MicroRNA395 mediates regulation of sulfate accumulation and allocation in Arabidopsis thaliana

Gang Liang1,2, Fengxi Yang1 and Diqu Yu1,*

1Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China, and
2Graduate University of Chinese Academy of Sciences, Beijing 100049, China

Received 8 February 2010; revised 12 March 2010; accepted 19 March 2010; published online 27 April 2010.
*For correspondence (fax +86 871 5160916; e-mail ydq@xtbg.ac.cn).

SUMMARY
Sulfur is a macronutrient that is necessary for plant growth and development. Sulfate, a major source of sulfur, is taken up by plant roots and transported into various tissues for assimilation. During sulfate limitation, expression of miR395 is significantly up-regulated. miR395 targets two families of genes, ATP sulfurylases (encoded by APS genes) and sulfate transporter 2;1 (SULTR2;1, also called AST68), both of which are involved in the sulfate metabolism pathway. Their transcripts are suppressed strongly in miR395-over-expressing transgenic Arabidopsis, which over-accumulates sulfate in the shoot but not in the root. APS1 knockdown mutants accumulate twice as much sulfate as the wild-type. By constructing APS4-RNAi transgenic plants, we found that silencing the APS4 gene also results in over-accumulation of sulfate. Even though miR395-over-expressing transgenic plants over-accumulate sulfate in the shoot, they display sulfur deficiency symptoms. Additionally, the distribution of sulfate from older to younger leaves is impaired in miR395-over-expressing plants, similar to a SULTR2;1 loss-of-function mutant. The aps1-1 sultr2;1 APS4-RNAi triply repressed mutants phenocopied miR395-over-expressing plants. Our research showed that miR395 is involved in the regulation of sulfate accumulation and allocation by targeting APS genes and SULTR2;1, respectively.

Keywords: miR395, sulfate assimilation, sulfate transport, APS, SULTR2;1, Arabidopsis thaliana.

INTRODUCTION
Sulfur, one of the six macronutrients that are indispensable for the growth and development of plants, is incorporated into cysteine, methionine, glutathione and other sulfur-containing metabolites. Sulfur plays critical biochemical roles in a number of cellular processes, such as redox cycles, detoxification of heavy metals, and metabolism of secondary products (Hell, 1997; Leustek and Saito, 1999; Saito, 2004). Acquisition of sulfur by plants is becoming more and more important for the agricultural industry due to the decrease of sulfur emissions from industrial sources and the consequent limited inputs from deposition (Buchner et al., 2004). Sulfur has key roles in plant growth and vigor, and hence crop yield (Leustek and Saito, 1999); thus it is important to characterize the processes of sulfate uptake, transport and assimilation in plants.

Inorganic sulfate, a major source of sulfur, is acquired by plant roots, but most sulfur exists in the form of reduced compounds which can not be absorbed by roots. Sometimes, plants may suffer from a lack of sulfate supply. To survive under sulfate-limited conditions, plants have evolved a network of sulfate transporter proteins that are responsible for the acquisition of sulfate from soil, delivery of sulfate to the plastid for assimilation or to the vacuoles for sulfate storage, and long-distance transport from source to sink (Buchner et al., 2004; Maruyama-Nakashita et al., 2004; Hawkesford and De Kok, 2006). In Arabidopsis thaliana, acquisition of sulfate from the soil is attributed to two high-affinity sulfate transporters, encoded by the genes SULTR1;1 and SULTR1;2. SULTR1;1 is inducible and is responsible for the uptake of sulfate under low-sulfate conditions, while SULTR1;2 encodes the protein that performs constitutive uptake of sulfate from the soil in roots (Yoshimoto et al., 2002). The acquired sulfate is transported to various locations for the synthesis of diverse primary and secondary metabolites. The phloem-localizing sulfate transporter encoded by SULTR1;3 mediates the re-distribution of sulfur from source
to sink organs (Yoshimoto et al., 2003). The protein encoded by SULTR3;5 enhances the sulfate transport ability of SULTR2;1 in the root vasculature (Kataoka et al., 2004a). The vascular sulfate transporters SULTR4;1 and SULTR4;2 control the sulfate contents of vacuoles in the roots of Arabidopsis thaliana (Kataoka et al., 2004b). Although most of these sulfate transporters are inducible under sulfate limitation (Takahashi et al., 2000; Yoshimoto et al., 2002; Kataoka et al., 2004b), the molecular mechanisms regulating them remain to be determined. Sulfur limitation 1, an EIL (ETHYLENE-INSENSITIVE3-LIKE) family transcription factor encoded by SLIM1, is the only well-characterized regulator involved in the up-regulation of sulfur starvation-responsive genes (Maruyama-Nakashita et al., 2006).

