Expression Pattern Suggests a Role of MiR399 in the Regulation of the Cellular Response to Local Pi Increase During Arbuscular Mycorrhizal Symbiosis

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Many plants improve their phosphate (Pi) availability by forming mutualistic associations with arbuscular mycorrhizal (AM) fungi. Pi-repleted plants are much less colonized by AM fungi than Pi-depleted plants. This indicates a link between plant Pi signaling and AM development. MicroRNAs (miR) of the 399 family are systemic Pi-starvation signals important for maintenance of Pi homeostasis in *Arabidopsis thaliana* and might also qualify as signals regulating AM development in response to Pi availability. MiR399 could either represent the systemic low-Pi signal promoting or required for AM formation or they could act as counter players of systemic Pi-availability signals that suppress AM symbiosis. To test either of these assumptions, we analyzed the miR399 family in the AM-capable plant model *Medicago truncatula* and could experimentally confirm 10 novel MIR399 genes in this species. Pi-depleted plants showed increased expression of mature miR399 and multiple pri-miR399, and unexpectedly, levels of five of the 15 pri-miR399 species were higher in leaves of mycorrhizal plants than in leaves of nonmycorrhizal plants. Compared with nonmycorrhizal Pi-depleted roots, mycorrhizal roots of Pi-depleted *M. truncatula* and tobacco plants had increased Pi contents due to symbiotic Pi uptake but displayed higher mature miR399 levels. Expression levels of *MtPho2* remained low and PHO2-dependent Pi-stress marker transcript levels remained high in these mycorrhizal roots. Hence, an AM symbiosis-related signal appears to increase miR399 expression and decrease PHO2 activity. MiR399 overexpression in tobacco suggested that miR399 alone is not sufficient to improve mycorrhizal colonization supporting the assumption that, in mycorrhizal roots, increased miR399 are necessary to keep the *MtPho2* expression and activity low, which would otherwise increase in response to symbiotic Pi uptake.

Phosphorus (P) is second to nitrogen as the most-limiting element for plant growth. P not only is a major component of fundamental macromolecules, it also plays an important role in

energy transfer, regulating enzymatic reactions, and different metabolic pathways. P is taken up by plant roots as inorganic phosphate (Pi), which is one of the least plant-available nutrients in the soil. To maintain optimal yield, a majority of agricultural systems depend on the application of P fertilizers. Their energy-intensive production depends on nonrenewable resources, which could be depleted by as early as 2060 (Vance et al. 2003).

Owing to the poor soil availability of Pi, plants have evolved a variety of adaptive strategies to improve Pi acquisition. These include altered root morphology, exudation of organic acids, phosphatases, and nucleases in order to solubilize inorganically and organically bound Pi and the establishment of a symbiotic relationship with arbuscular mycorrhizal (AM) fungi (Harrison 1999; Hause and Fester 2005; Parniske 2008). AM fungi are present in nearly all terrestrial ecosystems, forming mutualistic symbiosis with the roots of approximately 80% of vascular plants. They often improve Pi uptake and plant growth. The ability of AM fungal hyphae to grow beyond the root's Pi-depletion zone and deliver Pi to the host plant is thought to be the main basis for their positive effects on Pi uptake (Smith and Read 1997). The establishment of an AM symbiosis requires a complex developmental program of both plant and AM fungus. During the last years, plant mutants impaired at different steps of the AM colonization process have been identified and revealed insight into the plant developmental program and cell reprogramming during AM symbiosis (Parniske 2008). Additional insight in the analysis of molecular events during AM symbiosis has been achieved recently by the identification of plant Pi transporters that are either specifically induced in response to AM symbiosis (Glassop et al. 2005; Harrison et al. 2002; Nagy et al. 2005; Paszkowski et al. 2002) or strongly upregulated in mycorrhizal roots but having a basal expression in nonmycorrhizal roots (Chen et al. 2007; Maeda et al. 2006; Rausch et al. 2001). This induction of specific Pi transporters in mycorrhizal roots and the parallel downregulation of conventional Pi-uptake systems suggests that, in mycorrhizal roots, two Pi-uptake pathways are present: the direct Pi-uptake pathway involving Pi transporters expressed in the epidermis and in root hairs, and a mycorrhizal uptake pathway that involves the uptake of Pi from the soil by AM fungal transporters located in extraradical fungal hyphae and a subsequent transport to the intraradical hyphae in which it is provided to the plant (Bucher 2007). Interestingly, components of the direct Pi-uptake pathway are often downregulated in response

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to AM symbiosis leading to a reorganization of Pi uptake (Bucher 2007; Smith et al. 2003). This is intriguing because other P-dependent regulatory mechanisms seem to operate in the other direction, i.e., a high Pi status of the plant restricts AM symbiosis development. Recently, it was reported that lyso-phosphatidylcholine (LPC) acts as a mycorrhizal signal by inducing the mycorrhiza-specific *StPt3* promoter in potato roots (Drissner et al. 2007). However, a high-Pi signal is epistatic to this LPC signal, making Pi-repleted roots LPC insensitive, and the expression of *StPt3* and mycorrhizal Pi uptake is repressed in Pi-repleted roots (Nagy et al. 2008).

