Expression analysis suggests potential roles of microRNAs for phosphate and arbuscular mycorrhizal signaling in Solanum lycopersicum

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MicroRNAs (miRNAs) have emerged as a class of gene expression regulators that play crucial roles in many biological processes. Recently, several reports have revealed that microRNAs participate in regulation of symbiotic interaction between plants and nitrogen-fixing rhizobia bacteria. However, the role of miRNAs in another type of plant–microbe interaction, arbuscular mycorrhizal (AM) symbiosis, has not been documented. We carried out a microarray screen and poly(A)-tailed reverse transcriptase-polymerase chain reaction (RT-PCR) validation for miRNA expression in tomato (Solanum lycopersicum) under varying phosphate (Pi) availability and AM symbiosis conditions. In roots, miRNA158, miRNA862-3p, miRNA319, miRNA394 and miR399 were differentially regulated under three different treatments, Pi sufficient (+P), Pi deficient (−P) and AM symbiosis (+M). In leaves, up to 14 miRNAs were up- or down-regulated under either or both of the Pi treatments and AM symbiosis, of which miR158, miR319 and miR399 were responsive to the treatments in both roots and leaves. We detected that miR395, miR779.1, miR840 and miR867 in leaves were specifically responsive to AM symbiosis, whereas miR398 in leaves and miR399 in both roots and leaves were Pi starvation induced. Furthermore, miR158 in roots as well as miR837-3p in leaves were responsive to both Pi deprivation and AM colonization. In contrast, miR862-3p in roots was responsive to Pi nutrition, but not to AM symbiosis. Moreover, the group of miRNA consisting miR319 and miR394 in roots and miR158, miR169g*, miR172, miR172b*, miR319, miR771 and miR775 in leaves were up- and down-regulated by Pi starvation, respectively. The data suggest that altered expression of distinct groups of miRNA is an essential component of Pi starvation-induced responses and AM symbiosis.

Introduction

Phosphorus (P) is the most immobile element among the three main macronutrients required by plants. Plants have evolved a series of strategies to cope with P deficiency, including establishment of symbiotic associations with arbuscular mycorrhizal fungi (AMF). More than 80% terrestrial vascular flowering plants are able to form symbiotic relationship with the AMF, and there are fossil evidences suggesting the existence of AM symbiosis for more than 460 million years (Harrison 2006). Moreover, evidence indicates that even some of

Abbreviations – AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; miRNA, microRNA; nt, nucleotide; P, phosphorus; PHR1, Phosphate Starvation Responses 1; Pi, phosphate; PIP, plasma membrane intrinsic protein; RA, relative abundance; RT-PCR, reverse transcriptase-polymerase chain reaction; TIP, tonoplast intrinsic protein; WPI, weeks post-inoculation.
the non-mycorrhizal plants of today accounting for about 10% of the plant species were mycorrhizal 100 million years ago (Brundrett 2002).

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs that have been found in both animals and plants. The mature miRNAs are usually approximately 21 nucleotides (nt) in length and are conserved across plant species (Jones-Rhoades et al. 2006). They function as negative post-transcription regulators by specific binding and cleavage of their target miRNAs, or by repression of target mRNA translation (Jones-Rhoades et al. 2006). In recent years, an increasing number of miRNAs have been isolated/identified and functionally characterized (Jones-Rhoades et al. 2006). A significant amount of work has been focused on unraveling the pivotal role that miRNAs played in plant growth and development, including nutrient stresses (Chiou 2007).

In Arabidopsis, miR395 was up-regulated during sulfate starvation, and its accumulation was regulated by the transcription factor SLIM1 (Kawashima et al. 2009). MiR395 targeted not only the mRNAs of three members of the ATP sulfurylase (APS) gene family (Allen et al. 2005, Jones-Rhoades and Bartel 2004), but also the sulfate transporter SULTR2;1 (Kawashima et al. 2009). The miR399 is a Phosphate Starvation Responses 1 (PHR1) dependent phosphate (Pi) starvation-enhanced miRNA in plants (Bari et al. 2006, Rubio et al. 2001). It targets and directs the cleavage at the 5′ UTR of the mRNA of an ubiquitin-conjugating E2 enzyme-UBC24, and its overexpression resulted in enhanced Pi uptake and translocation of Pi within the plant (Chiou et al. 2006, Fuji et al. 2005, Sunkar and Zhu 2004). In addition, chimeric Arabidopsis or tobacco plants that were generated by micrografting revealed the translocation of miR399 from the miR399 overexpressed scions to the wild-type rootstocks (Lin et al. 2008, Pant et al. 2008). These results indicated that phloem transported miRNA from shoot to root could act as a long-distance signal for the regulation of P homeostasis (Lin et al. 2008, Pant et al. 2008).