After uptake, sulfate is either stored in the vacuole of the cell or is further assimilated in plastids. The sulfate assimilation pathway provides reduced sulfur for the synthesis of cysteine, methionine and glutathione. ATP sulfurylase is the first enzyme in the sulfate assimilation pathway. In Arabidopsis thaliana, there are four APS genes (APS1, APS2, APS3 and APS4) that encode ATP sulfurylase isoforms. The APS1, APS3 and APS4 proteins have been predicted to localize to plastids, whereas APS2 may function in the cytosol (Hatzfeld et al., 2000; Rotte and Leustek, 2000). Sulfate is activated by ATP sulfurylase to form adenosine 5′-phosphosulfate, which is subsequently reduced to form sulfur-containing metabolites (Leustek and Saito, 1999; Saito, 2004).

MicroRNAs are a class of small RNAs processed from non-coding double-stranded RNA precursors by DICER-LIKE1 (DCL1) RNase (Loudet et al., 2004). MicroRNAs negatively regulate their target genes by cleavage or by repressing translation via base pairing to nearly complementary sequences (Reinhart et al., 2002; Carrington and Ambros, 2003; Bartel, 2004). Several miRNAs that regulate root, leaf, or flower development by directing cleavage of transcription factor mRNAs have been identified. However, the most recent biochemical evidence revealed that miRNAs mediate gene silencing not only by transcriptional cleavage but also by translational repression, and these two processes are independent (Brodersen et al., 2008; Lanet et al., 2009). miR172 and miR156 were previously found to regulate target transcripts by translational repression (Chen, 2004; Gandikota et al., 2007). Identification of novel miRNAs and prediction of their targets has suggested that miRNAs are involved in physiological processes (Bari et al., 2006; Chiou et al., 2006) as well as plant development (Bartel and Bartel, 2003). In addition, expression of some miRNAs was found to be regulated by abiotic stresses, and may be involved in adaptation to these stresses (Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Adai et al., 2005; Axtell and Bartel, 2005; Lu et al., 2005; Sunkar et al., 2005).

It has been suggested that miR399 is highly induced by phosphate starvation and regulates phosphate acquisition and allocation by targeting UBC24/E2 (Fujii et al., 2005; Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006). Recent research indicated that miR395 is induced by sulfur starvation, and targets two groups of genes that function coordinately in the same metabolic pathway (Jones-Rhoades and Bartel, 2004; Allen et al., 2005; Chiou, 2007). We are interested in understanding how plants regulate the uptake and translocation of sulfate during sulfate starvation stress. The significant induction of miR395 and the responses of its targets during sulfate starvation suggest that miRNA395 responds to sulfur starvation. We demonstrate that miR395 regulates the accumulation of sulfate in the shoot by targeting APS genes and the relocation of sulfate between leaves by cleaving SULTR2;1.

RESULTS

Expression of miR395 and its target genes during sulfate limitation

miR395 is a conserved microRNA that has been identified in diverse species, including monocotyledons and dicotyledons. Six miR395 species (a–f), arising from six primary transcripts, have been identified in Arabidopsis (miRBase, release 11.0) (Griffiths-Jones, 2004). During sulfate limitation, the expression of miR395 is highly induced (Figure 1a,b) (Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009), but the relative accumulation of unrelated miR167 is not affected (Figure S1). Under various nutrient deprivation conditions (such as for C, N, P, K, Fe, Cu, S), only sulfate deficiency induced expression of miR395 (Hsieh et al., 2009). This evidence shows that miR395 specifically responds to sulfate starvation.

Previous studies have indicated that miR395 targets APS genes (APS1, APS3 and APS4) and SULTR2;1 in Arabidopsis (Jones-Rhoades and Bartel, 2004; Allen et al., 2005; Kawashima et al., 2009). To investigate the responses of its targets to sulfate limitation, we examined their transcripts levels under various sulfate concentration conditions. The target transcripts show various responses to sulfate deficiency (Figures 1b and 2). APS1 shows no significant response to sulfate limitation (Figure 1b), but quantitative RT-PCR analysis showed that the level of APS1 transcripts decreases slightly both in the root and shoot. APS3 transcripts increase in the root and shoot, and APS4 transcripts are significantly suppressed in both the root and shoot. The level of SULTR2;1 mRNA declines in the shoot, but increases in roots.