Knowledge about how plants are able to transfer Pi-starvation signals and maintain Pi homeostasis has been obtained recently by identifying components of Pi-starvation signaling in *Arabidopsis* (Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Franco-Zorrilla et al. 2007; Pant et al. 2008). The MYB transcription factor PHR1 plays a central role in signaling of primary Pi responses (Rubio et al. 2001). This is emphasized by the fact that approximately 90% of a set of 64 Pi starvation-inducible transcripts show attenuated induction in *Arabidopsis phr1* mutants (Bari et al. 2006), demonstrating the central importance of PHR1 in the Pi-starvation response. PHR1

binds to a *cis* element with an imperfect palindromic sequence (GNATATNC) (Rubio et al. 2001) and induces the expression of many Pi starvation-inducible genes, including microRNAs (miR) of the 399 family (Bari et al. 2006). Members of this miR family are able to bind and cleave the *PHO2* transcript (Allen et al. 2005) or to cause translational repression of *PHO2* (Bari et al. 2006; Chiou et al. 2006; Pant et al. 2009). The ubiquitin-conjugating enzyme *PHO2* negatively regulates the expression of a subset of Pi starvation-inducible genes, including several *Phl1* family Pi transporter genes (Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006). Hence, miR399 can be regarded as Pi-starvation signals leading to *PHO2* suppression and thus derepressing the Pi-starvation response and Pi-uptake capacity. MiR399 activity can be quenched by noncoding RNA *IPS1* through a novel mechanism termed target mimicry (Franco-Zorrilla et al. 2007). MiR399 also serve as phloem-mobile long-distance signals communicating Pi status between shoots and roots (Lin et al. 2008; Pant et al. 2008).

An important aim of this work was to investigate a potential role of miR399 as regulators in the establishment of AM symbiosis that occurs in Pi-depleted AM-capable plants, such as *Medicago truncatula* or tobacco (*Nicotiana tabacum*). For this purpose, miR399-overexpressing tobacco plants were characterized concerning their mycorrhizal phenotype under low- and high-Pi conditions. In addition, we characterized the miR399 family in the model legume *M. truncatula*. Analysis of miR399 primary transcripts and mature miR399 levels suggest the existence of a mycorrhiza-induced signal leading to increased miR399 transcription in leaves and to increased levels of mature miR399 in mycorrhizal roots.

RESULTS

AM symbiosis development is suppressed by Pi in *Medicago truncatula*.

Since miR399 represent a systemic Pi-starvation signal, it can be speculated that miR399 is the systemic signal leading to enhanced AM formation in Pi-depleted plants. In order to study the regulation of miR399 during AM symbiosis, *M. truncatula* plants were grown with or without *Glomus intraradices* inoculation and were fertilized with either 20 μ M or 1 mM Pi. These two Pi concentrations were selected after an initial experiment in which *M. truncatula* plants were fertilized with 0, 0.02, 1, or 2 mM Pi and were grown for 5 weeks. After 5 weeks, Pi concentrations in leaves did not differ significantly between the 0-mM and the 20- μ M treatments or between the 1- and 2-mM treatments (data not shown). Hence, we selected 20 μ M and 1 mM as contrasting Pi fertilization treatments in our experiment. All experiments were carried out using at least five independent replicates. All plants were harvested after 5 weeks. The resulting phenotype of the plants is shown in Supplementary Figure S1. Pi contents of roots and shoots were measured. Plants that were fertilized with 1 mM Pi showed clearly increased Pi concentrations in both roots and shoots (Fig. 1). Mycorrhizal plants at low-Pi conditions had increased root Pi concentrations. This is most likely a consequence of

Fig. 1. Soluble phosphate (Pi) concentrations in Pi-repleted (1 mM) and Pi-depleted (20 μ M) leaves and roots of mycorrhizal (MYC) and nonmycorrhizal (NM) *Medicago truncatula* plants. Data represent the mean $\pm$ standard deviation of five independent replicates containing two plants each. Values labeled with the same letter are not significantly different.

Table 1. Mycorrhizal parameters of *Medicago truncatula* roots^a

Myc	Pi condition	F (%)	M (%) $\pm$ SD	m (%) $\pm$ SD	A (%) $\pm$ SD	a (%) $\pm$ SD
–	1 mM or 20 μ M Pi	0	0	0	0	0
+	1 mM Pi	100	57.59 $\pm$ 7.95	57.59 $\pm$ 7.95	42.98 $\pm$ 7.54	75.30 $\pm$ 14.8
+	20 μ M Pi	100	76.49 $\pm$ 5.78	76.60 $\pm$ 5.82	69.92 $\pm$ 6.77	87.34 $\pm$ 2.42

^a Estimation of mycorrhizal parameters was carried out according to Trouvelot and associates (1986). Mean values and standard deviations of five biological replicates of each treatment are shown. Myc = inoculation with *Glomus intraradices*, Pi = phosphate, F = frequency of colonization, M = colonization intensity of the whole root system, SD = standard deviation, m = colonization intensity within colonized root areas, A = arbuscule frequency within the whole roots system, and a = arbuscule frequency within arbuscule-containing root areas.

increased cellular Pi due to symbiotic Pi transfer from fungus to the plant, since *MtPID* as a PHO2-independent Pi stress marker shows decreased expression at low Pi in mycorrhizal roots as compared with nonmycorrhizal roots (discussed below). Pi concentration in shoots was higher in nonmycorrhizal plants than in mycorrhizal plants, probably an effect of increased shoot growth of mycorrhizal plants receiving the 20- μ M Pi treatment. Staining root samples of all four treatments revealed no mycorrhizal structures in the noninoculated samples, whereas inoculated roots displayed typical structures of a *G. intraradices*–*M. truncatula* AM symbiosis, namely intercellular hyphae, arbuscules, and vesicles. Comparison of mycorrhizal parameters between the inoculated roots of the two Pi treatments revealed that fertilization with 1 mM Pi led to a 20% decrease in all parameters investigated, except for F (overall colonization frequency), which was 100% in both cases (Table 1). Hence, the high Pi conditions used in this experiment mainly suppressed arbuscule frequency and colonization intensity. In parallel, we quantified AM symbiosis by measuring three diagnostic markers

for arbuscules and fungal colonization: i) *MtPt4*, a mycorrhiza-specific Pi transporter (Javot et al. 2007), ii) *MtGst1*, a glutathione-S transferase-like mycorrhiza-specific gene (Wulf et al. 2003). and iii) ribosomal RNA of *Glomus intraradices* (Isayenkov et al. 2004). Using quantitative real-time polymerase chain reaction (qRT-PCR), transcripts of the two mycorrhiza-specific *M. truncatula* genes were not detected in samples of nonmycorrhizal roots. All three markers were highly expressed in mycorrhizal roots of both Pi treatments, with significantly lower expression in roots fertilized with 1 mM Pi (Fig. 2). In accordance with data of the mycorrhiza estimation after root staining (described above), this indicates that the 1 mM Pi treatment suppresses AM development.

