Small RNAs in response to phosphate availability

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Uncovering small RNA-mediated responses to phosphate-deficiency 
in *Arabidopsis* by deep sequencing

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**Abstract**

Recent studies have demonstrated the important role of plant miRNAs under nutrient deficiencies. In this study, deep sequencing of *Arabidopsis thaliana* small RNAs was conducted to reveal microRNAs (miRNAs) and other small RNAs that were differentially expressed in response to phosphate (Pi) deficiency. About 3.5 million sequence reads corresponding to 0.6-1.2 million unique sequence tags from each Pi-sufficient or -deficient root or shoot sample were mapped to the *Arabidopsis* genome. We showed that upon Pi deprivation, the expression of miR156, miR399, miR778, miR827 and miR2111 was induced, whereas the expression of miR169, miR395 and miR398 was repressed. We found crosstalks coordinated by these miRNAs under different nutrient deficiencies. In addition to miRNAs, we identified one Pi starvation-induced DCL1-dependent small RNA derived from the long terminal repeat of a retrotransposon and a group of 19-nucleotide small RNAs corresponding to the 5' end of tRNA and expressed at a high level in Pi-starved roots. Importantly, we observed an increased abundance of *TAS4*-derived trans-acting siRNAs (ta-siRNAs) in Pi-deficient shoots and uncovered an autoregulatory mechanism of *PAPI/MYB75* via miR828 and *TAS4*-siR81(-) that regulates the biosynthesis of anthocyanin. This finding sheds light on the regulatory network between miRNA/ta-siRNA and its target gene. Of note, a substantial amount of miR399* accumulated under Pi deficiency. Like miR399, miR399* can move across the graft junction, implying a potential biological role for miR399*. This study represents a comprehensive expression profiling of Pi-responsive small RNAs and advances our understanding of the regulation of Pi homeostasis mediated by small RNAs.
Introduction

Plants acquire mineral nutrients which are channeled into diverse biochemical and metabolic pathways essential for the completion of their life cycle. Phosphorus (P), one of the essential macronutrients of plants, is often not readily accessible to plants because of the low availability of phosphate (Pi) in the soil, the major acquired form of P (Marschner, 1995). To overcome this limitation, plants have evolved a number of responses to conserve and mobilize internal Pi and to enhance the acquisition of external Pi (Raghothama, 1999; Poirier and Bucher, 2002; Lin et al., 2009). Recently, the molecular basis underlying these adaptive responses has been an intense subject of research.

It is now recognized that small regulatory RNAs contribute to the complexity of gene regulation. MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) of 21-24 nucleotides (nts) long are two major classes of small regulatory RNAs in plants, functioning as negative regulators of gene expression (Brodersen and Voinnet, 2006; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006). miRNAs are processed from single-stranded RNA precursors capable of forming imperfectly complementary hairpin structures by RNase III enzyme DICER-LIKE1 (DCL1) or DICER-LIKE4 (DCL4). In most cases, plant miRNAs downregulate gene expression by direct cleavage of their target transcripts, although translational repression has been proposed to be common, too (Brodersen et al., 2008). In contrast, siRNAs are generated from perfectly complementary long double-stranded RNA precursors, requiring the activity of RNA-dependent RNA polymerase.

Endogenous siRNAs can be further classified into trans-acting siRNAs (ta-siRNAs), natural-antisense transcript-derived siRNAs (nat-siRNAs) and heterochromatin siRNAs (hc-siRNAs) according to their genomic origin and the
interaction of distinct components (Vaucheret, 2006; Vazquez, 2006). ta-siRNAs are phased 21-nt RNA molecules whose synthesis is triggered by the cleavage of miRNA on the TAS transcript (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). They act in-trans to downregulate the expression of unrelated loci from which they are produced.

The biological functions of miRNA cover a broad range of physiological processes. In addition to their involvement in normal plant growth and development, the expression of many miRNAs is regulated by various biotic and abiotic stresses, which may contribute to the development of adaptive responses to overcome unfavorable conditions (Sunkar and Zhu, 2004; Navarro et al., 2006; Chiou, 2007; Lu et al., 2008; Li et al., 2008a). Recent findings have also demonstrated the important role of several miRNAs under nutrient deficiencies. miR395, miR398 and miR399 are upregulated under sulfur (S), copper (Cu) and Pi deficiency, respectively. miR395 is involved in S assimilation and allocation by adjusting the activity of ATP sulfurylase (APS) and restricting the spatial expression of a sulfate transporter (AtSULTR2;1) responsible for root-to-shoot transport of sulfate (Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009). Other than a role in alleviating oxidative stresses (Sunkar et al., 2006), miR398 modulates the expression of Cu/Zn superoxide dismutase according to the availability of Cu (Yamasaki et al., 2007). Moreover, cell-specific expression of miR169 is crucial in the development of nitrogen (N)-fixing nodules in *Medicago truncatula* (Combier et al., 2006).

miR399 regulates Pi homeostasis by controlling the expression of PHO2 encoding a ubiquitin-conjugating E2 enzyme, UBC24 (Fujii et al., 2005; Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006). It acts as a positive regulator to activate the Pi uptake and translocation when Pi supply is insufficient, while PHO2 functions as a negative regulator to suppress these activities in order to prevent Pi excess when external Pi is...
ample. Notably, recent results from reciprocal grafting suggest that miR399 may serve as a long-distance signal from shoots to suppress PHO2 expression in roots as a way of communication between the Pi status of shoots and the Pi transport activity of roots (Lin et al., 2008; Pant et al., 2008). The long-distance movement of miR399 could be an early response to Pi deficiency. The shoot-derived miR399 is responsible for the degradation of PHO2 in the roots where the miR399 is not readily expressed (Lin et al., 2008).