The miRNAs are also involved in regulation of plant–microbe symbiosis. Combier et al. (2006) reported that in the model legume Medicago truncatula, miR169 was responsible for the post-transcriptional repression of MtHAP2-1, a gene encoding a transcription factor belonging to the CCAAT-binding family. Either overexpression of miR169 or RNA interference (RNAi) mediated knock-down of MtHAP2-1 in M. truncatula would lead to defective nodule cells differentiation (Combier et al. 2006). More recently, miR166, which has two copies in the MtMiR166a precursor, was shown as the post-transcriptional repressor of a novel family of transcription factors associated with nodule development, the class-III homeodomain-leucine zipper (HD-ZIP III) genes. Plants overexpressing miR166 showed a characteristic phenotype with reduced number of nodules as well as lateral roots (Boualem et al. 2008). Twenty conserved and thirty-five novel miRNA families have been identified in soybean roots inoculated with B. japonicum; among them a group of miRNAs was differentially regulated upon inoculation (Subramanian et al. 2008). These results offered the first insights into the miRNAs involved symbiotic signaling pathway in plants.

It has been suggested that AM signaling pathway involves a series of factors such as kinases, ion channels and transcription factors (Harrison 2006). However, the direct evidences for the involvement of these factors as well as miRNAs are lacking. In this work, we surveyed the expression of miRNAs in the roots and leaves of tomato plants that were highly infected with AMF or of tomato plants that were supplied with high and low Pi in an attempt to identify conserved miRNAs that may function in the stable plant–AMF symbiotic system. A preliminary screening for miRNAs using microarray technology was performed and the candidate regulatory miRNAs were selected, followed by validation of their expression patterns by poly(A)-tailed reverse transcriptase-polymerase chain reaction (RT-PCR). Our results revealed that 16 conserved miRNAs are differentially regulated by the Pi and/or AM symbiosis, thus paving the way for further functional characterization of miRNAs involved in Pi and AM symbiosis signaling pathways.

Materials and methods

**Plant material and culture condition**

Seeds of tomato (Solanum lycopersicum L. cv. Micro-Tom) were surface sterilized with a solution of 10% commercial bleach (0.525% sodium hypochlorite) for 5 min, washed three times with sterile water and placed on a wet sterile filter paper in a petri dish. The dish was placed in dark at 28°C for 2–3 days. After germination, seedlings were placed in sterilized quartz-sand until cotyledons were fully emerged. The seedlings were grown for 10 days with application of a 1/4 strength nutrient solution described below. The nutrient solution used for sand culture contains 2 mM KNO3, 1 mM NH4NO3, 0.5 mM Ca(NO3)2, 0.25 mM CaCl2, 0.5 mM MgSO4, 20 mM Fe-EDTA, 9 μM MnCl2, 46 μM H3BO3, 8 μM ZnSO4, 3 μM CuSO4 and 0.03 μM (NH4)2MoO4. Nutrient solution for Pi-sufficient plants was supplemented with 1 mM NaH2PO4, and for Pi-deficient and AMF colonized plants, 50 μM NaH2PO4 was provided.
A sand-based mycorrhizal inoculum containing *Glomus intraradices* was used for colonization (courtesy of Prof. Xiangui Lin from the Soil Science Institute of Nanjing, CAS). Each plantlet was inoculated with 3 g inoculum placed in the sterilized sand around the roots. One set of the plants provided with 50 μM Pi was colonized with the AM fungi, whereas another set was cultivated with the autoclaved inoculum that served as control.

The solution pH was adjusted to 5.5. The plants were grown in a chamber with a 14-h light period at 25–27°C and a 10-h dark period at 18–20°C for 7 weeks.