Characterization of the plant Pi status using diagnostic markers for Pi stress.

As mentioned above, AM symbiosis led to increased Pi concentration in roots of Pi-depleted plants. To get further information about the Pi status of the plants used in this study, we

Fig. 2. Relative expression levels of mycorrhiza marker genes of *Medicago truncatula* (*MtPt4*, *MtGst1*) and *Glomus intraradices* rRNA, determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Phosphate (Pi)-depleted (–P) and Pi-repleted (+P) mycorrhizal (MYC) and nonmycorrhizal (NM) plants were harvested at 5 weeks postinoculation. Relative expression levels of target genes were determined by qRT-PCR using *MtEfl* as reference gene. Values shown are mean $\pm$ standard deviations of five biological replicates and three technical repetitions of each qRT-PCR reaction.

Fig. 3. Relative expression levels of phosphate (Pi) signaling and Pi-stress markers in mycorrhizal and nonmycorrhizal roots of Pi-repleted and Pi-depleted plants. Pi-depleted (–P) and Pi-repleted (+P) mycorrhizal (MYC) and nonmycorrhizal (NM) plants were harvested at 5 weeks postinoculation. Relative expression levels of target genes were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using *MtEfl* as reference gene. Values shown are mean $\pm$ standard deviations of five biological replicates and three technical repetitions of each qRT-PCR reaction.

quantified transcript levels of marker genes for Pi stress and homeostasis by qRT-PCR using roots of mycorrhizal and non-mycorrhizal plants of both Pi treatments. The markers include *MtPho2*, *MtPhr1*, and *Mt4* involved in Pi homeostasis, as well as three Pi-stress markers, i.e., *MtPID*, *MtSpx*, and *MtUn* (Hohnjec et al. 2005). The orthologs of *MtSpx* and *MtUn* have been shown to be PHO2-dependent in *Arabidopsis*, whereas the potential *MtPID* ortholog in *Arabidopsis* is PHO2-independent (Bari et al. 2006). In agreement with previous reports, *MtPhr1* transcripts were not significantly dependent on Pi (Muller et al. 2007), whereas *MtPho2* transcript levels decreased in Pi-depleted plants (Fig. 3). Remarkably, *MtPho2* transcript levels did not differ significantly between mycorrhizal and nonmycorrhizal plants. The three Pi-stress markers *MtPID*, *MtSpx*, and *MtUn* are strongly induced under Pi starvation. Notably, *Mt4* transcripts were detected also under 1-mM Pi conditions, which is in contrast to previous reports that *Mt4* was not detectable by Northern blot under Pi-sufficient conditions (Burleigh and Harrison 1997), indicating that the here-applied 1-mM Pi treatment could still represent mild Pi starvation for plants. The locally enhanced Pi in mycorrhizal roots of Pi-depleted plants led to some repression of *MtPID* as compared with nonmycorrhizal roots, which is most probably a response to the locally increased Pi in mycorrhizal roots. The transcript levels of two other MtPHO2-dependent Pi-stress markers, *MtSpx* and *MtUn*, were not changed in response to mycorrhizal symbiosis in Pi-depleted plants, reflecting the unaltered *MtPho2* levels. In summary, although AM symbiosis led to elevated Pi in roots (Fig. 1), this did not affect *MtPho2* transcript levels. In conformity with this finding, expression of MtPHO2-dependent Pi-stress markers was also unaltered in mycorrhizal roots.

Fig. 4. Validation of miR399-mediated *MtPho2* transcript cleavage. **A**, modified RNA-ligase mediated-rapid amplification of cDNA ends (RLM-RACE) using cDNA of mycorrhizal roots revealed one single polymerase chain reaction (PCR) product **A**, M = DNA size marker, lane 1 = amplification product after RLM-RACE. **B**, Sequencing of five cloned PCR products confirmed a predicted miR399 target site and transcript cleavage at position 611/612 of the *MtPho2* cDNA in each of the five PCR products. Bold letters indicate conserved nucleotides within the *Medicago truncatula* mature miR399 sequences.

Identification of novel *MIR399* genes in *M. truncatula*.

For a comprehensive analysis of the miR399 family with regard to AM symbiosis, we aimed for a more thorough identification and characterization of *MIR399* genes in the model legume *Medicago truncatula*. We developed a miRNA prediction pipeline based on homology searches in the *M. truncatula* genome coupled to folding analysis of predicted RNA molecules. Based on the predicted free energy on secondary structures for known plant pre-miRNAs from miRBase, a cut-off of -28 kcal/mol was introduced to distinguish true miRNAs from false positives. Sequences passing this requirement were trimmed back to the base of the outmost predicted stem. Trimmed sequences had to be at least 60 nucleotides in length, with a predicted structure containing a stem of at least 20 base-pairs overlapping the region of similarity to the mature query sequence. Predicted miRNA precursor structures that contained more than three stem-loop branches were discarded. Applying this pipeline to mature plant miR399 sequences from miRBase Registry (release 13) resulted in the identification of 10 additional putative *MIR399* genes in the *M. truncatula* genome. As expected, these *MIR399* genes reside in so-far anonymous intergenic regions. Candidate miR399 sequences resulting from the prediction were manually compared with miR399 sequences deposited at the novel miRBase release 14 and were annotated. All of our predicted miR399 sequences appeared to be present in miRBase release 14 but without experimental confirmation. In order to verify our 10 in silico-predicted as well as the five previously (miRBase release 13) annotated *miR399* transcripts, we designed specific primers for each of these 15 pri-miR399. qRT-PCR analysis using cDNA of roots of Pi-depleted plants revealed specific amplification products for each of the primer pairs tested (data not shown). Supplementary Table S1 shows an overview of all our 15 experimentally confirmed *MIR399* genes from *M. truncatula*.

Confirmation of *MtPho2* as a miR399 target in *M. truncatula*.