New technologies of high-throughput sequencing have become powerful tools to disclose the large inventory of small RNA species in plants. These deep sequencing strategies may discover new miRNAs or novel small RNA classes and provide quantitative profiling of small RNA expression (Lu et al., 2005; Rajagopalan et al., 2006; Nobuta et al., 2007; Heisel et al., 2008; Zhu et al., 2008). In this study, Solexa sequencing was employed to profile the changes of small RNA expression in response to Pi deficiency. Very recently, an independent study in which P-responsive miRNAs were identified using quantitative RT-PCR of primary transcript of miRNAs followed by small RNA sequencing was reported (Pant et al., 2009). In our study, we not only profiled the expression of small RNAs, but also looked into their regulatory mechanism and potential function. Importantly, we found two unique classes of small RNAs upregulated by Pi deficiency and uncovered an autoregulatory mechanism of PAP1/MYB75 via miR828 and TAS4-siR81(-) that regulates the biosynthesis of anthocyanin. Changes in the abundance of specific groups of Pi-responsive small RNAs may mediate the activity or expression of target genes that are important in the development or regulation of adaptive responses, enabling plants to survive in a low-Pi environment.
Results and Discussion

Overview of small RNA profiles in response to Pi deficiency

Four small RNA libraries from Pi-sufficient roots (R+Pi), Pi-deficient roots (R-Pi), Pi-sufficient shoots (S+Pi) and Pi-deficient shoots (S-Pi) were constructed by Solexa high-throughput sequencing technology. After adaptor trimming, the numbers of sequence reads and unique sequence signatures from the raw data were calculated and then mapped to the *A. thaliana* genome (Supplemental Table S1). In summary, the sequencing yielded 4.8-5.1 million raw reads from each library and ~90% of the reads remained after trimming the adaptor sequences. Approximately 3.5 million sequence reads (~77-83% of trimmed sequence reads) corresponding to 0.6-1.2 million unique sequence signatures could be mapped perfectly onto the genome (TAIR8). Only those perfectly mapped sequences were analyzed further. The sequence reads that mapped to single and multiple genomic locations were defined, respectively, as single-hit reads and multiple-hit reads. One third of mapped sequences were single-hit reads in the root and one half in the shoot, respectively.

The length of the mapped sequences ranged from 16 to 27 nts. We first examined the correlation between the length of small RNAs and the proportion of total sequence reads or unique sequence signatures (Fig. 1). Similar distributions were observed in +Pi and -Pi libraries. Consistent with the earlier reports, there were two major peaks at 21 nt and 24 nt in the total sequence reads of shoot libraries (Fig. 1A). Unexpectedly, an additional peak at 19 nt was observed in the root libraries. This peak has not been noticed in the previous deep sequencing databases probably because the pure root sample has not been examined. Further analysis revealed that the majority of these 19-nt sequences originated from tRNAs (see below). When the unique sequence signatures were examined, the patterns of all 4 libraries were nearly identical (Fig.
The 24-nt small RNAs were dominant in either their reads or unique sequences, indicating that they are rich in sequence diversity.

Over-representation of tRNA-derived small RNAs in the Pi-starved roots

Mapped sequences were classified according to the annotation of the genome (Supplemental Table S2). Most sequences were mapped onto the non-annotated intergenic regions (30.12% - 42.30%) and to a lesser extent onto MIRNA genes and protein-coding genes. Strikingly, a significantly higher proportion of small RNAs was mapped to tRNA genes in the root libraries (24.29% - 34.32%) than in the shoot libraries (3.33% - 5.58%). The percentage in the -Pi root library (34.32%) was even higher than that in the +Pi root library (24.29%). When the origin of these small RNAs was further analyzed (Supplemental Table S3), we did not find any correlation between the abundance of small RNAs from specific tRNA species and their codon usage. We were surprised to find that a 19-nt sequence cleaved from the 5' end of Glycine-tRNA\textsuperscript{TCC} represented over 80% of tRNA-derived small RNAs in the roots and accounted for up to 18.44% and 27.70% of total sequence reads in the +Pi and -Pi root libraries, respectively, compared to only 1.00% - 1.79% in the shoot libraries (highlighted in Supplemental Table S3). For validation, we employed RNA gel analyses using the probes from 3 different segments corresponding to the 5' and 3' ends and the central anticodon loop of tRNA (Fig. 2B). The small RNAs derived from Aspartic acid-tRNA\textsuperscript{GTC} were also examined using the 5' end probe. Consistent with the sequencing data, the 19-nt RNAs from Gly-tRNA\textsuperscript{TCC} and Asp-tRNA\textsuperscript{GTC} corresponding to the 5' end of tRNA were highly accumulated in the roots but much less in the shoots (Fig. 2A). The -Pi roots showed the maximum accumulation. When probing with the 5' or 3' end probe, additional bands at 30-40 nt likely representing tRNA halves cleaved at the anticodon loop were observed preferentially in the roots.
(Fig. 2A). These results revealed a spatial and temporal expression pattern of small RNAs derived from the specific cleavage on tRNA molecules rather than random degradation and may represent a novel processing of small RNAs.