Seeds of rice (*Oryza sativa* ssp. *Japonica* cv. Nipponbare) were surface sterilized and cultured as previously described with minor modification (Li et al. 2006). After 7 days of growth, the plants were transferred to nutrient solution, either with 0.3 mM Pi (Pi sufficient, +P) or with no Pi supplied (Pi deficient, −P). The solution pH was adjusted to 5.5, and the solution was replaced every 2 days. The plants were harvested for RNA isolation after 14 days.

**Detection and analysis of AMF colonization**

The development of mycorrhizal fungal colonization in the root cortical cells was monitored regularly at 10-day intervals, until 7 weeks post-inoculation (WPI). Part of the tomato roots 7 WPI was used for microarray experiments and RNA isolation, and the rest were taken for observation of infection.

Root segments were treated with 10% (w/v) KOH solution at 85°C for 3 h and then the medium was acidified with 1% HCl solution for 10 min, followed by three washes with distilled water. The roots were then stained with trypan blue and examined for mycorrhizal fungal colonization under a binocular microscope (Leica, DMR, Germany) by the magnified line intersect method (David-Schwartz et al. 2001, McGonigle et al. 1990).

**Measurement of P concentration of plant tissues**

The measurement of P concentration was conducted as previously reported (Chen et al. 2007, Murphy and Riley 1962). Briefly, the dried crushed plant tissue powder (0.05 g) was digested with 5 ml of 98% H2SO4 and 3 ml of 30% hydrogen peroxide. After cooling, the digested sample was diluted to 100 ml with distilled water. The P concentration in the solution was measured using the molybdate-blue method with absorbance read at 700 nm on a 722 UV–visible spectrophotometer (JingHua Technology Instrument, Jing Hua, China).

**miRNA microarray experiments and preliminary screening**

Total RNAs were isolated from roots and leaves of tomato with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA labeling was performed with miRCURY™ Array Power Labeling kit (Cat #208032-A, Exiqon). The labeling reaction was performed at 16°C for 1 h. Hybridization was performed at 56°C for 16 h. After hybridization, the slides were washed using miRCURY Array, wash buffer kit (Cat #208021, Exiqon), and then dried by centrifugation at 1000 rpm for 5 min.

Clustering arrays was scanned with a scanner (Genepix 4000B). Data were extracted from the TIFF images using GENEPIX PRO 6.0 software (CapitalBio). Low intensity spots were removed, for which fewer than 30% of the signal pixels exceeded the median background plus two times its standard deviation (SD). Then, normalization was performed based on the mean array intensity for inter-array comparison. For each miRNA probe, four replicate spots were set on a microarray. The mean intensity value of each probe was used for cluster analysis. The raw data were log 2 transformed and median centered by arrays and genes using the Adjust Data function of CLUSTER 3.0 software and then further analyzed with hierarchical clustering with average linkage. To determine the differentially expressed miRNAs, significance analysis of microarrays (SAM, version 2.1) was carried out using two-class unpaired comparison in the SAM procedure. All hybridizations were normalized by total intensity with a criterion of normalized value more than 50.

The miRNA populations from +P and −P + M plants were compared with −P plants. The miRNAs with a fold change >1.5 and q value <0.001 were adopted as candidates that were differentially expressed in responses to P nutrition and/or AM symbiosis.

**RNA extraction and detection of miRNA expression by poly(A)-tailed RT-PCR**

Total RNA was extracted from plant tissues with TRIZol reagent (Invitrogen, Carlsbad, CA). The poly(A)-tailed RT-PCR was done as previously reported with minor modification (Fu et al. 2005). Considering the limited sequence information of tomato miRNAs (half of the candidates were not deposited in any tomato miRNA database), the poly(A)-tailed RT-PCR may not reflect the exact sequences of tomato miRNAs. However, this validation method may tolerate one or two mismatches on the miRNA sequences depending on the location of mismatches (Table S1) and still shows the expression trend of the miRNAs.

Four micrograms of total RNA was used in a 25-μl system for adding poly(A) tails with poly(A) polymerase.
(Ambion). Reverse transcription was performed using the poly(A)-tailed small RNA of plant tissues and 1.5 μg of RT primer (5’-CGA ACA TGT ACA GTC CAT GGA TAG-d(T)₃₀ (A, G or C) (A, G, C or T)-3’) with 500 U of M-MuLV reverse transcriptase (Fermentas, Ontario, Canada) in a 50-μl reaction system.

