function of miR399, we generated transgenic *Arabidopsis* plants overexpressing miR399. DNA fragments corresponding to the precursor fold-back structure of miR399 genes were amplified from *Arabidopsis* genomic DNA. miR399b, miR399c, and miR399f were selected because they had been cloned previously (Sunkar and Zhu, 2004). In these plants, miR399 was highly expressed even in the presence of high levels of Pi, under which the expression of miR399 is not normally detectable (Figure 3). Moreover, the expression of miR399 in transgenic plants was much stronger than that caused by Pi starvation in wild-type plants. Detection of the compatible amount of miR399 signal in the Pi-starved wild-type plants required longer exposure times (data not shown).

A high level of miR399 accumulation in transgenic plants indicates the successful expression and processing of precursor RNA. By contrast, E2 mRNA decreased to a barely detectable level even in miR399-overexpressing plants grown under high-Pi

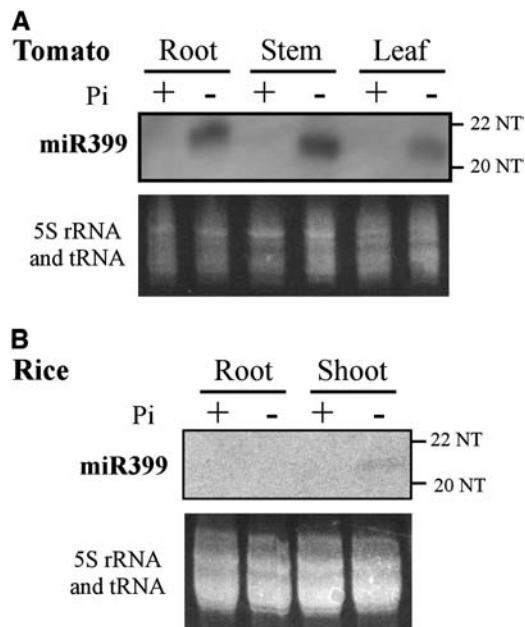


Figure 2. RNA Gel Blot Analyses of miR399 Homologs Grown under High-Pi (+Pi) or Low-Pi (-Pi) Conditions.

Homologs in tomato (**A**) and rice (**B**) were analyzed. 5S rRNA and tRNA staining is shown as the loading control. NT, nucleotides.

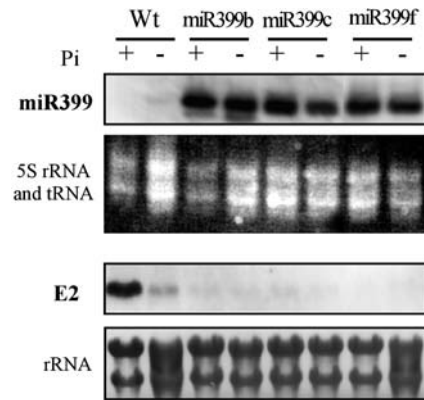


Figure 3. RNA Gel Blot Analyses of miR399 and E2 in Wild-Type and miR399-Overexpressing Plants (miR399b, miR399c, and miR399f).

RNA was isolated from liquid-cultured root samples grown in high-Pi (+Pi) or low-Pi (-Pi) medium. 5S rRNA and tRNA and 25S and 18S rRNA staining are shown as loading controls.

conditions (Figure 3). The overproduction of miR399 correlated well with the decreased E2 mRNA level in transgenic plants, further supporting the hypothesis that miR399 regulates E2 mRNA abundance.

Overexpression of miR399 Results in Overaccumulation of Pi in the Shoot

miR399-overexpressing plants grown in soil or hydroponically with a high Pi supply showed chlorosis or necrosis on the leaf margins, predominantly in mature leaves (Figure 4A). Interestingly, the Pi concentration in leaves of these transgenic plants was fivefold to sixfold that of wild-type plants (Figure 4B). The excessive amount of P in miR399-overexpressing plants was contributed mainly by the accumulation of inorganic Pi. P usually constitutes ~0.2% of plant dry matter (Schachtman et al., 1998), and Pi toxicity develops when P constitutes >1% of plant dry matter (Marschner, 1995). In our study, P levels in the leaves of miR399-overexpressing plants were 1.8 to 2.0%. Necrosis or chlorosis on the margins of mature leaves has been documented in plants with Pi toxicity (Delhaize and Randall, 1995; Shane et al., 2004a, 2004b). Together, these findings suggest that the chlorosis phenotype observed in the miR399-overexpressing plants resulted from the overaccumulation of Pi. We did not observe the chlorosis phenotype in plants grown in low Pi or in wild-type plants. Despite the high Pi accumulation in leaves of miR399-overexpressing plants, the Pi concentration in the roots was similar to that in the roots of wild-type plants (Figure 4B).