Studies in microorganisms or mammalian cells have suggested that the tRNA-derived small RNAs are accumulated in a developmentally-regulated manner and become dominant in specific tissues or under specific stress conditions (Lee and Collins, 2005; Haiser et al., 2008; Jochl et al., 2008; Thompson et al., 2008; Li et al., 2008b; Pant et al., 2009; Thompson and Parker, 2009). These small RNAs could be associated with the quality control of protein synthesis or regulation of gene expression. Moreover, a recent report showed that tRNA half molecules present in the phloem sap of pumpkin were able to inhibit translational activity in vitro (Zhang et al., 2009). The authors proposed that these phloem-delivered tRNA halves may be a long distance signal to coordinate the metabolic status between source and sink tissues. The differential accumulation of tRNA-derived small RNAs between roots and shoots observed here may represent the consequence of such long-distance movement.

**Expression profiling of miRNAs in response to Pi deprivation**

Forty-five and 55 differentially expressed MIRNA genes with greater than 1.5-fold relative change in sequence counts were identified in roots and shoots, respectively (Fig. 3 and Supplemental Table S4). MIR399s, MIR778, MIR827 and MIR2111 were clearly upregulated, whereas MIR169s, MIR395s and MIR398s were downregulated in -Pi roots and/or shoots. The upregulation of various species from the MIR399 family by -Pi was consistent with our previous observations (Lin et al., 2008), suggesting the reliability of our sequencing data. Among all MIRNA genes, the MIR399 family showed the highest degree of induction in both tissues.
The expression of miRNAs with relative changes greater than a ratio of 3 (highlighted in bold in Fig. 3) was validated by RNA gel analysis. To examine whether the response of these miRNAs was specific to -Pi or not, the RNA isolated from different nutrient deficiencies, including N, Pi, K, S, Cu and Fe were inspected at once (Fig. 4). miR156 was highly expressed but unaffected by nutrient deficiencies in the shoots; however, it was upregulated by -Pi, -N or -K with highest induction under -N. miR156 regulates a group of SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) transcription factors involved in juvenile-to-adult vegetative phase transition and other development processes (Wu and Poethig, 2006; Gandikota et al., 2007; Schwarz et al., 2008; Wang et al., 2008). The physiological role for the upregulation of miR156 in the -Pi, -N or -K root remains to be explored.

On the other hand, miR399, miR778 and miR827 were specifically upregulated by -Pi (Fig. 4A). It is interesting to note that several distinct small RNA sequences were generated by cleavage at different positions of the miR778 precursor (Supplemental Fig. S1). Because the corresponding miRNA* sequence could be detected for each cleaved RNA duplex, we suggest to rename miR778 as miR778-5p and the other two forms derived from the miR778 precursor as miR778-3p.1 and miR778-3p.2, respectively. Multiple processing of the miR778 precursor has not been reported previously, probably due to the low recovered sequence reads. All miR778 species were upregulated by -Pi with the highest sequence count of miR778-3p.1 (Supplemental Table S4). The target genes of miR778-5p encode SET domain-containing proteins which are involved in the regulation of histone methylation (Ebbs and Bender, 2006). Although the target genes of miR778-3p.1 and miR778-3p.2 await identification, generation of multiple miRNAs from the same precursor has increased the complexity for the miR778 regulation networks.
The target genes of miR827 encode SPX domain-containing proteins. In yeast cells, SPX domain-containing proteins participate in Pi transport or sensing (Lenburg and O'Shea, 1996). One of the target genes, At1g02860, encodes a nuclear-localized ubiquitin E3 ligase containing the RING and SPX domains, which was recently characterized as NLA (Nitrogen Limitation Adaptation) involved in the N recycle during N limitation (Peng et al., 2007) or as BAH1 (Benzoic Acid Hypersenstive1) involved in immune responses (Yaeno and Iba, 2008). Here, we validated the targeting of miR827 to At1g02860 mRNA by RLM 5'-RACE analysis (Supplemental Fig. S2A) and observed the downregulation of At1g02860 under -Pi (Supplemental Fig. S2B). At1g02860 may be regulated by many factors and have multiple functions in response to diverse external stimuli. The miR827-mediated regulation of At1g02860 under -Pi conditions may imply an interaction between N and P, although miR827 only responds to -Pi but not -N (Fig. 4A).

The expression of several downregulated miRNAs was also confirmed (Fig. 4B). miR169 was downregulated in -Pi, -N or -S roots but to a lesser extent in the shoots. Downregulation of miR169 was much more severe under -N. miR395 was downregulated in -Pi or -N roots and shoots. miR398 was downregulated by -Pi, -N, -K or -Fe in both roots and shoots. In contrast, miR395 and mR398 were upregulated by -S and -Cu, respectively, which is in agreement with previous findings (Jones-Rhoades and Bartel, 2004; Yamasaki et al., 2007). These observations suggest that miRNAs can coordinate crosstalk among different nutrient deficient responses. Noticing the upregulation of APS4 and SULTR2;1 under -Pi (Supplemental Fig. S2C), we speculate that the suppression of miR395 under -Pi may somewhat contribute to this effect. Upregulation of APS4 and SULTR2;1 may result in the increase of sulfate assimilation and translocation for sulpholipid biosynthesis, a compensation for the reduced phospholipids during -Pi. miR169 and its target gene, HAP2/NFYA
transcription factor, are critical in the development of nodules (Combier et al., 2006). Additionally, miR169a and NFYA5 have been shown to be involved in drought tolerance (Li et al., 2008a). NFYA5 functions upstream to activate several drought-responsive genes, including those involved in oxidative stress.