The amplification of miRNAs was performed for 35 cycles at an annealing temperature of 58°C using miRNA specific primers and primer (5’-CGA ACA TGT ACA GTC CAT GG-3’). The PCR products were analyzed on 12% polyacrylamide gel with Ethidium bromide (EB) staining. Then the gel slices containing DNA fragments of about 80 bp were excised and the DNA was purified using polyacrylamide gel electrophoresis (PAGE) purification kit (Biotek). The DNA fragments were directly subcloned into pMD19-T vector (TaKaRa) and sequenced. The relative abundance (RA) of each gene was calculated semi-quantitatively using the expression intensity of the Actin gene as their internal standard by software IMAGE-PRO PLUS (version 5.0, Media Cybernetics).

Prediction of miRNA targets

To predict potential targets of miRNA candidates, we utilized miRU (Zhang 2005), an online tool for prediction of plant miRNA potential target (http://bioinfo3.noble.org/miRNA/miRU.htm). Tomato miRNA sequences were used as query when their sequences are identical to Arabidopsis, otherwise the probe sequences derived from Arabidopsis miRNAs on the microarray were used. The default parameters were used for prediction which adapted the lowest stringency levels to capture more potential targets. The default input options were: score for each 20 nt = 3; G–U wobble pairs = 6; indels = 1 and other mismatches = 3. The dataset is from the TIGR Tomato Gene Index 10.

After the miRU prediction, a re-screening of the predicted potential targets following a series of standard criteria reported previously was performed (Qiu et al. 2007, Rhoades et al. 2002, Yin et al. 2008).

Results

AM infection rate and effect of AM colonization on Pi acquisition

To study the significance of miRNAs in tomato Pi and AM symbiosis pathways, we first established a tomato AM infection and colonization system. Inoculation of G. intraradices with the low Pi (0.05 mM) roots of tomato for 7 weeks resulted in formation of arbuscular mycorrhiza in about 70–85% of the root segments. The three main AMF structures, intraradical hyphae, vesicles and arbuscules, were clearly observed in the infected roots (Fig. 1A). The AMF colonization increased the total P uptake by 20% (Fig. 1B), even though the increase of P concentration in the plant was not significant, possibly because of the dilution effect of P by enhanced growth (Fig. 1B). As expected, supply of 1 mM Pi to the plant resulted in the highest P concentration and total P uptake.

Regulation of Pht1 transporters by Pi starvation and AM colonization

It has been well-documented in tomato that Pi deficiency enhanced expression of two Pi transporter genes, LePT1 in both roots and leaves and LePT2 in roots, whereas AM colonization induced expression of LePT4 in the roots (Liu et al. 1998, Muchhal and Raghothama 1999, Nagy et al. 2005, Xu et al. 2007). In this study, we used LePT1, LePT2 and LePT4 as marker genes for Pi deficiency and AMF colonization in the tomato plants. We sampled the highly colonized roots and leaves at 7 WPI and extracted total RNA for transcripts’ analysis. Besides the expected expression patterns of LePT1 and LePT2 under high P (+P) and low P (−P) supply conditions in the roots and leaves (Fig. 2), we observed that colonization of AMF in the low P roots slightly decreased expression of LePT1 and LePT2 in roots and suppressed expression of LePT1 greatly in the leaves (the very faint PCR product bands of LePT1 in high P and AMF colonized leaves are under the detection limit of the software for band intensity evaluation) (Fig. 2).

Preliminary screening of Pi and AM responsive miRNAs using miRNA microarray analysis

We screened the miRNA microarray (Product #208002-A, Lot #20478.06, Version: 9.2, Exiqon) containing probes against conserved miRNAs from plants, animals and microbes, among which 203 probes are against Arabidopsis miRNAs. A series of standards as described in the section Materials and methods was performed.

A total of 23 miRNAs with plant only derived sequences expressed either in the roots, or in the leaves or both that might recognize P nutrition and or AM colonization derived signals (Fig. 3) were identified for further validations by poly(A)-tailed RT-PCR.