We next considered whether overaccumulation of Pi in the leaves resulted from increased uptake of Pi. Seedlings overexpressing miR399 exhibited greater Pi uptake than did wild-type plants (Figure 5A). During Pi uptake, more ^{33}P was allocated from roots to shoots in miR399-overexpressing seedlings than in wild-type plants, which resulted in a higher shoot-to-root ratio of ^{33}P (Figure 5B). This observation is consistent with the high Pi accumulation in shoots but normal concentrations in roots of

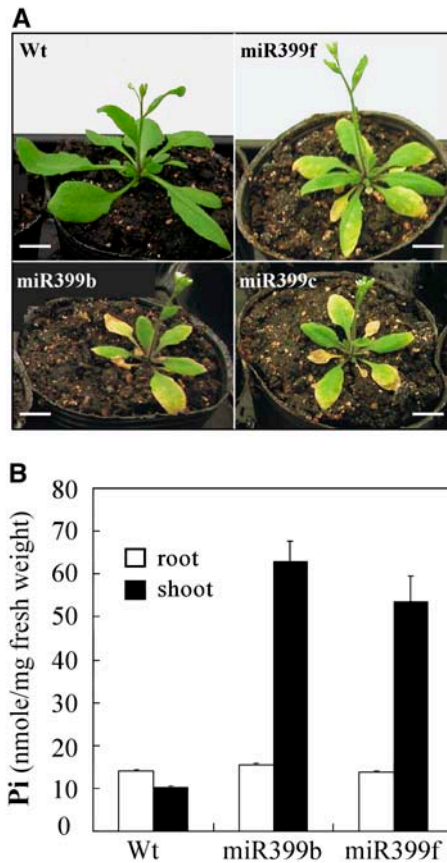


Figure 4. Pi Toxicity in miR399-Overexpressing Transgenic Plants.

(A) Chlorosis and necrosis in the leaf margins of miR399-overexpressing plants. Bars = 1 cm.

(B) Pi concentration in roots (white bars) and shoots (black bars) of wild-type or miR399-overexpressing plants grown hydroponically under high-Pi conditions. Error bars indicate SD ($n = 3$).

miR399-overexpressing plants (Figure 4B) and indicates that overexpression of miR399 affects not only Pi acquisition by roots but also the allocation of Pi between roots and shoots.

Overexpression of miR399 Impairs Pi Remobilization

Pi is initially taken up by roots, loaded into xylem, and subsequently transported to leaves (Marschner, 1995; Schachtman et al., 1998). Because Pi is a mobile element, it can remobilize from old to young tissues during senescence or when plants experience Pi deficiency (Mimura et al., 1996; Jeschke et al., 1997; Himmelblau and Amasino, 2001). The remobilization of Pi is crucial for supporting the continuous growth and development of young leaves. Thus, Pi starvation symptoms typically start in old rather than young leaves. One of the most recognized symptoms of Pi starvation is old, purple leaves caused by the accumulation of anthocyanin (Marschner, 1995). Unlike those of wild-type plants, the old leaves of miR399-overexpressing plants remained green when young leaves started to show Pi starvation symptoms (accumulation of anthocyanin) (see Supplemental Figures

2A and 2B online). Low levels of anthocyanin accumulation were associated with high Pi concentration in the old leaves of miR399-overexpressing plants (see Supplemental Figure 2C online). Similar distribution patterns of Pi were also observed under high-Pi conditions (see Supplemental Figure 2D online). Thus, we hypothesized that remobilization of Pi from old to young leaves could be impaired in miR399-overexpressing plants.

A detailed time-course analysis was also performed. In wild-type plants, the Pi concentration in the cotyledons and the first two leaves decreased, whereas that in younger leaves increased over the growth period (Figure 6A), which suggests the efficient remobilization of Pi from old to young leaves. By contrast, the Pi concentration in cotyledons and the first two leaves of miR399-overexpressing plants increased continually during the growth period (Figure 6A). Autoradiograms of leaf images obtained from pulse-chase labeling experiments also indicated strong ^{33}P

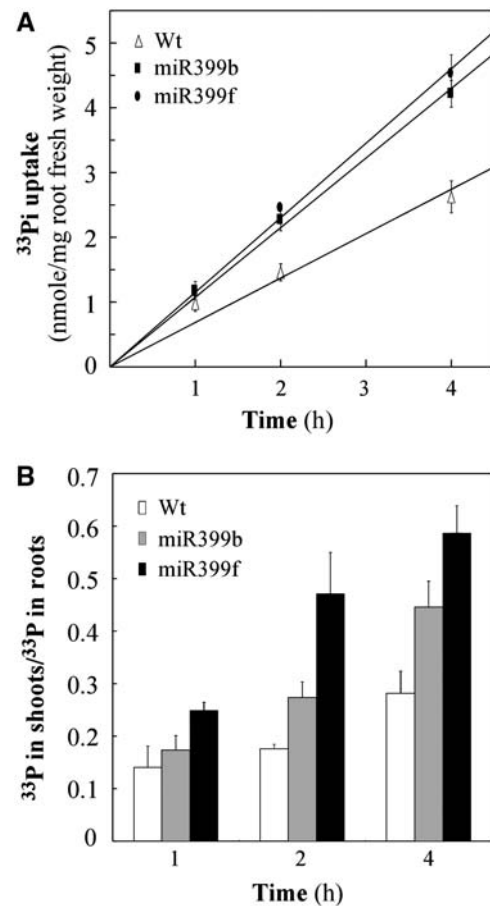


Figure 5. Increased Pi Uptake in miR399-Overexpressing Transgenic Plants.