The target genes of miR398 encoding Cu/Zn superoxide dismutase are also involved in the response to oxidative stress (Sunkar et al., 2006). Several nutrient deficiencies, such as N, Pi, K and S, can trigger oxidative stresses (Shin et al., 2005; Schachtman and Shin, 2007). Changes of miR169 and miR398 expression could be involved in adjusting plant ability in order to survive the oxidative stress caused by nutrient deficiencies. Interestingly, the opposite expression of miR398 in response to Cu and Fe deficiencies may reflect an elegant regulation of the preferential expression of different superoxide dismutase (SOD), Cu/Zn-SOD or Fe-SOD, coupling with different metal cofactors. These Pi-responsive miRNAs and the potential functions of corresponding target genes relative to adaptive responses to -Pi were summarized in Table 1.

Upregulation of miR399, miR778 and miR827 and dowregulation of miR398 by -Pi were also observed in a recent study in which a quantitative RT-PCR platform was established to profile the primary transcript of miRNAs (pri-miRNA) (Pant et al., 2009). The changes of these pri-miRNAs agreed with those of their mature miRNAs in response to -Pi, suggesting that they are mainly regulated at the transcriptional level. However, changes in miR156 and miR395 were not discovered in their system probably because of differences in growth conditions or sample sources (Table 1). To examine whether the regulation of these miRNAs depends on the PHR1 (PHOSPHATE STARVATION RESPONSE 1) MYB transcription factor, which activates a group of Pi starvation-induced genes, including MIR399s (Rubio et al., 2001; Bari et al., 2006), we analyzed the existence of P1BS cis-element, the binding
site of PHR1, within the 2 kb sequences upstream of miRNA hairpins. In addition to MIR399s, the P1BS element could be found in the upstream sequences of several species of MIR156 and MIR169, MIR398c and MIR827 genes (Table 1), suggesting the possible regulation of these genes by PHR1.

Characterization of the Pi-responsive miRNA2111 family

We identified a miRNA gene family consisting of two genes (miR2111a and miR2111b) from the non-annotated sequence clusters, which were not reported as miRNAs during the time of our analysis. They were specifically upregulated by -Pi not by any of the other nutrient deficiencies tested (Fig. 5A). They possessed a potential hairpin secondary structure and could produce a small RNA duplex with 2-nt overhang at the 3’ end (Fig. 5C), the primary criteria of miRNA definition (Meyers et al., 2008). Because a substantial amount of both complementary strands of the RNA duplex was detected, it was difficult to differentiate miRNA from miRNA*, and thus we named them miR2111a-5p, miR2111a-3p, miR2111b-5p and miR2111b-3p. The sequences of miR2111a-5p and miR2111b-5p were identical, while those of miR2111a-3p and miR2111b-3p differed by 4 nts.

Although this family was also identified by other groups very recently (Fahlgren et al., 2009; Pant et al., 2009), its biogenesis and target genes have not been studied. The biogenesis of these miRNAs was analyzed using various mutants defective in the production of different classes of small RNAs (Fig. 5B). Here, we showed that the accumulation of these miRNAs was significantly reduced in the Pi-starved hen1 and dcl1 mutants. Nevertheless, some accumulation of miR2111 was still observed in the dcl1 mutant, suggesting that miR2111 can also be processed by another DCL, probably DCL4 in the absence of DCL1.
The gene At3g27150, encoding a kelch domain-containing F-box protein, was predicted to be a target of miR2111-5p. The cleavage of the mRNA of At3g27150 was confirmed by RLM 5'-RACE analysis (Fig. 5D). Interestingly, we did not observe the negative correlation between the expression of miR2111-5p and At3g27150. Instead, the steady-state level of At3g27150 mRNA was moderately induced by -Pi (Fig. 5E) regardless of the enhanced expression of miR2111-5p. Several reports have shown a positive temporal correlation between the expression of a miRNA and its target gene in spite of the negative regulatory role of miRNAs (Llave et al., 2002; Achard et al., 2004; Kawashima et al., 2009). The consequences of different miRNA regulatory circuits were classified into 4 categories, spatial restriction, mutual exclusion, dampening and temporal regulation, in which the final expression level of the miRNA and its target gene may not be correlated (Voinnet, 2009). We hypothesize that miR2111-5p may regulate the spatial expression of At3g27150 by restricting its expression in specific cells and/or fine-tune the expression of At3g27150 to maintain an optimal expression level. Alternatively, the possibility of translational repression can not be ruled out. Nevertheless, regulation by miR2111 may be only one of many regulatory routes of At3g27150 because multiple regulatory pathways integrated from different levels are usually involved in gene regulation. It is important to note that the target genes of miR399, miR827 and miR2111-5p encode proteins involved in ubiquitin-mediated protein degradation, suggesting a crucial role of post-translational regulation of protein abundance in the control of -Pi responses.