In order to confirm that *MtPho2* represents a target for miR399 in *M. truncatula* as it is described for *A. thaliana*, we carried out a modified rapid amplification of cDNA ends (RACE) experiment. The 5' end of the *MtPho2* cDNA was amplified from mycorrhizal roots grown under Pi-depleted conditions using a gene-specific primer, and one single band was obtained (Fig. 4). Cloning and sequencing of the PCR product revealed a miR399 binding and transcript cleavage site at position 611/612 of the *MtPho2* cDNA sequence.

In order to investigate if miR399 might have targets other than *Pho2* in *M. truncatula*, we used two target-prediction tools, the UEA Plant Small RNA Toolkit (Moxon et al. 2008) and the MiRU: Plant microRNA Potential Target Finder (Zhang 2005) (Supplementary Table S2). Both prediction tools confirmed *MtPho2* as putative target after searching the DFCI (Dana Farber Cancer Institute) *Medicago truncatula* Gene Index 9.0 for miR399 targets. A second sequence (BG444416), a singleton with similarities to the *MtPho2* 5' untranslated region, was predicted to be a miR399 target from both prediction tools. A total of 10 other putative target sequences were predicted but none of them by both prediction tools.

Mature miR399 accumulate to higher levels in mycorrhizal roots as compared with nonmycorrhizal roots under low Pi fertilization.

We next wanted to determine whether pri-miR399 and mature miR399 levels are correlated with Pi and AM symbiosis status in *M. truncatula*. Pri-miR399 were quantified in leaves and roots of mycorrhizal and nonmycorrhizal plants of both 1-mM and 20- μ M Pi fertilization treatments (Fig. 5). Most pri-

MiR399 were only very weakly detectable in Pi-repleted plants. Highest pri-miR levels were measured in leaves of Pi-depleted plants. Interestingly, five pri-MiR399 (miR399b, c, d, m, and o) were significantly higher in leaves of mycorrhizal Pi-depleted plants as compared with nonmycorrhizal plants. In roots, pri-miRNA399 levels were generally lower than in leaves, supporting the belief that leaves are the major site for miR399 transcription (Bari et al.; 2006; Pant et al.; 2008). Un-

der low-Pi conditions, AM symbiosis in most cases led to decreased miR399 transcript levels mirroring the improved local Pi availability.

To investigate the abundance of mature miR399 molecules, we applied a stem-loop primer-based qRT-PCR. Sequence variation within the 15 mature miR399 sequences (Fig. 6) allowed us to design different stem-loop RT primers in order to distinguish four groups of mature MiR399 by PCR (miR399_GC,

Fig. 5. Relative expression levels of 15 miR399 primary transcripts in roots and leaves of mycorrhizal and nonmycorrhizal phosphate (Pi)-depleted or Pi-repleted plants. Pi-depleted (-P) and -repleted (+P) mycorrhizal (MYC) and nonmycorrhizal (NM) plants were harvested at 5 weeks postinoculation. Relative levels of miR399 primary transcripts were determined by quantitative reverse transcription-polymerase chain reaction using *MtEfl* as reference gene. Values are mean $\pm$ standard deviations of five biological replicates and three technical repetitions.

miR399_TT, miR399_TT/j, and miR399_GT/p). RNA of each tissue was reverse-transcribed using the appropriate RT primer. In parallel, identical amounts of RNA were used for a standard RT reaction, to allow normalization of the mature miR399 levels against reference transcripts. The expression levels of mature miR399 subfamilies were calculated as relative expression levels compared with *MtEfl* as reference (Fig. 7). In Pi-repleted plants, mature miR399 were very low and did not differ significantly between shoots and roots. In contrast, all four types of mature miR399 could be detected at high levels in leaves and roots of Pi-depleted plants. Surprisingly, mycorrhizal roots displayed higher mature miR399 levels of all four subfamilies measured. We measured a statistically significant (two-way

Fig. 6. Sequences of mature miR399s grouped in families according to stem-loop reverse transcription (RT) primers used for mature miR399 quantification by quantitative RT-polymerase chain reaction. Conserved nucleotides are marked in gray. The 15 mature miR399 sequences displayed three different nucleotide combinations, namely TT, GC, and GU at positions 14 and 15. Since miR399j has a different nucleotide at position 1 than other members of the miR399_TT family, it could be specifically quantified using an individual RT primer (miR399_TT/j).

analysis of variance, $P = 0.001$) increased miR399j level in mycorrhizal roots as compared with nonmycorrhizal roots; for the three other miR399 subfamilies, no significant changes were observed in mycorrhizal roots. Hence, although AM symbiosis leads to locally improved Pi availability, the accumulation of mature miR399 is not decreased. In contrast, we observed a significant increase in mature miR399j. This and the expression pattern of *MtPho2* and the Pi-stress markers led us to assume that miR399 might be involved in suppressing a PHO2 increase in mycorrhizal roots.

Fig. 7. Relative expression levels of four classes of mature miR399 in roots and leaves. Phosphate (Pi)-depleted (-P) and -repleted (+P) mycorrhizal (MYC) and nonmycorrhizal (NM) plants were harvested at 5 weeks postinoculation. Levels of mature miR399 of the subfamilies miR399_TT, miR399_GC, miR399_GT, and miR399_j were determined, and relative miR399 abundance was measured by quantitative reverse transcription (RT)-polymerase chain reaction after cDNA synthesis using specific stem-loop (RT) primers for each miR399 subfamily. The values shown are mean $\pm$ standard deviation of five biological replicates and three technical repetitions.

Fig. 8. Reciprocal grafting of miR399-overexpressing (OX) and wild-type (WT) tobacco plants. **A**, Aspect of a 62-day-old grafted tobacco plant. **B**, Phosphate (Pi) contents in leaves of 8-week-old, hydroponically grown WT (black bars), OX (white bars), and reciprocally grafted chimeras (hatched bars). Plants were fertilized with 1.5 mM Pi. Root (R) and shoot (S) genotypes of the chimeric plants are depicted below the graph. Data are mean values $\pm$ standard deviation of five replicates.