(A) Pi uptake activities of wild-type plants (open triangles) and miR399-overexpressing plants (miR399b, closed squares; miR399f, closed circles). Error bars represent SD ($n = 3$).

(B) Shoot-to-root ratios of the ^{33}P taken up by wild-type plants (white bars) and miR399-overexpressing plants (miR399b, gray bars; miR399f, black bars) from (A).

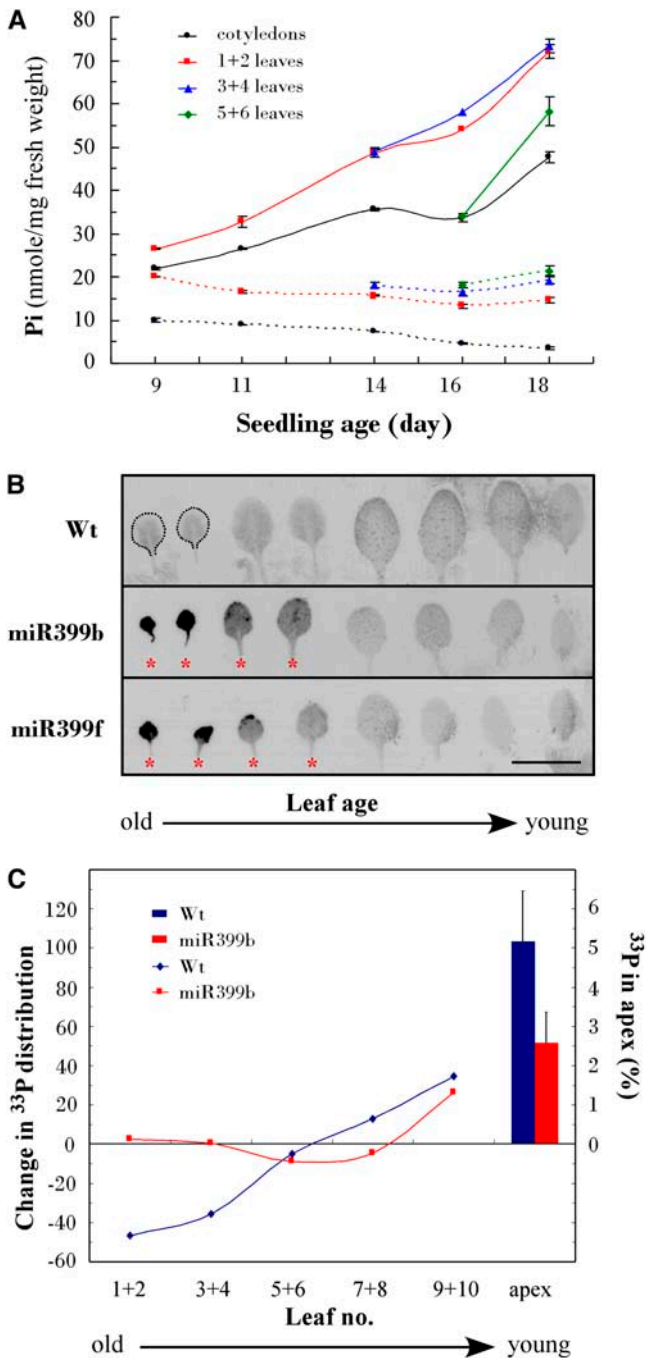


Figure 6. Remobilization of Pi within leaves.

(A) Changes in Pi concentration in the leaves of wild-type plants (dotted lines) or miR399f-overexpressing plants (solid lines). Individual leaves were collected at the indicated times beginning with 9-d-old seedlings. Leaves from 10 plants were pooled, and two proximal leaves were collected as one sample for the Pi assay (cotyledons, black circles; first and second leaves, red squares; third and fourth leaves, blue triangles; fifth and sixth leaves, green diamonds). Error bars represent SD ($n = 3$).

(B) Autoradiograms of leaf images obtained from pulse-chase labeling experiments. The first two leaves of wild-type plants are outlined be-

signals in old leaves of miR399-overexpressing plants after 10 d of radioactive-free chase (Figure 6B). High accumulation of ³³P radioactivity was associated with a chlorosis or necrosis phenotype on these leaves (Figure 6B, red asterisks). Quantitative analyses further revealed little change in the ³³P distribution in old leaves of miR399-overexpressing plants during a 6-d chase period (Figure 6C). By contrast, the ³³P distribution in the old leaves of wild-type plants (Figure 6C, leaves 1 to 4) decreased 35 to 46% over the same period. Moreover, the distribution of ³³P in the newly emerging apices of wild-type plants was twice that of miR399-overexpressing plants (Figure 6C). Together, these data support the view that overexpression of miR399 interferes with the remobilization of Pi from old to young leaves.