The homologues of the miR2111 gene could be identified in many other dicotyledon plant species, such as *Brassica rapa*, *Medicago truncatula*, *Lotus japonicus* and *Vitis vinifera* (Supplemental Fig. S3), but could not be found in monocotyledon plants. This observation suggests that either the miR2111 gene had become lost in monocotyledons or, more likely; it had emerged after the separation of
the dicotyledons from the monocotyledons. The sequence of miR2111-5p is more conserved than that of miR2111-3p. The 4 miR2111 genes in *M. truncatula* are clustered in the same direction within a range of 40 kb. Interestingly, there are only 32 nts apart from the middle 2 hairpins which are likely derived from a polycistronic transcript. Conservation of the miR2111 species implies the importance of miRPi1-mediated regulation for the survival under the -Pi environment.

**Pi-responsive small RNAs derived from LTR of retrotransposon**

A cluster of small RNA originated from the long terminal repeat (LTR) of *Copia95* retrotransposon was identified as showing a differential expression pattern in response to -Pi (Fig. 6A). This small RNA cluster is located in the intergenic region of At5g27990 and At5g28000 containing a multiple array of truncated LTR either as inverted or tandem repeats, suggesting the occurrence of several duplication events (Figure 6C). The arrangement of inverted repeats allows the formation of a hairpin structure. The length of this hairpin is relatively long, at ~250 nts. Unlike those miRNA-like small RNAs recently found in rice grains (Heisel et al., 2008; Zhu et al., 2008), which are cleaved from a long hairpin, the small RNAs generated from this LTR hairpin are not phased. smRPi1<sup>LTR</sup>, the most abundant small RNA from the hairpin stem, accumulated specifically in -Pi roots but not in other nutrient-deficient tissues (Fig. 6A). DCL1 is essential for the biogenesis of smRPi1<sup>LTR</sup> and its accumulation was not reduced in the *dcl2,3,4* triple mutants nor in the *rdr1, rdr2* and *rdr6* mutants (Fig. 6B). smRPi1<sup>LTR</sup> is likely generated from the cleavage of a single-stranded rather than a double-stranded RNA precursor because the passenger strand of its duplex was identified from the hairpin stem with two mismatched base pairs. Because we were unable to detect smRPi1<sup>LTR</sup> in the Landsberg accession, we hypothesize that smRPi1<sup>LTR</sup> is a newly-evolved small RNA due to rapid
rearrangement of LTR and may represent an intermediate small RNA species from the transition of siRNAs to miRNAs. AtCopeg1 (Copia evolved gene 1, At2g04460), a protein-coding gene originating from a truncated AtCopia95 retrotransposon, was recently shown to be highly upregulated by -Pi (Duan et al., 2008). It will be interesting to elucidate the relationship between AtCopeg1 and smRP1\textsuperscript{LTR} in terms of co-evolution or cross-regulation.

**Upregulation of miR828 and TAS4-derived siRNAs by Pi deficiency and autoregulation of PAPI/MYB75**

The sequencing results revealed the upregulation of ta-siRNAs derived from TAS4 and miR828 which initiates the cleavage of TAS4 RNA in -Pi shoots (Supplemental Table S4). These ta-siRNAs were upregulated in the shoots by -Pi as well as -N (Fig. 7A). TAS4-siR81(-) targets a group of MYB transcription factors, PAP1/MYB75, PAP2/MYB90 and MYB113, that are involved in the biosynthesis of anthocyanin (Rajagopalan et al., 2006). It is well documented that plants accumulate anthocyanin under -Pi, in which these MYB transcription factors are upregulated (Misson et al., 2005). The negative regulatory role of siRNA seemed to be contradictory to the positive correlation between the expression of TAS4-siR81(-) and its target genes under the same conditions. This prompted us to carry out further investigation. TAS4 and MIR828 T-DNA knockout lines and a PAPI/MYB75 activation mutant, production of anthocyanin pigment 1-Dominant (pap1-D) (Borevitz et al., 2000), were examined. As predicted, there was no detectable TAS4-siR81(-) in the tas4 mutant, and neither miR828 nor TAS4-siR81(-) were detected in the mir828 mutant (Fig. 7B). The accumulation of miR828 and TAS4-siR81(-) was already observed in the +Pi shoots of pap1-D mutant and increased under –Pi (Fig. 7B). This suggests that PAP1/MYB75 can positively
regulate \textit{MIR828} and/or \textit{TAS4} genes either by activating their expression directly or indirectly or by suppressing a negative regulator of \textit{MIR828} and/or \textit{TAS4} via a different feedback loop. Analyses of the 1 kb upstream sequences of \textit{MIR828} and \textit{TAS4} genes revealed multiple MYB binding sites (predicted by PLACE database, http://www.dna.affrc.go.jp/PLACE/index.html) and one PAP1 cis-regulatory element, \((C/T)(A/C)NCCAC(A/G/T)N(G/T)\) (Dare et al., 2008) at the 305 bp and 747 bp upstream of the \textit{MIR828} hairpin precursor and \textit{TAS4}, respectively. Thus, PAP1 likely acts directly on the \textit{MIR828} and \textit{TAS4} promoters to activate their transcription. On the other hand, the transcript level of \textit{PAP1/MYB75}, \textit{PAP2/MYB90} and \textit{MYB113} was elevated in the \textit{tas4} and \textit{mir828} mutants under both +Pi and -Pi conditions (Fig. 7C). This could be explained by the relief of targeting of \textit{TAS4-siR81(-)}. Consistent with the increased expression of these MYB transcription factors, the anthocyanin accumulation was enhanced in the shoots of \textit{tas4} and \textit{mir828} mutants under both +Pi and -Pi conditions (Fig. 7D).