Increased miR399 levels in mycorrhizal tobacco roots.

To test the hypothesis that MiR399 are regulators of Pi homeostasis during AM symbiosis and to investigate the influence of a constant Pi-starvation signal on AM symbiosis, we transformed tobacco plants with a construct leading to overexpression of miR399 (ath-miR399d) (Bari et al. 2006). Here, we used tobacco as an AM-capable plant model that can be easily transformed. In *Arabidopsis*, overexpression of miR399d leads to Pi accumulation in shoots under P-replete conditions and, thus, a *pho2* phenocopy (Bari et al. 2006; Chiou et al. 2006). To see whether the ath-miR399d is biologically active in tobacco, we performed reciprocal grafting experiments with wild type (WT) and miR399-overexpressing (OX) tobacco plants (Fig. 8). High levels of Pi were found in shoots of miR399-OX plants. Moreover, grafting a WT shoot on OX roots or grafting a miR399-OX shoot on WT roots also led to Pi overaccumulation in shoots, demonstrating that i) miR399 overexpression in roots is sufficient to induce Pi accumulation in shoots and ii) that miR399 also is a long-distance signal in tobacco. Thus the observed phenotype is identical to the observed *pho2* or miR399-OX phenotype in *Arabidopsis* (Bari et al. 2006; Lin et al. 2008; Pant et al. 2008), showing that ath-miR399d is biologically active in tobacco. Thus, miR399-OX tobacco plants display a constant high level of miR399 as systemic Pi-starvation signal and represent a suitable tool to analyze this miR399-born Pi-starvation signal concerning its involvement in the correlation between Pi status and AM-symbiosis development.

To analyze the effect of miR399 overexpression on AM symbiosis, plants were inoculated with *G. intraradices*, and roots were harvested 6 weeks after inoculation. To confirm the miR399 OX, we measured the mature miR399d expression by stem-loop PCR. Overexpression was confirmed for all transgenic plants (Supplementary Fig. S2). Apparently, the applied PCR conditions and primers were also able to detect endogenous tobacco mature miR399 expression. We observed a much stronger accumulation of mature miR399 in mycorrhizal Pi-depleted WT roots as compared with nonmycorrhizal roots (Fig. 9), although soluble Pi contents of the leaves did not differ significantly (data not shown). This finding is consistent with our results in *M. truncatula*, in which strongest accumulation of mature miR399j was also detected in mycorrhizal roots under Pi-deplete conditions.

MiR399 overexpression does not influence mycorrhizal colonization in tobacco.

We next investigated whether miR399 overexpression leads to a phenotype with regard to root colonization by *G. intraradices*. A recent characterization of tobacco Pi-transporter genes revealed the identification of NtPt3 (Pht1;3) as AM-upregulated as well as two transporters, NtPt4 and NtPt5 (Pht1;4 and Pht1;5, respectively), as AM-specifically expressed (Chen et al. 2007). We used the expression of these AM-regulated transporters as markers for an established AM symbiosis in the tobacco roots (Fig. 10). In contrast to the non-AM regulated transporter NtPt1, all three mycorrhiza-regulated Pi transporters were strongly increased in mycorrhizal roots of Pi-depleted plants. Expression levels in mycorrhizal roots of Pi-repleted plants were lower, mirroring the lower fungal colonization rate. This molecular phenotype did not differ between WT and miR399-OX plants. We next investigated the visual phenotype of miR399-OX plants with regard to root colonization by *G. intraradices*. To exclude local effects of Pi fertilization on the AM fungus, we used split-root systems. This experimental setup allowed the analysis of the systemic effect of Pi fertilization in one compartment on mycorrhizal colonization in the second compartment. Three weeks after inoculation with

G. intraradices, plants were harvested and roots of the inoculated compartment were stained for mycorrhizal structures. Evaluation of colonization intensity following methods described by Trouvelot and associates (1986) revealed a clear repression of colonization intensity in WT and in miR399-OX plants in Pi-repleted plants. Fertilization with 1 mM Pi decreased overall colonization intensity (F) by 50% as compared with plants fertilized with 20 μ M Pi (data not shown). Arbuscule frequency was decreased more than 100-fold in Pi-repleted plants in both miR399-OX and WT plants. Hence, we could not observe an effect of miR399 per se with respect to establishment of AM-symbiosis in tobacco.

DISCUSSION

The aim of this work was to determine the correlation between miR399 expression, Pi homeostasis, and AM development in plants. A negative correlation between Pi status and development of AM symbiosis has been described in different plant-AM fungus combinations (Menge et al. 1978; Nagy et al. 2008; Sanders 1975). This is consistent with this study using *M. truncatula* or tobacco plants cultivated in split-root systems. In the latter case, fertilization with high Pi in one root compartment was able to suppress the AM symbiosis in the second root compartment. This indicates that either a systemic Pi availability signal suppresses AM symbioses or that a systemic Pi-starvation signal promoting symbiosis development is lacking. Evidence for a high Pi signal that is able to suppress AM symbiosis is provided by the fact that Pi-repleted roots become insensitive to the mycorrhizal signal LPC and the induction of mycorrhiza-inducible Pi transporters is repressed (Nagy et al. 2008). However, the Pi signal leading to suppression of the LPC signal is unknown.