A Loss-of-Function E2 Mutant Exhibits the Same Phenotype as the miR399-Overexpressing Plants

We obtained an E2-null mutant (SAIL_47_E01) (Figures 7A and 7B) with T-DNA inserted in the second exon of the E2 gene (Figure 1A). This mutant displayed the phenotypes of Pi toxicity resulting from overaccumulation of Pi in the shoots (Figures 7A and 7C) and defects in the remobilization of Pi within leaves (Figure 7D), which phenocopy the miR399-overexpressing plants. The negative regulation of E2 mRNA by miR399s and the identical phenotype of the E2 T-DNA knockout mutant and the miR399-overexpressing plants establish a direct link between miR399 regulation of E2 and Pi homeostasis in plants.

DISCUSSION

Induction of miR399 Downregulates E2 during Pi Starvation

The contrary expression patterns of miR399 and the E2 gene in response to Pi starvation suggest that a decreased level of E2 transcript could result from the direct cleavage of the E2 transcript by miR399. Importantly, this cleavage occurs when plants sense the shortage of Pi. 5' rapid amplification of cDNA ends experiments identified cleaved transcripts of E2 corresponding to predicted target sequences in the 5' UTR, which supports the cleavage of the E2 transcript by recognizing miR399 (Allen et al., 2005). Overexpression of the E2 gene without 5' UTR miR399 target sites revealed that this E2 transcript was no longer recognized and cleaved by miR399 during Pi deficiency (Fujii et al., 2005). Furthermore, we showed that miR399-overexpressing plants and the E2 T-DNA knockout mutant displayed similar phenotypes (Figures 4A and 7). Together, these results strongly demonstrated the targeting of E2 mRNA by miR399 in planta. Here, we provide another example for the upregulation of miRNA by nutrient starvation in addition to the previous report on the

cause of faint signals. Leaves with the chlorosis or necrosis phenotype are marked with red asterisks. Bar = 1 cm.

(C) Change in ³³P distribution in the leaves of wild-type plants (blue line) and miR399b-overexpressing plants (red line) by pulse-chase labeling. Also indicated is the amount of ³³P accumulated in the apex as a proportion of that in whole shoots (wild type, blue bar; miR399b, red bar). Error bars represent SD ($n = 3$).

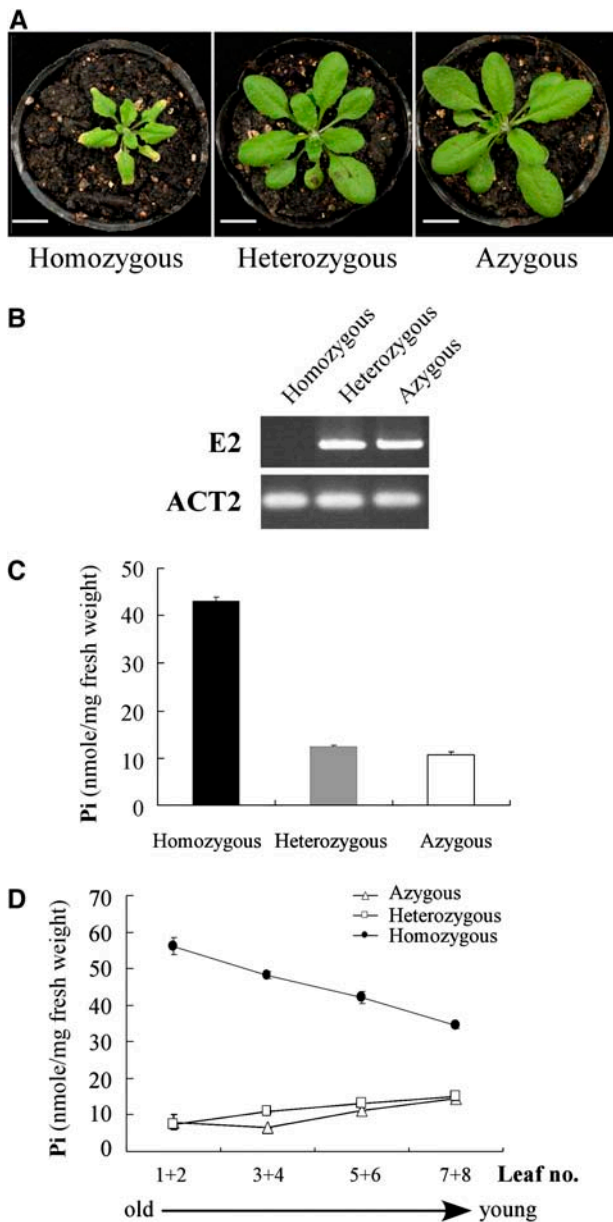


Figure 7. Phenotypes of the At2g33770 (E2) T-DNA Insertion Line.