These results uncovered an autoregulatory mechanism of \textit{PAP1/MYB75} via the \textit{miR828} and \textit{TAS4-siR81(-)}, in which an adequate expression level of \textit{PAP1/MYB75}, \textit{PAP2/MYB90} and \textit{MYB113} and a proper accumulation of anthocyanin are maintained during -Pi (Fig. 7E). In this model, Pi deficiency results in upregulation of those MYB transcription factors, which subsequently activate the biosynthesis of anthocyanin. On the other hand, the increased level of \textit{PAP1/MYB75} can also lead to production of \textit{TAS4-siR81(-)} via the activation of \textit{miR828} and/or \textit{TAS4}, which antagonizes its own expression and other MYB transcription factors. Whether the upregulation of \textit{miR828} can be bypassed by the activation of \textit{PAP1/MYB75} remains to be studied. Because anthocyanin is a common stress pigment, we hypothesize that this autoregulation machinery may not be restricted to -Pi and could also be applied to other stress conditions in which anthocyanin is accumulated.
**miRNA399* and its movement**

In addition to miR399s, we observed a considerable amount of miR399* accumulated in -Pi tissues. The read number of different miR399* species was about half of miR399 (e.g. miR399f*) or even higher than that of miR399 (e.g. miR399a* and miR399d*). Such high accumulation of miR399* could not be neglected because it was more abundant than many other miRNAs. RNA gel analyses confirmed the existence of miR399* with the high abundance in miR399d* and miR399f*, predominantly in the roots (Fig. 8A), which was consistent with the high level of their primary transcripts and of mature miR399d and miR399f (Lin et al., 2008).

We and others have previously shown that miR399 can move from shoots to roots and function as a long-distance signal in response to -Pi (Lin et al., 2008; Pant et al., 2008). We found that like miR399f, miR399f* could be detected in the wild-type rootstock grafted with the miR399f-overexpressing scions (Fig. 8B). This result indicates that both miR399f and miR399f* can move across the graft junction from scions to rootstocks. We hypothesize that miR399* may have certain physiological relevance, possibly functioning in concert with miR399.

There may be several possible functions of miR399*. First, miR399* may be loaded into the RISC to target different genes. Many miRNA* species from *Drosophila* were reported to associate with the Argonaute protein and have inhibitory activity (Okamura et al., 2008). Several genes were predicted to be the putative targets of miR399* species; however, we were unable to validate them by RLM 5'-RACE analysis. Thus, whether these miR399* species possess gene silencing activity remains uncertain. Secondly, given the ability of miR399 and miR399* to move across the graft junction, we suspect that miR399* may assist the long-distance movement of miR399 by forming a miR399/miR399* duplex in the phloem stream.
Several miRNA* species were also detected in the phloem saps of *Brassica napus* (Buhtz et al., 2008; Pant et al., 2009). Although the authors concluded that they remain in the single-stranded form, based on the *in vitro* RNase digestion assay, they could not exclude the possibility of separation from double-stranded RNA duplex during the RNA extraction. An *in vivo* non-destructive analysis needs to be developed to differentiate the double-stranded small RNA molecules from single-stranded. Alternatively, miR399* may regulate or buffer the targeting efficiency of miR399 on *PHO2* mRNA via the formation of RNA duplex.

**Conclusion**

In summary, we have presented an extensive survey of the small RNAs showing differential expression in response to -Pi. These Pi-responsive small RNAs and their target genes are likely involved in the development or regulation of adaptive responses to -Pi. Interestingly, while miR399, miR778, miR827 and miR2111 responded specifically to -Pi, miR156, miR169, miR395 and miR398 also responded to other nutrient stresses. These responses involve upregulation or downregulation of distinct miRNAs, which coordinate and balance the demands of different nutrients. We hypothesize that metabolic adjustment, carbon assimilation or oxidative stresses may have an effect on the crosstalk among different nutrients as we observed here. Although there is still much more to learn about the biological significances of these Pi-responsive small RNAs, this work has opened a new avenue for the functional study of small RNA-mediated gene regulation in -Pi responses.
Materials and Methods

Plant materials and growth conditions

A. thaliana (Col-0) wild-type plants and mutants (mir828, SALK_097788; tas4, SALK_066997; pap1-D, CS3884) (Borevitz et al., 2000; Alonso et al., 2003) were grown hydroponically as described (Aung et al., 2006; Chiou et al., 2006). Seventeen-day-old seedlings were subjected for -Pi treatment by transferring them from +Pi (250 µM KH2PO4) into Pi-free 1/2 Hoagland solution. Roots and shoots were collected separately after 5-day treatment. Control samples were maintained in +Pi and collected at the same time. For treatments with different nutrient deficiency, the specific nutrient element was withdrawn from the nutrient solution for 5 days. To compensate for the ion originally associated with the omitted nutrient element, it was supplied as the form of chlorite salt at equivalent concentration except the treatments of -Cu and -Fe in which they were omitted from the solution.

RNA preparation and detection and high-throughput small RNA sequencing

Total RNA was isolated using Trizol reagent (Invitrogen). Four RNA samples from +Pi or -Pi roots or shoots of wild-type plants were used to generate small RNA libraries by the Solexa sequencing technology (Illumina). Small RNA gel blot analysis was carried out as described (Chiou et al., 2006) with modification according to Pall et al. (Pall et al., 2007). Quantitative RT-PCR analysis was performed as described (Lin et al., 2008). The sequences of probes and PCR primers are listed in Supplemental Table S5.