These four target genes have been identified as being cleaved by miR395 in Arabidopsis thaliana (Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009). However, we observed that, during sulfate starvation, APS1 and APS4 transcripts and shoot SULTR2;1 are down-regulated, but APS3 and root SULTR2;1 transcripts are up-regulated even though miRNAs usually negatively regulate their target...
genes. miR159, miR172 and miR2111 have been shown to be in temporal accordance with their targets (Llave et al., 2002; Achard et al., 2004; Hsieh et al., 2009). Recent studies also revealed that the positive correlation between root SULTR2;1 mRNA and miR395 during sulfate starvation is due to non-overlapping tissue-specific expression (Kawashima et al., 2009). It is likely that APS3 mRNA avoids cleavage by miR395 in a manner similar to SULTR2;1.

**Over-expression of miR395 reduces the levels of its target transcripts**

In Arabidopsis, there are two forms of mature miR395 (miR395a,d,e and miR395b,c,f) that differ by only one nucleotide (Figure S2). To investigate whether they target the same genes, we generated two kinds of transgenic plants over-expressing miR395d and miR395f, respectively. Expression of the DNA sequence containing the precursor foldback structure of miR395d or miR395f was driven by the constitutive 35S promoter. This construct was then introduced into Arabidopsis ecotype Columbia (Col) by Agrobacterium-mediated transformation (Clough and Bent, 1998).

In the miR395-over-expressing plants, miR395 is highly expressed in roots and shoots (Figure 3a). Moreover, expression of miR395 in the transgenic plants is much stronger than that caused by sulfate starvation in wild-type plants. It has been shown that authentic plant miRNAs

![Figure 1. Expression of miR395 and its target genes.](image)

(a) Specific expression of miR395 upon sulfate starvation. MiR395 promoter–GUS plants were grown on MS medium with sulfate (+S, left) or MS medium without sulfate (−S, right).

(b) Northern blot analyses of miR395 and its target genes in wild-type plants. The roots and shoots from 10-day-old seedlings grown on MS medium with the indicated sulfate content (0, 20, 1500 or 2000 μM) were used for the extraction of RNA. rRNA staining is shown as a loading control. The extracted RNA (20 μg) was used for Northern blotting.

![Figure 2. Expression of miR395 targets in wild-type and miR395-over-expressing plants.](image)

Real-time quantitative RT-PCR analysis of APS1, APS3, APS4 and SULTR2;1. Plants were grown for 10 days on MS medium with 1500 μM sulfate (+S) or MS medium without sulfate (−S). RNA was isolated from roots and shoots, respectively. The quantitative RT-PCR analysis was repeated for three biological replicates, each of which consisted of three technical replicates. The error bars represent SD from triplicate samples. Student’s t-test indicated that the values marked by two asterisks are significantly different from the corresponding wild-type value (P < 0.01; n = 3).
miRNA395 and sulfate homeostasis

Figure 3. Phenotypes of miR395-over-expressing plants. (a) Northern analysis of miR395-over-expressing plants. (b) Small stature and slight chlorosis of miR395-over-expressing plants. Plants were grown for 3 weeks in soil. (c) More lateral roots were produced by miR395-over-expressing plants. Plants were grown vertically for 2 weeks on MS medium containing 20 μM sulfate. (d) Shoot biomass of plants. Plants were grown for 3 weeks in soil. Values are the means of three replicates of 20 plants. Error bars indicate SD (n = 3). The two asterisks indicate statistical significance at P < 0.01 compared with the wild-type (Student’s t test). (e) Sulfate concentration of miR395-over-expressing plants. Plants were grown for 3 weeks in soil. Shoots and roots were separated and used for the determination of sulfate content. Error bars indicate SD (n = 10). The two asterisks indicate statistical significance at P < 0.01 compared with the wild-type (Student’s t test).

miRNA395 and sulfate homeostasis

© 2010 The Authors

cleave their targets with high specificity by analyzing the transcriptomes of plants over-expressing different miRNAs (Schwab et al., 2005). Additionally, study of miRNA evolution suggested that ancient miRNAs underwent long-term selection to cleave their targets specifically (Allen et al., 2004; Fahlgren et al., 2007). As miR395 is an ancient miRNA, we determined expression of its target genes in miR395-over-expressing plants. APS1, APS3, APS4 and shoot SULTR2;1 transcripts were suppressed when the miR395-over-expressing plants were grown under sulfate-sufficient (+S) or sulfate-deficient (−S) conditions (Figure 2).

The over-accumulation of miR395d or miR395f correlates well with the decreased target transcripts levels (except for root SULTR2;1), further supporting our hypothesis that miR395 regulates the abundance of target transcripts and that the two forms of miR395 target the same genes.