(A) Chlorosis or necrosis was observed on the leaf margins of homozygous lines but not in the heterozygous or azygous lines. Bars = 1 cm. **(B)** RT-PCR analysis confirmed the lack of expression of E2 in the homozygous T-DNA knockout line. **(C)** and **(D)** The leaves of homozygous lines (black bar) accumulated more Pi than those of heterozygous (gray bar) or azygous (white bar) lines **(C)** and were also defective in the remobilization of Pi within leaves (azygous lines, open triangles; heterozygous lines, open squares; homozygous lines, closed circles) **(D)**. Error bars represent SD ($n = 3$). Plants were all grown under high-Pi conditions.

induction of miR395 by sulfate starvation (Jones-Rhoades and Bartel, 2004).

The suppression efficiency of miR399 on the accumulation of the E2 transcript was different between roots and shoots. Although miR399 accumulated to approximately an equal amount in both roots and shoots during Pi starvation (Figure 1B), the suppression was more evident in roots (Figure 1C). Differences in tissue or cell compartmentation of miR399 and E2 in shoots may account for the inefficient targeting. Detailed characterization of the localization of miR399 and E2 may provide additional information.

In addition to the E2 gene, two other genes, At3g54700 and At4g09730, are also predicted to be targets of miR399 (Jones-Rhoades and Bartel, 2004; Adai et al., 2005). However, at least four mismatches of base pairing occur between miR399 and these two genes, so they are less likely to be targets. In fact, their transcript levels were not affected by the overexpression of miR399 (data not shown), which suggests that they may not be targets of miR399 or that they are regulated by miR399 at other levels.

Appropriate Regulation of E2 Expression by miR399 Is Required for the Maintenance of Pi Homeostasis

Overexpression of miR399 suppressed the accumulation of E2 mRNA (Figure 3) and caused chlorosis or necrosis on the leaf margins (Figure 4A). The association between this chlorosis phenotype and increased Pi concentration (Figure 4B) suggests that the phenotype is a symptom of Pi toxicity. Although the real cause of Pi toxicity is still not understood, the mature leaves usually accumulate more Pi than young leaves and are the initial sites to show toxicity symptoms (Shane et al., 2004a, 2004b). Overaccumulation of Pi in the mature leaves of miR399-overexpressing plants is marked by (1) increased uptake of Pi by roots (Figure 5A), (2) greater translocation of Pi from roots to shoots (Figure 5B), and (3) retention of Pi in mature leaves (Figure 6). Thus, the regulation of E2 expression by miR399 is critical for maintaining Pi homeostasis at the level of Pi acquisition, allocation, and remobilization in *Arabidopsis*. In a parallel study, overexpression of E2 lacking the 5' UTR miR399 target sites altered Pi starvation responses, such as reductions in the inhibition of primary root growth and upregulation of a Pi transporter gene, *PHT1;1* (Fujii et al., 2005). These results further support the importance of miR399-directed regulation of E2 in the development of Pi starvation responses.

Pi remobilization from old to young leaves was impaired by the overexpression of miR399 (Figure 6). This defect in Pi remobilization within leaves suggests that the export of Pi out of the mature leaves or the loading of Pi into the phloem may be downregulated or impaired in miR399-overexpressing plants. Although this finding was originally identified by visualizing the initiation place of anthocyanin accumulation in Pi-deficient plants (see Supplemental Figures 2A and 2B online), the impaired Pi remobilization was also observed in plants grown under high-Pi conditions (see Supplemental Figures 2C and 2D online). Measurement of Pi movement, however, revealed that the defect in Pi remobilization under low-Pi conditions was not as severe as that in plants grown under Pi-sufficient conditions (Figure 6).

Consistent with this observation, enhanced Pi uptake activity in miR399-overexpressing plants (Figure 5) was less evident in plants grown under low-Pi conditions (data not shown). In general, the overall differences between wild-type and miR399-overexpressing plants were more striking under high-Pi conditions. This observation may be explained by miR399 being normally induced under low-Pi conditions in wild-type plants.

It is important to note that the miR399-overexpressing lines miR399b, miR399c, and miR399f showed similar phenotypes in terms of the suppression of E2, Pi toxicity, and defects in Pi remobilization, which suggests that these three miR399s may be equally efficient in regulating the expression of E2 despite minor sequence variations. Nevertheless, differences in targeting efficiency for these miR399 variants may not be determined because of the overproduction of miR399. In addition, whether different variants have differing degrees of expression in Pi starvation or different tissue specificity remains to be studied.