Analysis of sequencing data (For details, see Supplemental Materials and Methods)
The identical adapter-trimmed reads were grouped as unique sequences with associated counts of the individual sequence read. Each unique sequence was aligned to the *Arabidopsis* genome (TAIR8, http://www.arabidopsis.org/) and only perfectly mapped sequences were retained and analyzed further.

We defined a sequence cluster as a group of same-strand sequences that were mapped to similar genomic locations; and the union of the mapped regions of the individual sequences of the cluster can form a consecutive non-gapped region. For every cluster, the number of sequence reads from each of the four samples was counted separately and used for differential expression analysis.

We used two approaches to perform the differential expression analysis, a cluster-based approach for finding novel differentially expressed gene models and a gene-model-based approach for analyzing known gene models, especially for miRNA genes and tasiRNA genes. All sequence clusters or genes of interest were compared between two libraries for obtaining differentially expressed ones by using statistics test.

For each of the differentially expressed sequence clusters that were not annotated, the sequence including the cluster union and 200-bp flanking genomic sequence extending from each side was extracted and folded by RNAfold (Hofacker, 2003). A sequence cluster was considered to be a novel miRNA if it met the following 4 criteria: 1) the structure forms a hairpin, 2) the sequence cluster is located in the duplex region of the hairpin structure, 3) there is another sequence cluster located on the other strand of the duplex, and 4) the most abundant sequences from each of the 2 clusters can form a duplex with 2-nt overhang at the 3’ end. The targets for the novel miRNAs were predicted using methods described elsewhere (Allen et al., 2005). Validation of the target gene was carried out by RLM 5’-RACE analysis using GeneRacer kit (Invitrogen).
Anthocyanin analysis

Anthocyanin analysis was measured according to Lange et al. (Lange et al., 1971)

Accession Numbers

The small RNA sequence data was submitted to Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number of GSE17741. Accession numbers of miRNAs are available at miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml).

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Supplemental data

Supplemental Figure S1. Multiple processing of the miR778 precursor
The small RNAs mapped on the miR778 precursor form 4 RNA duplexes with 3’ double- or single-nucleotide overhangs. The read counts from the -Pi root library are indicated.

Supplemental Figure S2. Expression of the target genes of miR395 and miR827 in response to Pi deprivation
(A) RLM 5’-RACE analysis of miR827 cleavage on At1g02860 mRNA.
(B) Quantitative RT-PCR analysis of At1g02860 mRNA.
(C) Quantitative RT-PCR analyses of two miR395 target genes, At5g43780 (APS4) and At5g10180 (SULTR2;1).

Supplemental Figure S3. Evolutionary analysis of MIR2111
(A) Orthologues of AtMIR2111 in different plant species.
(B) Predicted hairpin structures of MIR2111 orthologues.

Supplemental Table S1. Statistics of small RNA sequences from four libraries

Supplemental Table S2. Categories of small RNA origins

Supplemental Table S3. Normalized abundance of tRNA-derived small RNAs

Supplemental Table S4. Normalized abundances of miRNA (a) and ta-siRNA and smRpi1LTR (b) as well as their differential expression

Supplemental Table S5. Sequences of probe and primer used in this study

Supplemental Materials and Methods
Table 1. Summary of validated Pi-responsive miRNAs and ta-siRNAs and the potential function involved under Pi deficiency

| miRNA/ta-siRNA | Target genes |
|----------------|--------------|
| Family | Species | Expression | Gene product | Expression | Potential role |
| miR169 | a, b, c, d, e, f, g, i, j, k, l, m, n | Down | HAP2 transcription factors (At1g17590, At1g54160, At1g72830, At3g05690, At3g20910, At5g06510, At5g12840) | Up | Involved in antioxidative stress |
| miR395 | b, c, f | Down | ATP Sulfurylase (APS1, APS3, APS4) (At3g22890, At4g14680, At5g43780), Sulfate transporter (SULTR2;1) (At5g10180) | Up | Increase sulfate translocation and utilization for sulpholipid biosynthesis |
| miR398 | a, b, e | Down | Cu/Zn superoxide dismutase (CSD1, At1g08830; CSD2, At2g28190), Cytochrome c oxidase (At3g15640) | Unchanged | Scavenge reactive oxygen species |
| miR402 | Down | DEMETER-LIKE protein 3 (DML3) (At4g34060) | | | Undetermined |
| miR156 | a, b, c, d, e, f, g | Up (root) | SPL transcription factors (At1g53160, At2g33810, At3g15270, At5g43270, At1g27360, At1g27370, At1g69170, At2g42200, At3g57920, At5g50570, At5g50670) | Down | |
| miR399 | a, b, c, d, f | Up | Ubiquitin conjugase E2 (UBC24/PHO2) (At2g33770) | Down | Pi uptake and translocation |
| miR778 | 5p | Up | SET domain-containing protein (At2g22740, At2g35160) | | |
| miR827 | a-5p, b-5p, 3p.1, 3p.2 | Up | Ubiquitin E3 ligase with RING and SPX domains (NLA/BAH1) (At1g02860), SPX domain-containing protein (At1g63010) | Down | Nutrient recycle |
| miR2111 | a-3p, b-3p | Up | Kelch repeat-containing F-box protein (At3g27150) | Up | |
| miR828 | Up (shoot) | TAS4 | | | Anthocyanin biosynthesis |

The Pi-responsive miRNAs reported by Pant et al., 2009 are underlined.