Five miRNAs were differentially regulated in roots during Pi starvation and AM colonization

We performed poly(A)-tailed RT-PCR using the total RNA extracted from either the roots or leaves of tomato at 7 WPI. The data showed that transcripts abundance
of miR158 and miR862-3p in the roots was reduced under both Pi deficiency (−P) and AM symbiosis (+M) as compared with that under Pi sufficient condition (+P) (Fig. 4A). AM colonization in low P roots (+M/−P) further decreased the expression of miR158 (Fig. 4A).

In contrast, miR319 and miR394 tended to be up-regulated by Pi starvation in the roots (Fig. 4B), whereas their levels decreased under AM symbiosis as well (Fig. 1). Similarly, expression of the well-characterized miR399 was also increased by P deficiency in roots, which was consistent with published reports (Chiou et al. 2006, Fujii et al. 2005).

**Pi and AM symbiosis positively impact miRNA expression in leaves**

We tested the abundance of the mature fragments of miR172, miR771, miR169g*, miR158, miR319, miR172b* and miR775 in tomato leaves (Fig. 5). They were all down-regulated under Pi deficiency compared with that under Pi sufficient condition. The AM inoculation enhanced their expression nearly to the levels observed under high Pi supply condition (Fig. 5).

**Some miRNAs in leaves are up-regulated only under AM colonization**

It would be interesting to identify the AM symbiosis-specific induced genes, especially the ones with upstream regulatory functions, such as transcription factors and miRNAs. Here, we detected more mature fragments of miR395, miR779.1, miR840 and miR867 in leaves, particularly under AM colonization (Fig. 6). Therefore, we predict that these four miRNAs in leaves might also be involved in AM symbiosis signaling and independent of P signaling in tomato.
miRNAs in leaves up- or down-regulated by Pi deprivation and AM symbiosis

In the leaves, expression of miR399 was up-regulated under Pi deficient conditions as that in the roots (Fig. 4B), whereas its expression was not regulated by AM colonization as some other Pi-starvation-inducible genes (Burleigh and Harrison 1999) (Fig. 7 and 2). Interestingly, another well-characterized miRNA, miR398, showed a very similar expression pattern in leaves as compared with that of miR399 (Fig. 7). These results indicated that miR398 is also a Pi starvation-up-regulated miRNA in leaves, and both miR398 and miR399 are not strongly responsive to AM symbiosis.

In contrast to miR399 and miR398, miR837-3p was abundant in the leaves under high Pi and greatly down-regulated by Pi deficiency (Fig. 7). Its expression in the AMF colonized plants was barely detectable (Fig. 7).

Fig. 2. Expression of LePT1, LePT2 and LePT4 in response to Pi availability and AM fungal colonization. RT-PCR was performed with total RNA from roots and leaves of tomato. Actin was used for cDNA normalization. The number below the band indicates RA of each gene with respect to the loading control actin. ND indicates not detected.

Fig. 3. miRNA candidates selected by preliminary screening of miRNA microarray data. Preliminary screening was performed as described in the section Materials and methods. Gray circle, miRNAs selected from roots; white circle, miRNAs selected from leaves.

Fig. 4. Expression of miR158 and miR862-3p (A), as well as miR319, miR394 and miR399 (B) in response to Pi availability and AM fungal colonization. RT-PCR was performed with poly(A)-tailed total RNA from tomato roots. Actin was used for cDNA normalization. The number below the band indicates RA of each gene with respect to the loading control actin. ND indicates not detected.

Fig. 5. Expression of miR172, miR771, miR169g*, miR158, miR319, miR172b* and miR775 in response to Pi availability and AM fungal colonization. RT-PCR was performed with poly(A)-tailed total RNA from tomato leaves. Actin was used for cDNA normalization. The number below the band indicates RA of each gene with respect to the loading control actin. ND indicates not detected.
Fig. 6. Expression of miR395, miR779.1, miR840 and miR867 in response to Pi availability and AM fungal colonization. RT-PCR was performed with poly(A)-tailed total RNA from tomato leaves. Actin was used for cDNA normalization. The number below the band indicates RA of each gene with respect to the loading control actin.

Fig. 7. Expression of miR399, miR398 and miR837-3p in response to Pi availability and AM fungal colonization. RT-PCR was performed with poly(A)-tailed total RNA from tomato leaves. Actin was used for cDNA normalization. The number below the band indicates RA of each gene with respect to the loading control actin. ND indicates not detected.