MiR399 has been shown to act as a systemic Pi-starvation signal (Lin et al. 2008; Pant et al. 2008). Hence, one might speculate that MiR399 represent a systemic low-Pi signal pro-

Fig. 9. Relative abundance of mature miR399 in roots of wild-type tobacco plants. Phosphate (Pi)-depleted (P-) and -repleted (P+) mycorrhizal (MYC) and nonmycorrhizal (NM) wild-type tobacco plants were harvested at 6 weeks postinoculation. Abundance of mature miR399d was measured by quantitative reverse transcription-polymerase chain reaction after cDNA synthesis using a miR399d-specific stem-loop (RT) primer. Three individual mycorrhizal plant were used for both Pi regimes (1 through 3). Two nonmycorrhizal plants of P+ and P- were pooled (1/2). The values shown are mean $\pm$ standard deviation from two technical repetitions. White bars represent expression under Pi-depleted conditions.

moting or required for AM formation. On the other hand, they also could act as counter players of systemic Pi availability signals that suppress AM symbiosis. To test each of these assumptions, we analyzed the miR399 family in the AM-capable plant

model *Medicago truncatula* and could experimentally confirm 10 novel *MIR399* genes in this species. Recently, a bioinformatic approach for miR prediction from expressed sequence tags and genome survey sequences suggested five novel mem-

Fig. 10. Relative expression levels of tobacco phosphate (Pi) transporter genes. Pi-depleted (P-; left panel) and -repleted (P+; right panel) mycorrhizal (MYC; gray bars) and nonmycorrhizal (NM; white bars) plants were harvested at 6 weeks postinoculation. The expression of *NtPt2* as non-arbuscular mycorrhizal (AM) regulated Pi transporter as well as the expression of *NtPt3*, *NtPt4*, and *NtPt5* as AM-regulated Pi transporters was measured. Individual plants were labeled by numbers. Two nonmycorrhizal plants of P+ and P- were pooled (NM 3/4). Relative expression levels of target genes were determined in roots by quantitative reverse transcription-polymerase chain reaction using actin as reference gene. Values shown are mean $\pm$ standard deviations of two technical repetitions.

bers of the miR399 family in *M. truncatula* (Zhou et al. 2008). Of this data set, only one predicted miR399 (named 399i by Zhou and associates [2008]) is overlapping with our experimentally confirmed miR399 genes (miR399 miRBASE 14). In addition, a deep-sequencing approach of small RNAs from *M. truncatula* root apices and nodules revealed 13 MIR399 genome loci, of which six precursor sequences are identical to our dataset (Lelandais-Briere et al. 2009). Hence, it is very likely that the miR399 family has even more members in *M. truncatula*. With 15 so-far confirmed members, the miR399 family currently represents the largest miRNA family in *M. truncatula*. This compares well to the number in rice and poplar, in which 11 and 12 members have been identified, respectively (Lindow et al. 2007). In *A. thaliana*, the miR399 family has only six members. Possibly, the low number of MiR399 in *A. thaliana* is related to the fact that *A. thaliana*, in contrast to *M. truncatula*, rice, and poplar, is not capable of enhancing its Pi uptake through symbiosis with AM fungi.

All 15 pri-miR399 members were strongly accumulating in Pi-depleted plants with higher transcript levels in leaves as compared with roots. This implies that leaves are the major sites of miR399 transcription. This was also observed in *A. thaliana* (Bari et al. 2006; Pant et al. 2008), in which the transcriptional induction of miR399 in leaves seems to be in response to a root-born systemic effect that has yet to be identified (Lin et al. 2008). In roots, we observed a decrease of pri-miR399 in mycorrhizal plants, implying that this is an effect of the AM-mediated improved local Pi availability. This is also in accordance with Lin and associates (2008), who postulated a

local root-born signal regulating pri-MiR399 in roots. Surprisingly, we found five pri-MiR399 further induced in leaves of Pi-depleted mycorrhizal plants. On the other hand, in the *M. truncatula*–*G. intraradices* system under Pi-deplete conditions, we observed slightly decreased soluble Pi contents in shoots of mycorrhizal plants, which is most likely an effect of increased shoot fresh weights (data not shown). The phenomenon that AM symbiosis can dominate a plant's Pi supply without increasing total soluble Pi concentration in shoots has been described earlier (Smith et al. 2003). Moreover, the soluble Pi content is just a rough indicator of the actual Pi status; a precise determination of the Pi status requires ashing of the material or FTICR (Fourier transform ion cyclotron resonance mass spectrometry) to also determine chemically bound and insoluble Pi. Hence, whether the small change of soluble shoot Pi in the observed range and concentration has a direct impact in miR399 expression remains to be evaluated. In addition, as mentioned earlier, transcription levels of MiR399 in leaves are at least partially dependent on root-born signals, and the Pi contents in mycorrhizal roots were higher than in nonmycorrhizal roots. Hence, although we currently cannot exclude the possibility that the observed decrease in soluble Pi content in leaves of mycorrhizal plants causes an induction of miR399 transcription, it seems to be unlikely.

Mature miR399 accumulation was higher in roots than in shoots, consistent with their role as signaling molecules transported from shoots to roots, in which they negatively regulate *PHO2* (Lin et al. 2008; Pant et al. 2008). We could experimentally confirm a miR399-mediated cleavage of the *MtPho2* tran-

Fig. 11. A hypothetical model for the role of miR399 during arbuscular mycorrhizal (AM) symbiosis. AM symbiosis leads to a local phosphate (Pi) increase in colonized roots. Hence, PHR1 will be downregulated and, consistently, PHR1-depending Pi-stress markers and pri-miR399 synthesis are repressed. In parallel, a hypothetical mycorrhizal signal leads to increased pri-miR399 synthesis in shoots, which leads to increased accumulation of mature miR399 in mycorrhizal roots after phloem transport. Here, miR399 suppress PHO2. This leads to unaltered levels of *Pho2* and PHO2-dependent Pi-stress markers in mycorrhizal roots, although Pi is locally increased and miR399 transcript levels are decreased. This suggests that, in mycorrhizal roots, increased miR399 levels keep *MtPho2* expression and activity low, which otherwise would increase in response to symbiotic Pi uptake. One possible reason for this phenomenon is that PHO2 or other PHO2-dependent high-Pi responses might be involved in the suppression of AM symbiosis in Pi-repleted plants. Thus, an induction of PHO2 due to a symbiotically provided Pi increase in colonized roots has to be prevented by miR399 action in order to sustain mycorrhizal symbiosis. Yet unknown pathways are indicated as dashed lines.

script at position 611/612 in mycorrhizal *M. truncatula* roots, indicating that the miR399-PHO2 regulation pathway is conserved in *M. truncatula* and active in mycorrhizal roots. Recently, four miR399-cleavage sites in *MtPho2* transcript were found in RNA of whole Pi-stressed seedlings (Jagadeeswaran et al. 2009). Hence, it might be anticipated that miR399-mediated *MtPho2* cleavage patterns are tissue specific.