Only those species with statistically significant changes in expression are shown. Bold letters indicate the existence of P1BS cis-element (GNATATNC), a binding site for PHR1, in the 2 kb sequences upstream of miRNA hairpins. There are P1BS elements in the upstream sequences of MIR827 and TAS4 genes.

Change of small RNA expression to Pi deficiency.

The target genes are based on the ASRP database (http://asrp.cgrb.oregonstate.edu/) except miR2111 (this study). The target genes with altered expression by Pi deficiency are indicated in bold.

Change of target gene expression to Pi deficiency. The results were based on the microarray analysis from Geneinvestigator (https://www.genevestigator.ethz.ch/gv/index.jsp) except for the target genes of miR399 (Chiou et al., 2006), miR2111-5p and TAS4-siR81(-) (Fig. 5 and 7), miR395 and miR827 (Fig. S2), whose expression was analyzed by quantitative RT-PCR. Changes larger than twofold are indicated.

Potential role of target gene under Pi deficiency.
Because of its low abundance, the expression of miR402 was validated by quantitative RT-PCR of primary transcript.

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Figure legends

Figure 1. Global profiling of small RNAs.
Correlation between the small RNA length and the proportion of total sequence reads (A) or unique sequence signatures (B).

Figure 2. Over-representation of tRNA-derived small RNAs in the Pi-starved roots.
A, Small RNA gel analyses of tRNA-derived small RNAs in +Pi or -Pi roots or shoots. The tRNA regions corresponding to the 5’ and 3’ end probes are designated by the black line and the anticodon probe is designated by the grey line in B. The black arrows in A and B indicate the 5’ cleaved products and corresponding cleavage site in tRNA, respectively. The vertical grey lines in A indicate the products of tRNA halves from the cleavage at the anticodon loop (the grey arrow in B).

Figure 3. Differentially expressed MIRNA genes in response to Pi deficiency in roots (A) and shoots (B).
The significantly differentially expressed MIRNA genes with greater than 1.5-fold relative change are shown. The miRNAs with a relative change ratio greater than 3 are highlighted in bold.

Figure 4. Validation of differentially expressed miRNAs under different nutrient deficiencies.
Small RNA gel analyses of Pi starvation-upregulated miRNAs (A) and Pi starvation-downregulated miRNAs (B). miR778 was detected using the probe corresponding to miR778-3p.1 and -3p.2

Figure 5. Characterization of miR2111 in response to Pi deficiency.
Small RNA gel analyses of miR2111-5p and miR2111-3p under different nutrient deficient conditions (A) and in small RNA biogenesis mutants (B).
C, Hairpin secondary structures of miR2111a and miR2111b precursors.
D, RLM 5'-RACE analysis of miR2111-5p cleavage on At3g27150 mRNA.
E, Quantitative RT-PCR analysis of At3g27150 mRNA in response to -Pi. One of two biological replicates is presented, and the error bars indicate the SD of two technical replicates.
Figure 6. Pi starvation-induced small RNAs derived from LTR of retrotransposon.
Small RNA gel analysis of smRPi1LTR under different nutrient deficient conditions (A) and in small RNA biogenesis mutants (B).
C, Arrangement of Copia95 LTR in the intergenic region of At5g27990 and At5g28000 and the potential hairpin structure and the location of smRPi1LTR.

Figure 7. Autoregulatory mechanism of PAPI/MYB75 via miR828 and TAS4-siR81(-).
A, Small RNA gel analysis of TAS4-siRNAs under different nutrient deficient conditions.
B, Small RNA gel analysis of TAS4-siR81(-) and miR828 in the shoots of wild-type (wt) plants, tas4, mir828 and PAPI/MYB75 activation (pap1-D) mutants.
C, Expression of PAPI/MYB75, PAP2/MYB90 and MYB113 by quantitative RT-PCR analysis in tas4 and mir828 mutant. One of two biological replicates is presented, and the error bars indicate the SD of two technical replicates.
D, Alteration of anthocyanin accumulation in tas4, mir828 and pap1-D mutants. Error bars represent the SD (n = 5).
E, A proposed model of the autoregulatory pathway of PAPI/MYB75 via miR828 and TAS4-siR81(-).

Figure 8. Expression and long-distance movement of miR399*.
A, Small RNA gel analysis of miR399*.
B, Quantitative RT-PCR analysis of miR399f and miR399f* in the rootstocks of grafted plants. Combination of grafts is indicated as scion/rootstock. wt: wild-type plants, 399f: miR399f-overexpressing plants. The error bars represent SD (n = 4-5 independent grafted plants).
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Small RNA gel analysis of smRPi1LTR under different nutrient deficient conditions (A) and in small RNA biogenesis mutants (B). C, Arrangement of Copia95 LTR in the intergenic region of At5g27990 and At5g28000 and the potential hairpin structure and the location of smRPi1LTR.
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B, Small RNA gel analysis of TAS4-siR81(-) and miR828 in the shoots of wild-type (wt) plants, tas4, mir828 and PAP1/MYB75 activation (pap1-D) mutants.
C, Expression of PAP1/MYB75, PAP2/MYB90 and MYB113 by quantitative RT-PCR analysis in tas4 and mir828 mutant. One of two biological replicates is presented, and the error bars indicate the SD of two technical replicates.
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