Several features of the miR399-overexpressing phenotypes, including overaccumulation of Pi in shoots and increased uptake and allocation of Pi into shoots, are similar to those described for the *pho2* mutant (Delhaize and Randall, 1995; Dong et al., 1998). However, the impaired Pi remobilization within leaves has not been observed in *pho2*. The gene corresponding to the *pho2* mutation has not yet been reported. *PHO2* was mapped to chromosome 2 (Delhaize and Randall, 1995), near the E2 gene; thus, we sequenced the E2 gene of *pho2* and found a single nucleotide mutation causing a stop codon within the coding region (K. Aung, S.-I. Lin, C.-C. Wu, Y.-T. Huang, and T.-J. Chiou, unpublished data). Further comparison between miR399-overexpressing plants and *pho2* will provide a clearer picture of this association. A *pht2;1* null mutant also showed defects in Pi allocation between shoots and roots and remobilization of Pi within leaves (Versaw and Harrison, 2002). However, changes in *pht2;1* were relatively small compared with those in miR399-overexpressing plants. A recent report revealed that mutation of an inositol polyphosphate kinase gene, *IPK1*, also caused Pi toxicity (Stevenson-Paulik et al., 2005). Examining the expression of *PHT2;1* and *IPK1* in relation to that of miR399 and E2 in these plants is of interest.

A Working Hypothesis

Figure 8 illustrates our working hypothesis for the regulation of Pi homeostasis via the interaction between miR399 and E2. miR399 is not normally expressed under high-Pi conditions. Because E2 generally participates in protein degradation, the high expression of E2 in roots under high-Pi conditions may directly or indirectly be involved in the downregulation of specific Pi uptake and transport systems (Figure 8A). We consider that substantial amounts of E2 under high-Pi conditions are critical for preventing the overloading of Pi into plants, because suppression of E2 by overexpression of miR399 results in increased Pi uptake and Pi toxicity (Figure 8B). Overaccumulation of Pi in the shoots of miR399-overexpressing plants (Figure 4B) can be attributed to the increased uptake of Pi from the soil (Figure 5A) and a greater transport of Pi from roots to shoots (Figure 5B). Furthermore, the retention of Pi in mature leaves of transgenic plants (Figure 6) can accelerate Pi accumulation to toxic levels. Upon Pi starvation,

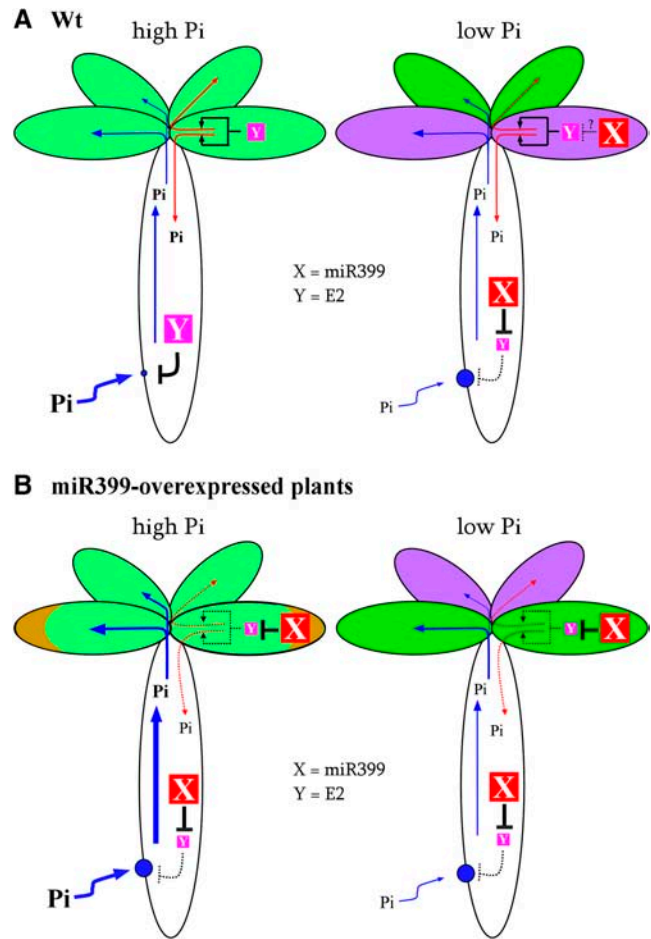


Figure 8. Working Hypothesis for the Regulation of Pi Homeostasis by miR399 and E2 in Wild-Type and miR399-Overexpressing Plants.

(A) Wild-type plants.

(B) miR399-overexpressing plants.

The solid blue circles indicate the Pi uptake system in roots. The blue and red lines indicate the translocation of Pi from roots to shoots and the remobilization of Pi out of old leaves, respectively. The dotted lines in **(B)** indicate the impaired Pi remobilization in miR399-overexpressing plants. Greater Pi uptake and transport activities are presented as large circles and thick lines. Purple leaves are the initiation sites of Pi starvation symptoms because of accumulated anthocyanin. Pi toxicity is shown as chlorosis in leaf margins (yellow in **[B]**). miR399 and E2 are X and Y, respectively. See text for a detailed description.