Surprisingly, we found an increase of mature miR399 in mycorrhizal roots as compared with nonmycorrhizal roots in *M. truncatula* and tobacco. In addition, five pri-MiR399 showed increased transcription levels in leaves of mycorrhizal plants. Hence, the observed expression pattern of pri- and mature miR399 suggests an AM symbiosis-related signal that appears to increase miR399 transcription in leaves and consequently increased abundance of mature miR399 in mycorrhizal roots. The increased miR399 abundance in mycorrhizal roots is mirrored by the expression pattern of Pi-stress marker genes; *MtPho2* transcripts as miR399 targets remained low although the Pi content is increased in mycorrhizal roots and, consistently, PHO2-dependent Pi-stress markers do not also change mycorrhizal roots. In contrast, transcript levels of *MtPID*, which is PHO2-independent, decreased in mycorrhizal roots. This suggests that, in mycorrhizal roots, increased miR399 levels keep *MtPho2* expression and activity low, which otherwise would increase in response to symbiotic Pi uptake. Figure 11 shows a hypothetical model for the regulation and role of miR399 during mycorrhizal symbiosis.

To further strengthen our hypothesis that miR399 members are suppressing high Pi signals in mycorrhizal roots rather than being a Pi-starvation signal capable, per se, of improving AM colonization, we characterized the mycorrhizal phenotype of miR399-OX tobacco plants. We decided to use tobacco as the AM-capable plant model, because it is more suitable for transformation approaches than *M. truncatula*. Micrografting experiments confirmed a strong Pi overaccumulation in leaves of ath-miR399d-OX tobacco plants, demonstrating that the ath-miR399d is biologically active in tobacco and that these OX plants are a suitable model to study the influence of a constant Pi-starvation signal on the AM-symbiosis. Transcription levels of mycorrhiza-regulated Pi transporters (Chen et al. 2007) as well as estimation of mycorrhizal structures by light microscopy revealed the same repression of mycorrhizal symbiosis under Pi-replete conditions in miR399-OX and WT plants. This is consistent with our assumption that an internal miR399-based low Pi signal alone is not enough to enhance AM development in Pi-repleted plants. Moreover, constant high miR399 levels are not able to bypass AM suppression in Pi-repleted plants. Both conclusions are in accordance to the observation that a high Pi signal is epistatic to the mycorrhizal signal LPC (Nagy et al.). However, the corresponding high Pi signal remains to be identified and whether additional upstream-acting components of the Pi-signaling pathway are involved in this phenomenon remains to be answered. Recent studies in *Arabidopsis* revealed at least four additional miR with strong Pi status-dependent expression (Pant et al. 2009). This finding indicates that the regulation of Pi stress and homeostasis is remarkably widespread. Moreover, one novel miRNA (miR2111) was also detected with Pi status-dependent abundance in rape-seed (*Brassica napus*) phloem sap, indicating that it might be a new potential long-distance signal that should be tested for its involvement in AM symbiosis establishment.

MATERIALS AND METHODS

Prediction of miR399 genes of *Medicago truncatula*.

Plant miRNAs exhibit great variation in precursor length, and frequently, only the mature sequence is conserved between

related species (Rhoades et al. 2002). Therefore, mature miR399 sequences were used as queries to find corresponding potential mature miR399 sequences in the *M. truncatula* genome. The program SSEARCH (Pearson 2000) was used for this purpose, with an *e*-value cutoff of 0.5. Genomic hits to mature miRNA399 were extended by 400 nucleotides in both directions. The folding potential of these sequences was assessed using RNAfold (Zuker et al. 1991).

Plant material, inoculation, and growth conditions.

Medicago truncatula cv. Jemalong line A17 seeds were incubated with sulfuric acid, followed by repeated washings with distilled water. Afterwards, surface sterilization was carried out using 6% (vol/vol) sodium hypochlorite for 8 min. After rinsing with sterile water, seeds were germinated on water agar at 4°C for 48 h, followed by a 3-day incubation at room temperature. Seedlings were placed in sand and expanded clay (1:2). Inoculation with *Glomus intraradices* (BEG141) was carried out using 10% (vol/vol) inoculum (Biorize, Dijon, France).

All plants were grown in growth rooms under a 16 h light (photosynthetically active radiation, 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), 8-h dark (22°C), 60% relative humidity regime. Plants were fertilized twice a week with 25 ml of Hoagland's solution (Hoagland and Arnon 1950) containing either 20 μM or 1 mM KH_2PO_4 . Plants were harvested 5 weeks after inoculation. For each experiment, five biological replicates containing two plants were harvested. Samples of the roots were stained with trypan blue in order to estimate colonization intensity according to Trouvelot and associates (1986).

Soluble Pi measurement.

Pi levels were measured using a colorimetric method (Ames 1966). Tissue was ground in liquid nitrogen to a fine powder, and 20 mg of tissue powder were incubated in 200 μl of 25 mM NaOH. Samples were incubated at 95°C for 30 s, and 200 μl of 25 mM HCl and 100 μl of Tween-20 were added. Samples were mixed and centrifuged for 4 min at 9,000 rpm. Supernatant (3 to 15 μl) was mixed with 15 μl of water, 100 μl of HCL, and 100 μl of color reagent (1 volume of 4.2% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$ in 5 N HCl, three volumes of malachite green dye in water). The reaction was incubated at room temperature for 15 min before 100 μl of 1.5% Tween-20 were added. The absorbance at 660 nm was measured after a second incubation of 15 min. The Pi concentration in the samples was determined against a calibration curve.

Generation of ath-miR399d overexpressing tobacco plants.