Many miRNAs are constitutively expressed in tomato

Among the 23 miRNA candidates selected from our microarray data (Fig. 3), miR861-5p in roots, and miR171, miR847, miR839 and miR862-5p in leaves showed either constitutive expression or slight changes under our treatments (data not shown). This might be because of the technical difference between miRNA microarray experiment and RT-PCR validation method. However, these results confirmed their presence in tomato. We detected no or very low amplification of miR847, miR169, miR779.1 and miR860 in the roots (data not shown). The reasons for this absence could be that (1) their abundance is too low to be detected in the tissues; (2) these miRNAs are not inducible under our treatment conditions and (3) some of them could be false positive of microarray experiment.

Predicted targets of the selected miRNAs

We used the plant miRNA target finder tool, miRU (http://bioinfo3.noble.org/miRNA/miRU.htm) (Zhang 2005) as well as a series of standard criteria for predicting the potential targets of the miRNAs regulated either by Pi nutrition or AMF colonization or both. In brief, the predicted target genes complementary to the miRNAs with four or fewer mismatches were selected. Mismatches at the 11th nt of miRNAs and gaps were not allowed, and the non-canonical G:U pair was considered as a mismatch.

A variety of potential miRNA targets was predicted, of which some targets had annotated functions, whereas the others were unknown proteins (Table 1). The different targets of the predicted tomato miRNAs include a group of proteins associated with regulatory and metabolic pathways (Table 1). In general, the predicted mRNA targets can be separated into three groups. The members of the first group are all genes encoding transcription factors. Transcription factors are vital for signaling process of plant growth, development and stress responses. The second group includes genes encoding proteins with biological importance. Among these annotated proteins, some are kinases (miR172 and miR867), whereas another is responsible for nucleic acid binding (miR867). These proteins may have a close link with P, indicating their potential involvement in the plant Pi starvation responses. The last group is consisting of unknown proteins.

Discussion

Delicate molecular mechanisms are required for plants to accomplish the physiological and developmental processes, as well as responses to environmental stimuli. There are increasing evidences suggesting that miRNAs are one essential member of these mechanisms (Chiou 2007, Chuck et al. 2008, Jones-Rhoades et al. 2006). It is of interest and importance to determine whether and how miRNAs play a role in plant adaptation to the environmental stimuli. A logical first step toward this aim is the identification of miRNAs that are differentially expressed in response to these stimuli. In this study, we identified a total of 16 miRNAs in tomato (2 in roots, 11 in leaves and 3 in both roots and leaves), and found that they were differentially regulated by either P nutrition or AM colonization or both, indicating that miRNAs are probably a component for the P nutrition and AM symbiosis signaling.
Table 1. List of the potential targets of the selected miRNAs in S. lycopersicum. The target genes were predicted by using of miRU, plant microRNA potential target finder (http://bioinfo3.noble.org/miRNA/miRU.htm). The number of mismatches between each miRNA and their target mRNA is indicated in parentheses.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Targeted protein</th>
<th>Target function</th>
<th>Targeted sequence</th>
</tr>
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<tbody>
<tr>
<td>miR158</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>miR169g*</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>miR172</td>
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<td>Unknown</td>
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<td>PHAP2A protein</td>
<td>Transcription factor</td>
<td>TC142472(3)</td>
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<td>Homolog to AHAP2</td>
<td>Transcription factor</td>
<td>TC147477(3)</td>
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<td></td>
<td>Similar to UPL5S1</td>
<td>Cell cycle</td>
<td>TC152549(3)</td>
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<td>HD-ZIP protein</td>
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<td>Similar to S-receptor kinase</td>
<td>Metabolism</td>
<td>TC151000(3), TC152100(3)</td>
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<td>miR172b*</td>
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<td>–</td>
<td>–</td>
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<td>Homolog to sulfate adenylyltransferase</td>
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<td>Similar to AT5g04420/T32M21-20</td>
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<td>Metabolism</td>
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<td></td>
<td>Weakly similar to elongation factor Tu family</td>
<td>Nucleic acid binding</td>
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Recently, miRNA395, miRNA398, and miRNA399 have been well characterized and linked with nutrient deficiency-induced stresses (Chiou et al. 2006, Kawashima et al. 2009, Yamasaki et al. 2009), of which miRNA399 is specifically induced by Pi starvation. This uncovered another branch of the complex Pi starvation signaling pathway. However, plants have evolved a set of strategies to adjust to environment with limited Pi, which involves alterations of root architecture, enhanced excretion of organic acid and acid phosphatase, and formation of symbiotic associations with AMF (Raghothama 1999). The manifestation of these alterations caused by Pi deprivation is fine controlled by diverse molecular mechanisms, in which miRNAs besides miR399 might also display a significant regulatory role. On the other hand, in a well-established plant–AM fungi symbiosis, plants supply the endo-symbiont with sugar to sustain their growth and development. AM colonization in turn can help plants with exploration of P far away from the rhizosphere, transform the unaccessible forms of P into Pi, and thus improve the P nutrition of plants (Harrison 2006). Therefore, plants might share a common signaling
pathway to recognize the AM signal and the Pi signal. As a matter of fact, the Pi and AM signaling pathways share a great deal of upstream regulatory factors and downstream structural genes as revealed previously by microarray analyses (Guimil et al. 2005).