increased miR399 expression represses E2 expression. With reduced E2 levels, the repression of Pi uptake is alleviated, and increased uptake of Pi from the soil occurs, which enables the plant to cope with the Pi deficiency (Figure 8A). Because of limited soil availability, overaccumulation of Pi does not occur. This hypothesis correlates with the recent observation that overexpression of E2 without 5' UTR miR399 target sites diminished the upregulation of the *PHT1;1* Pi transporter gene upon Pi deficiency (Fujii et al., 2005). Although the shoots of miR399-overexpressing lines contained higher Pi, their roots did not accumulate more Pi than those of wild-type plants (Figure 4B),

which suggests that remobilization of Pi from shoots to roots may also be affected by overexpression of miR399. Therefore, we propose that maintaining critical amounts of E2 in shoots is important for the remobilization of Pi out of mature leaves. This hypothesis is consistent with the observation that a low level of E2 mRNA in shoots was not affected substantially by the induction of miR399 during Pi deficiency (Figure 1C). It is interesting that expression patterns from the AtGenExpress microarray atlas showed higher expression of the E2 gene in senescing leaves during leaf development (Schmid et al., 2005). Promoter: reporter fusion analyses also suggested the expression of *PHT1;4* and *PHT1;5* in senescing leaves (Karthikeyan et al., 2002; Mudge et al., 2002). Whether these are associated with the recycling of Pi out of senescing leaves requires further studies.

In *Arabidopsis*, 37 E2 genes were predicted based on the existence of a conserved catalytic domain of ubiquitin conjugation (Bachmair et al., 2001). Compared with most E2 proteins, this miR399-regulated E2 is relatively large in molecular mass (100 kD versus 20 to 30 kD). In addition to the ubiquitin-conjugating domain consisting of ~150 amino acids close to the C terminus, it contains an extra ~650 amino acids at the N terminus. We hypothesize that this N-terminal region may be involved in the interaction with specific E3 or regulatory proteins to mediate the Pi homeostasis of plants. Recent results demonstrating the regulation of several Pi starvation responses by a SUMO E3 ligase (Miura et al., 2005) agree with our finding that posttranslational regulation by ubiquitin or ubiquitin-like molecules is involved in adaptive responses to Pi deficiency. Although there were distinct E1, E2, and E3 enzymes participating in ubiquitylation and sumoylation (Kurepa et al., 2003), it is of interest to examine the crosstalk between these two pathways, especially in response to changes of Pi status.

The mechanisms by which plants coordinate Pi acquisition and allocation to maintain Pi homeostasis are still largely unknown. In this report, we demonstrate that miR399-dependent changes in E2 gene expression affect Pi acquisition, Pi allocation between roots and shoots, and Pi remobilization within leaves. From these results, we conclude that precise control of miR399 expression and its interaction with E2 mRNA is critical to the maintenance of Pi homeostasis within plants. Our results shed light on the molecular mechanisms that regulate Pi homeostasis. Further analyses of upstream regulatory pathways of miR399s and downstream pathways of the E2 protein will provide further insights into the regulatory network that controls Pi homeostasis in the plant.

METHODS

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were surface-sterilized and germinated on agar plates with half-strength modified Hoagland nutrient solution containing 1 mM KH_2PO_4 (high Pi) or 10 μM KH_2PO_4 (low Pi), 1% sucrose, and 1% Bactoagar. Some *Arabidopsis* plants were grown in high-Pi medium for the indicated times before being transferred to low-Pi medium. Transgenic T2 plants were grown in the same conditions but with the addition of hygromycin. For hydroponic culture, 9-d-old seedlings were transferred from agar plates to nutrient solution. After an additional 5-d growth period in high-Pi solution, Pi starvation was initiated

by transferring seedlings to Pi-free solution. After 10 to 14 d, root and shoot samples were collected separately for Pi assay or RNA analyses. For liquid culture, 7-d-old seedlings were grown in high-Pi B5 liquid medium in flasks with continuous shaking at 90 rpm for 13 d. Pi in the nutrient solution was then omitted, and seedlings were allowed to grow for another 5 d. Root samples were collected for RNA isolation. The T-DNA insertion line SAIL_47_E01 was acquired from the ABRC. All growth conditions were at 22°C under a 16-h photoperiod with cool fluorescent light at 100 to 150 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Rice (*Oryza sativa*) and tomato (*Lycopersicon esculentum*) plants were grown in a hydroponic system with the same nutrient solution. Four-week-old tomato plants were maintained in the Pi-sufficient solution (0.5 mM KH_2PO_4) or transferred to Pi-free solution for 5 d. Rice plants were grown directly in high-Pi solution (0.1 mM KH_2PO_4) or no-Pi solution for 2 weeks. Roots, stems, and leaves were harvested separately.

RNA Gel Blot and RT-PCR Analyses

Total RNA was isolated with the use of TRIzol reagent (Invitrogen). Low-molecular-weight RNA was isolated with the use of the RNeasy midi kit (Qiagen) according to the manufacturer's instructions. RNA gel blot analysis was performed as described (Lin et al., 2005). For small RNA gel blots, 5 to 10 μg of small RNA or 50 μg of total RNA was separated on a 15% polyacrylamide gel with 7 M urea. RNA was electroblotted to a Nytran SuperCharge membrane (Schleicher & Schuell BioScience) and fixed by UV light cross-linking and baking for 1 h at 80°C. Antisense DNA oligonucleotide probes were end-labeled with T4 polynucleotide kinase (New England Biolabs) for 1 h at 37°C. Unincorporated ^{32}P was removed with the use of a ProbeQuant G25 microcolumn (Amersham Biosciences). The membrane was hybridized and washed as described (Yoo et al., 2004).