The construct for constitutively overexpressing the ath-miR399d was created by cloning a 289-bp fragment containing ath-miR399d precursor sequence into CaMV:35S promoter containing vector pMDC32 (Bari et al. 2006; Pant et al. 2008). *Agrobacterium tumefaciens* GV3101 containing the construct was used for transformation of tobacco (*Nicotiana tabacum* cv. SNN) plants. In brief, the cut pieces of the tobacco leaves were washed in bacterial suspension containing the construct and placed on Murashige Skoog (MS) media with 2% agar, followed by incubation for 2 days in the dark. The leaf pieces were then transferred into MS medium with 1.6% glucose + 6-benzyl-aminopurine (1 mg/ml) + naphthylacetic acid (0.2 mg/ml) + claforan (300 mg/liter) + hygromycin (10 mg/ml). The medium was changed every 7 to 10 days, and the regenerated calli were transferred into glass pots with the same medium. The shoots were cut and transferred onto MS media with 2% sucrose + claforan (250 mg/liter). The tobacco seedlings were transferred to the greenhouse and overexpression of ath-miR399d was confirmed by qRT-PCR.

Tobacco grafting.

MiR399 OX and WT tobacco plants were grown in hydroponic systems providing 1.5 mM Pi (Scheible et al. 2004). At 40 days old, plants were reciprocally grafted using silicon tubing. Grafted plants were covered to maintain high humidity until the graft junction had healed. Successfully grafted plants were used for Pi-content measurements when plants were 8 weeks old.

Mycorrhizal inoculation

of ath-miR399d overexpressing and WT tobacco.

Seeds of wild-type and ath-miR399d-overexpressing plants were surface sterilized by incubation for 2 min in 70% ethanol, were washed with sterile water, and treated for 6 min with 10% Na-hypochlorite. After a second washing step, seeds were germinated on wet filter paper in petri dishes at room temperature in the dark. To select for overexpressing plants, 20 µg of hygromycin per milliliter was added to the water. After 10 days, the seedlings were planted into quartz sand in a growth chamber and were fertilized every two days with Hoagland's solution containing either 20 µM Pi (–Pi) or 2 mM (+Pi). Half of the plants were inoculated with 10% (vol/vol) *Glomus intraradices* inoculum. Roots were harvested after 6 weeks and were used for qRT-PCR, in order to quantify the expression of mycorrhiza-regulated Pi-transporter genes. For light microscopy analysis, plants were transferred after 17 days to split root pots containing quartz sand and 10% (vol/vol) *Glomus intraradices* inoculum in one compartment. Roots in the nonmycorrhiza compartment were fertilized with Hoagland's solution containing either 20 µM Pi (–Pi) or 2 mM (+Pi). Three weeks after inoculation, plants were harvested. For the estimation of mycorrhiza parameters according to Trouvelot and associates (1988), we used four biological replicate plants per treatment (WT, overexpression mutant, –Pi, +Pi).

RNA extraction and qRT-PCR.

Roots and shoot tissue were harvested and frozen in liquid nitrogen. RNA was isolated using the mirVana miRNA kit (Ambion, Austin, TX, U.S.A.) and was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.). The RNA integrity was proven using a Bioanalyzer (Agilent, Palo Alto, CA, U.S.A.), and all RNA samples displayed RIN (RNA integrity numbers) >8. RNA samples were DNase treated using the TURBO DNA-free kit (Ambion), and 1 µg of RNA was reverse transcribed using superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). qPCR measurements were performed using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) using Power SYBR green (Applied Biosystems). cDNA samples were diluted 1:10, and 1 µl of diluted cDNA was used as template in 10-µl PCR reactions containing 2.5 µM primer. qRT-PCR was carried out in optical 384-well plates with the following PCR regime: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was carried out after each amplification to exclude unspecific amplifications from the analysis. Data analysis was performed using the SDS2.2.1 software (Applied Biosystems). The threshold was set at 0.2. As constitutively expressed *M. truncatula* reference gene, we used the transcription elongation factor EF1α (Wulf et al. 2003; Kakar et al. 2008) for the normalization of expression levels of the genes of interest. The amplification efficiencies (E) were calculated using the LinRegPCR program (Ramakers et al. 2003). For normalization, the reference gene cycle threshold (C_T) value was subtracted from the C_T value of the gene of interest, yielding a ΔC_T value. The relative expression levels of the target genes were calculated using the formula $(1 + E)^{\Delta C_T}$ (Pfaffl

2001). Three technical replicates and five independent biological replicates were measured in each experiment. For quantification of mature miR399, total RNA was reverse transcribed using stem-loop primers and MultiScribe reverse transcriptase (Applied Biosystems). In brief, 1 µg of RNA was mixed with 1 µl of 10 mM dNTPs, 1 µl of 2.5 µM stem-loop RT primer, and RNase-free water to a final volume of 36.5 µl, and the mixture was heated to 65°C for 5 min before being chilled on ice. Then, 10 µl of 5× first-strand buffer, 2 µl of 0.1 M dithiothreitol, 0.5 µl of RNase inhibitor, and 1 µl of MultiScribe reverse transcriptase were added. The reaction mixture was incubated for 30 min at 16°C, followed by an elongation reaction at 42°C for 30 min and an incubation at 85°C for 5 min. Mature miRNAs were quantified using qRT-PCR as described above.

miR399 target validation

by modified RNA ligase-mediated (RLM)-RACE.

RNA of mycorrhizal Pi-depleted roots was reverse transcribed using gene-specific primers (Pho2_outer: 5' to 3', CGTATGTA TTGCAATCAACAAC; Pho2_inner: 5' to 3', GATGAGTCGA TACCAACTTC), using the FirstChoice RLM-RACE kit (Applied Biosystems) according to the manufacturer's instructions, except that the RNA was directly ligated to the 5' RACE adapter without the RNA processing steps (calf intestine phosphatase and tobacco acid pyrophosphatase treatment). The PCR products were cloned into the TOPO TA cloning kit (Invitrogen) and were sequenced.

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