In this work, our results support the hypothesis that there are indeed common and specific signalings of the P nutrition and AM symbiosis processes. In roots, the expression of both miR158 and miR862-3p decreased under Pi starvation, and the expression level of miR158 was even lower under AM colonization. This indicated that miR158 is likely to be involved in the signaling of both normal P nutrition and AM mediated Pi uptake processes, whereas miR862-3p is expected to display its function under non-mycorrhizal conditions. In addition, the expression of miR319 and miR394 was only detectable under Pi starved condition. One may postulate that increased Pi derived from either the direct Pi uptake pathway or from the mycorrhizal uptake pathway suppressed the gene expression. However, it should be noted that when mycorrhizal fungi dominate Pi supply to plants, there is a functional loss of direct Pi uptake pathway in roots colonized by AM irrespective of growth responses (Smith et al. 2003). In addition, the improved P nutrition status was limited in comparison to high P supply in the tomato (Fig. 1). Therefore, we predict that miR319 and miR394 in roots (Fig. 4A) might be involved in AM symbiosis signaling Pi nutrition in an independent manner in tomato or respond to Pi in a dosage-dependent manner. It might be possible that an improvement of Pi nutrition as much as that offered by mycorrhizal fungi dominate Pi uptake pathway is sufficient to suppress their expression.

In leaves, up to 14 miRNAs were identified and found differentially regulated. They were grouped into three subclasses based on the similarity of their responses to our treatments. The first group consisted of miR172, miR771, miR169g*, miR158, miR319, miR172b* and miR775. These miRNAs in leaves were up-regulated by either sufficient level of Pi or AM symbiosis in general (Fig. 5). As miR319 and miR394 were commonly down-regulated by either sufficient level of Pi or AM symbiosis in roots (Fig. 4), we suggest that the miRNAs of the first group in leaves might also participate in AM colonization signaling independent of the Pi level or respond to Pi in a dosage-dependent manner. Interestingly, miR319 which might target a transcription factor (Table 1) showed opposite expression patterns in response to Pi nutrition in leaves and in roots (Fig. 4B and 5).

The miRNAs deposited in the second group, miR395, miR779.1, miR840 and miR867, had a comparable expression level under Pi sufficient and deficient conditions, and were specifically up-regulated by AM symbiosis, suggesting that an AMF derived signal might be responsible for their up-regulation. Of these miRNAs, miR395 was responsible for sulfate assimilation and translocation of plants (Kawashima et al. 2009). In the past decades, increasing evidences have revealed that AM fungi were able to help the host plants with acquisition of other mineral nutrients besides P, such as nitrogen (N) (Govindarajulu et al. 2005, Jin et al. 2005, Ortiz-Ceballos et al. 2007, Perner et al. 2008). More recently, AM fungi were reported to play a role in sulfur (S) transport to plants (Allen and Shachar-Hill 2009). Sulfate taken up by the fungus was transferred to mycorrhizal roots, which increased the root S contents under S supply at moderate levels (Allen and Shachar-Hill 2009). Therefore, to explore the potential role of miR395 for plant uptake of S in the mycorrhizal uptake pathway is also of interest. MiR840 was predicted to target water-stress induced tonoplast intrinsic protein (TIP) and a plasma membrane intrinsic protein (PIP)-type aquaporin (Table 1). As the AMF also supply their host plants with water in addition to nutrients (Parniske 2008), one can speculate that miR840 may be involved in the water transport between plants and AMF.