Over-expression of miR395 results in sulfur deficiency symptoms and over-accumulation of sulfate in the shoot

Under sulfate-sufficient conditions, miR395-over-expressing plants show slight chlorosis in their leaves and reduced growth (with lower shoot biomass) compared to wild-type plants (Figure 3b,d). In addition, miR395-over-expressing plants produce more lateral roots than wild-type plants under sulfate-deficient conditions (Figure 3c). These phenotypes are similar to the sulfur deficiency symptoms of Arabidopsis (Lopez-Bucio et al., 2003; Nikiforova et al., 2003). We further detected the expression of sulfur starvation-responsive genes in miR395-over-expressing plants. The results suggest that expression of four sulfur starvation-responsive genes, SULTR1;1, SULTR1;2, SULTR4;1 and SULTR4;2, is significantly enhanced under normal sulfate conditions, similar to the level in wild-type plants under sulfate starvation (Figure 4), further indicating that miR395-over-expressing plants are in a state of sulfur starvation.

As miR395 is induced specifically by sulfate starvation and its targets are involved in the sulfate metabolism pathway, over-expression of miR395 may change the sulfate content in plants. The sulfate concentrations of wild-type and

Figure 4. Expression of sulfate starvation-responsive genes in wild-type and miR395-over-expressing plants. Real-time quantitative RT-PCR analysis of SULTR1;1, SULTR1;2, SULTR4;1 and SULTR4;2. Plants were grown for 10 days on MS medium with 1500 μM sulfate (+S) or MS medium without sulfate (−S). RNA was isolated from shoots. The quantitative RT-PCR analysis was repeated for three biological replicates, each consisting of three technical replicates. The error bars represent SD. Student’s t test indicated that the values indicated by two asterisks were significantly different from the corresponding wild-type value (P < 0.01; n = 3).
miR395-over-expressing plants grown in soil were determined. Intriguingly, even though miR395-over-expressing plants display sulfur deficiency symptoms, the sulfate concentration of shoots was 7–8-fold higher than that of wild-type leaves but the sulfate concentration of their roots was lower than that in the roots of wild-type plants (Figure 3e). This contradiction between sulfur deficiency symptoms and sulfate over-accumulation implies that sulfate assimilation may be repressed in miR395-over-expressing plants.

Over-expression of miR395 impairs the relocation of sulfate between leaves

The remobilization of sulfate is crucial for supporting continuous growth and development of young leaves. During leaf senescence, nutrients are mobilized to seeds, storage organs or new vegetative growth, and the level of sulfur drops by more than 60% during leaf senescence in Arabidopsis (Himelblau and Amasino, 2001).

To understand the distribution of sulfate among leaves, we determined the sulfate concentrations of leaves of various ages from 3-week-old plants. In wild-type plants, the sulfate concentration increases with decreasing leaf age (Figure 5a). In contrast, the sulfate concentration decreases with decreasing leaf age in miR395-over-expressing plants (Figure 5a). This suggests that over-expression of miR395 impairs the distribution of sulfate between leaves.

To further investigate the translocation of sulfate, we measured the sulfate concentrations of the same leaves at various developmental stages. A detailed time-course analysis was also performed (Figure 5b). In wild-type plants, the sulfate concentration in all leaves decreased over the growth period, which suggests efficient remobilization of sulfate from old to new leaves. By contrast, the sulfate concentration in all leaves of miR395-over-expressing plants increased continually over the growth period, which implies that sulfate transport between leaves is impaired.

Loss of function of APS1 or/and APS4 leads to over-accumulation of sulfate in the shoot

A recent study showed that an APR2 mutant accumulated twice as much sulfate in the shoot than the wild-type (Loudet et al., 2007). APR2 functions downstream of APS in the sulfate assimilation pathway (Leustek and Saito, 1999; Saito, 2004). Mutation of the miR395-targeted APS genes may therefore also lead to over-accumulation of sulfate. To confirm our hypothesis, two independent insertion alleles for APS1 and APS3, in the Col-0 genetic background, were obtained from the Arabidopsis Biological Resource Center (ABRC) (Figure 6a). Homozygous lines were isolated by PCR, and the loss of transcripts was established by Northern blotting or RT-PCR (Figure 6b,c). No T-DNA insertion allele was available for the APS4 gene. We therefore used an RNAi approach to knock down the APS4 gene, and confirmed that only APS4, but not APS1 and APS3, was suppressed in the APS4-RNAi plants (Figure 6d and Figure S3a–c). To determine whether disruption of the genes for individual APS isoforms has any effect on sulfate accumulation, we determined the sulfate levels in the shoots of 3-week-old plants grown in soil. Both aps1-1 and aps1-2 mutants accumulate twice as much sulfate as wild-type plants, and APS4-RNAi transgenic plants accumulate five times more, but the sulfate content of the aps3 mutant was similar to that of wild-type plants (Figure 6e).