Construction of Transgene and *Arabidopsis* Transformation

The primers used for PCR amplification were 5'-ACAAGCCTTCATATG-GATCTTATAG-3' and 5'-GGAAGAGTGTACGTACCTTTGATTT-3' for miR399b, 5'-GCAAACCAAAAAGAAAACCA-3' and 5'-GCAGAACCG-GTTATAGACTGTG-3' for miR399c, and 5'-TTGCAGTAGAGATGAAAATACA-3' and 5'-CTTACTAACCAAAAACCAACAAATTA-3' for miR399f. The amplified fragments were 236, 192, and 198 bp long for miR399b, miR399c, and miR399f, respectively; they were cloned into pGEM-T easy vector (Promega) and confirmed by sequencing. The miR399 precursors were subcloned into pMDC32 (Curtis and Grossniklaus, 2003) harboring a dual cauliflower mosaic virus 35S promoter, a strong constitutive promoter, with the use of Gateway recombination technology (Invitrogen). Plasmids were then transformed into *Agrobacterium tumefaciens* strain GV3101pMP90. *Arabidopsis* transformation was by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with the use of 20 $\mu\text{g}/\text{mL}$ hygromycin.

P Content Assay

Pi and total P contents were analyzed as described (Ames, 1966) with minor modifications. Fresh tissue was frozen with liquid nitrogen and homogenized with extraction buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 1 mM β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) at a ratio of 1 mg of sample (fresh weight) to 10 μL of extraction buffer. A total of 100 μL of homogenized sample was mixed with 900 μL of 1% glacial acetic acid and incubated at 42°C for 30 min. Then, 100 μL of the mixture was transferred to a glass tube for total P assay. The rest of the solution was centrifuged at 13,000g for 5 min, and 300 μL of the supernatant aliquot was used in the Pi assay. The reaction containing 700 μL of assay solution (0.35% NH_4MoO_4 , 0.86 N H_2SO_4 , and 1.4% ascorbic acid) and 300 μL of sample was incubated at 42°C for 30 min. Pi

content was measured at A_{820} . For the total P assay, 30 μL of 10% $\text{Mg}(\text{NO}_3)_2$ in 95% ethanol was added to 100 μL of the aliquot. Samples were then dried and flamed to ash. After cooling, 300 μL of 0.5 N HCl was added to dissolve the samples at 65°C for 30 min. Total P content was measured as described for Pi by the addition of 700 μL of assay solution.

Pi Uptake Assay

Twelve-day-old seedlings were grown on half-strength Hoagland medium supplemented with 250 μM KH_2PO_4 and used for the Pi uptake assay. Roots of 10 seedlings were pooled as a biological sample and incubated in 3 mL of uptake solution, which was the same as the previous growth medium but with the addition of [^{33}P]orthophosphate (22.2×10^{-3} MBq/3 mL; Perkin-Elmer). After 1, 2, and 4 h, reactions were stopped by rinsing seedlings three times with the same solution but without radioactive ^{33}P . Root and shoot samples were weighed and lysed in 500 μL of 30% H_2O_2 and 200 μL of perchloric acid for 1 h at 70°C. A total of 10 mL of scintillation cocktail (Beckman Coulter) was added to each sample, and the activity was measured by scintillation counting.

Pulse-Chase Labeling

Seedlings were prepared as described for the Pi uptake assay. Thirteen-day-old seedlings were fed [^{33}P]orthophosphate for 4 h. A double amount of radioactivity was applied to wild-type plants to reach an approximately equal amount of ^{33}P uptake. After 10 d of chase in the nutrient solution free of radioactive Pi to allow new leaves to grow, images of plants were obtained by radiography. Quantitative analyses were performed by feeding 18-d-old seedlings with [^{33}P]orthophosphate for 1 d. After being washed, leaves of half of the seedlings were excised, and the radioactivity was measured. The rest of the seedlings were transferred to the same nutrient solution free of radioactive Pi. The nutrient solution was replaced every day. After 6 d of chase, leaves were excised and the radioactivity was measured. The distribution of ^{33}P within different leaves was measured as a proportion to that in the whole shoot. Changes in ^{33}P distribution were calculated as follows: (percentage after chase – percentage after pulse) \div (percentage after pulse).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative/GenBank data libraries under accession numbers AtUBC24, At2g33770, and DQ027037.

Supplemental Data

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

Supplemental Figure 1. RNA Gel Blot Analyses of miR399 Expression under Different Abiotic Stresses.

Supplemental Figure 2. Distribution of Anthocyanin and Pi within Leaves.

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