The expression of the members in the last group was either strongly increased (miR399 and miR398) or strongly decreased (miR837-3p) upon Pi starvation. MiR399 have been well characterized as reported previously (Aung et al. 2006, Bari et al. 2006, Chiou et al. 2006, Lin et al. 2008), and we have confirmed its conserved expression pattern in tomato (Fig. 4B and 7). Moreover, we observed that miR837-3p was completely suppressed by AMF inoculation in the leaves (Fig. 7), which prompts us to explore the potential role of miR837-3p played in the AM signaling.

MiR398 is another well-characterized miRNA induced by copper deficiency (Yamashita et al. 2007, 2009). It has been reported to target two Cu/Zn superoxide dismutases, CSD1 (localized to cytoplasm) and CSD2 (localized to chloroplast), and be involved in a broad range of plant responses to diverse biotic and abiotic stresses, such as oxidative stress, copper deficiency and addition of sucrose, paraquat, ozone or plant pathogen (Dugas and Bartel 2008, Jagadeeswaran et al. 2009, Sunkar et al. 2006, Yamashita et al. 2007, 2009).

Very recently, one of the three members of the Arabidopsis miR398 family, ath-miR398a, was found to be strongly down-regulated in response to Pi starvation (Pant et al. 2009), which is contrary to our findings in tomato. However, Jia et al. reported that miR398 responded completely opposite or differentially to ABA and NaCl stress in poplars (Populus tremula) and Arabidopsis, respectively (Jia et al. 2009). This highlights the possibility that evolutionarily conserved miRNAs...
might respond differently to a certain abiotic stress and thus play distinct roles in diverse species. In order to further confirm this possibility, we tested the abundance of the two members of miR398 family in rice (*O. sativa*), Osa-miR398a and Osa-miR398b. Osa-miR398a was dramatically induced by Pi starvation (Figure S1), which is consistent with that we found in tomato, whereas Osa-miR398b tended to be constitutively expressed (Figure S1). Furthermore, a genomic fragment of 2000 bp upstream in relation to each of the OsmiRNA398s precursor sequence together with that of AtmiRNA398s was retrieved from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), respectively, and then set to PLACE (a database of Plant cis-acting Regulatory DNA Elements, http://www.dna.affrc.go.jp/PLACE/) for the Pi starvation related cis-regulatory element analysis. W Box (Devaiah et al. 2007) motif is commonly distributed in the promoter regions surveyed (in OsmiRNA398a, OsmiRNA398b and AtmiRNA398a) (Table S2). Whereas most of these miRNAs are not up-regulated by Pi starvation, indicating W Box might not be functional in these genes. The PHR1 binding site, P1BS, (Rubio et al. 2001) is found in the 5' upstream region of both OsmiRNA398a and AtmiRNA398c. We suggest that the up-regulation of OsmiRNA398c in this study might be mediated by the P1BS motif, although whether P1BS leads to the up-regulation of AtmiRNA398c is unknown. Moreover, another potential Pi starvation-responsive cis-element identified before, PHO, (Hammond et al. 2003, Mukatira et al. 2001) is only presented in the 5’ upstream of AtmiRNA398c. Whether AtmiRNA398c responding to Pi stress through PHO also needs to be investigated.

In summary, in the present study, we used the miRNA candidates acquired by preliminary screening of the microarray data for further expression analysis. A subset of miRNAs in tomato was identified and found differentially regulated by either P nutrition or AMF colonization or both, confirming the hypothesis that miRNAs may play important roles in the complex signal transduction networks of P nutrition and/or AM derived signaling. In addition, it should be noted that there is a possibility that some early responding and/or novel miRNAs’ expression are not detected by the microarray analysis. The future work, therefore, will be focused on the identification of some early responding and/or novel miRNAs, if any, under our treatments, as well as the elucidation of the correlations between the miRNAs and their target genes and the functional characterization of the targets.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1:** The PCR primers used in this study.

**Table S2:** Sequences related to the Pi starvation-responsive motifs found at the upstream region of AtMiR398s and OsMiR398s.

**Figure S1:** miR398s in response to Pi starvation in *O. sativa*.

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