Although loss of function of a single isoform, APS1 or APS4, results in the over-accumulation of sulfate, the sulfate content is still lower than that of miR395-over-expressing plants (Figures 3e and 6e). It is probable that functional
redundancy exists among the three APS isoforms. We therefore created an aps1-1 aps3 double mutant and transformed 35S:APS4-RNAi into aps1-1 aps3 plants to form triply repressed mutants (Figure S4). As shown in Figure 6(e), the sulfate contents of the aps1-1 and aps1-1 aps3-1 mutants are approximately the same, but the aps1-1 aps3 APS4-RNAi triply repressed plants have similar sulfate concentrations to APS4-RNAi plants. These data suggests that loss of function of APS1 or APS4 results in over-accumulation of sulfate in the shoot, and APS4 is the main contributor to the accumulation of sulfate.

A loss-of-function mutant of sultr2;1 impairs the distribution of sulfate between leaves

Over-expression of miR395 impairs sulfate relocation from old to new leaves (Figure 5a, b). However, we wished to understand how over-expression of miR395 affects the distribution of sulfate between leaves. Among the targets of miR395, only SULTR2;1 is a sulfate transporter involved in the translocation of sulfate. Reporter gene expression showed that SULTR2;1 is located in the xylem parenchyma and phloem cells in leaves (Takahashi et al., 2000), implying that it may act in the transport of sulfate in leaves. To investigate whether SULTR2;1 is required for the translocation of sulfate between leaves, we obtained a sultr2;1 mutant with T-DNA inserted into the intron of the SULTR2;1 gene (Figure 7a, b). Determination of the sulfate contents of various leaves from 3-week-old sultr2;1 mutant plants indicated that the sulfate content of old leaves is higher than that of young leaves (Figure 7d). However, the mutant accumulates shoot sulfate to the same level as the wild-type in the whole plant (Figure 7c). This defect in the remobilization of sulfate between leaves is similar to the sulfate remobilization defect of miR395-over-expressing plants. The negative regulation of SULTR2;1 mRNA by miR395, and the similar phenotypes of the sultr2;1 knockout mutant and miR395-over-expressing plants, establish a direct link between miR395 regulation of SULTR2;1 and the relocation of sulfate between the leaves of Arabidopsis.

aps1-1 sultr2;1 APS4-RNAi plants exhibit phenotypes similar to those of miR395-over-expressing plants

Although lack of APSs results in over-accumulation of sulfate, sulfate is transported normally into new leaves from old (Figure 6f). In contrast, sultr2;1 mutants showed impaired distribution of sulfate between their leaves, but accumulated similar overall levels of sulfate to wild-type plants (Figure 7c). To determine whether the over-accumulation and impaired relocation of sulfate in the leaves of miR395-over-expressing plants are caused by the lack of both APS gene products and SULTR2;1, we created an aps1-1 sultr2;1 APS4-RNAi triply repressed mutant (Figure S4). We observed a similar sulfate over-accumulation and distribution between the leaves of aps1-1 sultr2;1 APS4-RNAi mutant plants to...
that for miR395-over-expressing plants (Figure 8a,b). These data suggest that miR395 regulates the accumulation and distribution of sulfate between leaves by repressing APS genes and SULT2;1, respectively.

DISCUSSION

miR395 regulates the accumulation of sulfate in the shoot by targeting APS genes

APSs are the first enzymes that participate in sulfate assimilation, and function to activate sulfate. Four APS genes have been identified in Arabidopsis (Hatzfeld et al., 2000). APS1, APS3 and APS4 are located in the chloroplast (Hatzfeld et al., 2000), but APS2 may function in the cytosol (Rotte and Leustek, 2000). RNA ligase-mediated (RLM) 5′ RACE experiments confirmed that the cleavage sites of the three APS genes correspond to the predicted miR395 target sequences (Kawashima et al., 2009), supporting the prediction that the three APS genes are targeted by miR395. Our results showed that APS1, APS3 and APS4 are suppressed in the roots and shoots of miR395-over-expressing plants (Figure 2). These results strongly support the targeting of APS1, APS3 and APS4 by miR395 in Arabidopsis.

Determination of sulfate content suggested that APS1 mutants accumulates twice as much sulfate in the shoot, but that APS3 mutants have sulfate levels similar to those of wild-type plants. The sulfate content of APS4-RNAi plants was five times higher than that of wild-type plants. These data suggest that inhibition of APS1 and APS4 expression leads to the over-accumulation of sulfate in shoots, and that it is loss of APS4 that contributes most to the over-accumulation of sulfate. Based on these data, we conclude that miR395 regulates the sulfate accumulation of shoots by cleavage of APS1 and APS4. Although APS3 is also cleaved by miR395, it is induced by sulfate starvation and its loss does not lead to sulfate over-accumulation, which suggests that APS3 may function during sulfate limitation when APS1 and APS4 are repressed. Interestingly, miR395 is induced specifically when plants suffer sulfate starvation, which indicates that miR395 could be a sulfate starvation signal. In fact, miR395 over-expression results in over-accumulation of sulfate, which suggests that miR395 facilitates the accumulation of sulfate by targeting APS genes during sulfate starvation.

Three factors lead to the sulfate over-accumulation in the shoot of plants: (i) reduction of sulfate assimilation, (ii) over-uptake of sulfate from soil, and (iii) increased transport of sulfate from roots to shoots. We have shown that three APS genes (the first enzymes in the sulfate assimilation pathway) are repressed in miR395-over-expressing plants (Figure 2), which then may lead to reduced sulfate activation for assimilation. On the other hand, SULTR1;1 and SULTR1;2, two high-affinity sulfate transporters responsible for the uptake of sulfate from soil to roots (Yoshimoto et al., 2002), are up-regulated in the root of miR395-over-expressing plants, which may result in over-influx of sulfate from the soil and contribute to the over-accumulation of sulfate in shoots (Figure 4). SULTR4;1 and SULTR4;2 are responsible for the efflux of sulfate from vacuole lumen and influence the capacity for vacuolar storage of sulfate in the root tissue (Kataoka et al., 2004b). It is likely that the sulfur starvation resulting from miR395 over-expression up-regulates the expression of SULTR4;1 and SULTR4;2 (Figure 4), which then down-regulate the storage of sulfate in the vacuoles of roots. At the same time, SULTR2;1 is up-regulated in the root of miR395-over-expressing plants (Figure 2), which may promote the transport of sulfate from the roots to the shoots (Takahashi et al., 2000).

miR395 regulates the translocation of sulfate between leaves by targeting SULT2;1

Sulfate is acquired from soil by roots, uploaded into the xylem, and then moves into the apoplastic continuum and the symplast, which brings sulfate to sink organs or tissues for reduction in the plastids or storage in the vacuoles.
the phloem may be down-regulated or impaired in miR395-over-expressing plants.

Fourteen 14 sulfate transporters have been identified in Arabidopsis. The transport of sulfate from source (cotyledons) to sink organs (euphylla) is attributed to the sulfate transporter SULTR1;3 (Yoshimoto et al., 2003). However, the sulfate transporter that functions in the translocation of sulfate from mature to new leaves in Arabidopsis has not been identified. The most probable candidate was SULTR2;1, which is expressed in the xylem parenchyma and phloem cells in leaves (Takahashi et al., 2000). Analysis of sulfate contents showed that allocation of sulfate from old to young leaves is impaired in the sultr2;1 mutant, indicating that SULTR2;1 is responsible for the sulfate translocation from mature into younger leaves.

RLM 5′ RACE results confirmed the presence of an miR395 cleavage site in SULTR2;1 mRNA (Kawashima et al., 2009), and SULTR2;1 was suppressed in the shoots of miR395-over-expressing plants (Figure 2), indicating that miR395s mediate cleavage of SULTR2;1 in Arabidopsis. Impaired redistribution of sulfate between leaves in miR395-over-expressing Arabidopsis was also observed in the sultr2;1 mutant (Figure 7d). In addition, we also observed that the miR395-over-expressing plants had higher SULTR2;1 transcript levels in the roots than wild-type plants do (Figure 2). It is likely that the sulfate starvation resulting from miR395 over-expression led to the increase of in the SULTR2;1 transcript level, which may facilitate the root-to-shoot transport of sulfate (Takahashi et al., 2000). These results show that miR395 participates in the redistribution of sulfate between leaves by targeting SULTR2;1.

**Temporal and spatial regulation of sulfate metabolism by miR395**

The up-regulation of miR395 depends on the sulfate status of the plant, and the expression patterns of its target genes are also closely correlated with sulfate status (Figures 1b and 2). Under sulfate starvation, APS1 and APS4 show a negative temporal correlation with miR395, as does leaf SULTR2;1 (Figure 1b). However, root SULTR2;1 displays a positive temporal correlation with miR395 during sulfate starvation, as does APS3 (Figure 1b). Recent research has also found a positive temporal correlation between the expression of some microRNAs and their target genes (Llave et al., 2002; Achard et al., 2004; Hsieh et al., 2009). Precise expression analysis suggested that miR395 was mainly expressed in phloem companion cells (Kawashima et al., 2009), and SULTR2;1 was mainly expressed in xylem parenchyma cells (Takahashi et al., 2000). This different spatial expression allows the positive temporal correlation between miR395 and SULTR2;1 in roots. We also observed that, in miR395-over-expressing plants, the expression of APS1, APS3 and SULTR2;1, but not that of APS4, increased under sulfate deficiency compared with sulfate sufficiency.

---

**Figure 8.** Phenotypes of aps1-1 sultr2;1 APS4RNAi plants. (a) Sulfate concentration of shoots from 21-day-old plants grown in soil. Error bars represent SD (n = 3). (b) Sulfate concentration of two proximal leaves from 21-day-old plants. aps1-1 sultr2;1 APS4RNAi plants are defective in the remobilization of sulfate between leaves. Error bars represent SD (n = 3).

(Buchner et al., 2004). Pulse-labeling experiments indicated substantial redistribution of sulfur from old leaves to younger leaves in other plant species, including barley (Hordeum vulgare), soybean (Glycine max) and poplar (Populus trichocarpa) (Smith and Lang, 1988; Adiputra and Anderson, 1992; Hartmann et al., 2000). Smith and Lang (1988) showed that sulfate is transported into mature leaves and is quickly exported via the phloem in soybean. However, there have been no reports on the distribution and transport of sulfate between the leaves of Arabidopsis. We found that the oldest leaves have the lowest sulfate content and the youngest leaves have the highest, and sulfate content of leaves decreases with increasing leaf age (Figure 5a,b), suggesting that sulfate is transported from old leaves to new leaves in Arabidopsis. However, sulfate remobilization from mature into younger leaves is disrupted in the miR395-over-expressing plants (Figure 5a,b). This defect in sulfate remobilization between leaves suggests that export of sulfate out of the mature leaves or loading of sulfate into the phloem may be down-regulated or impaired in miR395-over-expressing plants.
(Figure 2), which indicates that sulfate starvation induced their expression and they may avoid cleavage by miR395 through spatial restriction. Given the partial repression of APS1 and APS3 by miR395, we propose that they may be expressed in cell compartments different from those in which miR395 is expressed but with some overlap.

Of the three APS genes, only APS4 is suppressed strongly under sulfate starvation, which facilitates accumulation of sulfate in the shoot. The others are expressed and ensure assimilation of sulfate for plant development. The up-regulation of root SULTR2;1 by sulfate starvation may contribute to the transport of sulfate from the root to the shoot (Kataoka et al., 2004a). The spatial and temporal regulation of APS genes and SULTR2;1 may be crucial for plant growth and development, because their strong suppression by miR395 results in over-accumulation and impaired allocation of sulfate in shoots, and sulfur deficiency symptoms in miR395-over-expressing plants.

The miR395/APS-SULTR2;1 pathway is conserved in plants

miR395 has been found in several other plant species, such as Oryza sativa, Vitis vinifera, Populus trichocarpa and Sorghum bicolor (miRBase, release 1.0, http://microrna.sanger.ac.uk) (Griffiths-Jones, 2004). In Arabidopsis, six miR395 genes have been identified, and they are localized in clusters on two chromosomes (Jones-Rhoades and Bartel, 2004). It appears that they evolved from a process of genome-wide duplication, tandem duplication and segmental duplication, followed by dispersal and diversification (Maher et al., 2006). Analysis of the molecular evolution of the rice miR395 genes suggested that this gene family consists of four compact clusters with a total of 24 genes (Guddeti et al., 2005). These data suggest that miR395 is an evolutionarily conserved microRNA.

microRNA-mediated post-transcriptional gene regulation is an ancient gene regulation mechanism in plants (Floyd and Bowman, 2004). In our study, we showed that miR395 regulates sulfate metabolism by targeting APS genes and SULTR2;1 in Arabidopsis. To verify whether the mechanism of miR395 regulation is conserved, we predicted the putative target genes of miR395 in other plant species using web software (http://bioinfo3.noble.org/miRNA/miRU.htm and http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). In Oryza sativa, Medicago truncatula, Solanum lycopersicum and Sorghum bicolor, miR395 is predicted to target both ATP sulfurylases and sulfate transporters (Table S1). However, only ATP sulfurylases are predicted to be the targets of miR395 in Populus trichocarpa, Brassica napus, Zea mays and Vitis vinifera (Table S1). It is likely that sulfate transporters are not targeted by miR395 in the latter plant species, or are not predicted due to the limited cDNA database. Additionally, the predicted target genes from different species share a conserved miR395 consensus binding site (Figure S5a) and their gene structures are similar (Figure S5b). Thus, we believe that miR395 could also be involved in the sulfate metabolism pathway in other plant species, similarly to Arabidopsis.

A putative working model for the mechanism of sulfur sensing

The mechanisms involved in regulation of sulfate acquisition from soil, transport into various sink organs and redistribution in plants are unclear. Figure 9 shows putative working models for the regulation of sulfate accumulation and relocation by miR395 on the basis of our results. In this model, the up-regulation of miR395, SULT1R1;1, SULT1R2, SULT4;1 and SULT4;2 depends on SLIM1 under sulfate limitation conditions (Maruyama-Nakashita et al., 2006; Kawashima et al., 2009). Sulfur starvation induces miR395, which then targets APS genes, responsible for activation of sulfate in sulfate assimilation, and SULTR2;1, whose product acts in the redistribution of sulfate between leaves.

As shown in Figure 9(a), the induced miR395 cleaves the APS transcripts under sulfate starvation conditions (Figure 1b). However, APS3 is up-regulated, and the other APS transcripts may avoid cleavage by miR395 due to tissue-specific expression. As a result of the decrease in APS1 and APS4, sulfate activation is suppressed and the
production of sulfur-containing metabolites is reduced, which leads to the accumulation of sulfate. Up-regulation of APS3 may ensure the survival of plants during sulfate starvation.

As shown in Figure 9(b), sulfate is redistributed into new leaves from old leaves under sulfate-sufficient conditions (Figure 5a,b). Upon sulfur starvation, the increased expression of miR395 reduces the abundance of SULTR2;1 in leaves (Figure 1b). As a result of the reduced leaf SULTR2;1 levels, the transport of sulfate from old to new leaves is restricted, which facilitates the maintenance of sulfate in old leaves. In roots, SULTR2;1 is induced by sulfate starvation (Figure 1b) (Lappartient et al., 1999), implying that it may be responsible for the transport of sulfate from roots to shoots under sulfate limitation. Kawashima et al. (2009) suggested that SULTR2;1 is regulated dually under sulfate starvation. On the one hand, up-regulation of SULTR2;1 by sulfur starvation in the slim1 mutant suggests that sulfur starvation induces SULTR2;1 expression at the transcriptional level, independently of SLIM1. On the other hand, SULTR2;1 is down-regulated post-transcriptionally by miR395, and the induction of miR395 by sulfur starvation is dependent on SLIM1. SULTR1;1 and SULTR1;2 are induced by sulfate starvation (Figure 4) for uptake of sulfate from the soil, which enables the plants to cope with sulfur deficiency. The up-regulation of SULTR4;1 and SULTR4;2 enhances the efflux of sulfate from root vacuoles in order to supply the demand of sulfur-starved tissues.

Over-accumulation of sulfate in the shoots of miR395-over-expressing plants (Figure 3e) can be attributed to the increased uptake of sulfate from the soil by SULTR1;1 and SULTR1;2, and the reduced activation of sulfate by APSs. On the other hand, the lower sulfate level in the root of miR395-over-expressing plants (Figure 3e) could result from enhanced expression of SULTR4;1 and SULTR4;2 (Figure 4). The SULTR2;1 levels in the roots of miR395-over-expressing plants are higher than those of wild-type plants under sulfate-sufficient conditions, which may promote the transport of sulfate from roots to shoots (Kataoka et al., 2004a). This evidence suggest that appropriate regulation of APS genes and SULTR2;1 by miR395 is crucial for sulfate homeostasis in plants.

Our study has elucidated the mechanism involved in maintenance of sulfate homeostasis by miR395 in plants. Further investigation of the detailed signal transduction between sulfur starvation and miR395 will be helpful to understand the mechanism underlying sulfur utility of plants.

**EXPERIMENTAL PROCEDURES**

**Plant growth conditions**

*Arabidopsis thaliana* (ecotype Columbia) seeds were surface-sterilized with 20% bleach and washed three times (each time for 1 min) with sterile water. Sterilized seeds were suspended in 0.1% agarose and plated on MS medium. Plates were vernalized in darkness for 2 days at 4°C, and then transferred to a tissue culture room at 22°C under a 16 h light/8 h dark photoperiod. For determination of sulfate content, 7-day-old seedlings were planted in soil. For observation of root phenotypes, seedlings were grown on vertical MS agar medium containing 0.8% agar. Potted plants were grown in growth chambers at 22°C and 75% humidity under a 16 h light/8 h dark photoperiod.

For sulfate starvation experiments, Columbia plants were grown under long-day conditions (6 h light/8 h dark) on modified MS/agar medium, containing 0.7% agar, in which the sulfate containing salts of the MS media were replaced with their chloride counterparts and the medium was supplemented with 0, 20, 1500 or 2000 μM (NH₄)₂SO₄. Root and shoot samples for RNA isolation were collected from 10-day-old seedlings grown vertically.

**Plasmid construction**

The putative promoters of MIR395d and MIR395f were amplified from Arabidopsis genomic DNA using primers Pro-MIR395d-L, Pro-MIR395d-R, Pro-MIR395f-L and Pro-MIR395f-R (Table S2). Amplified sequences were subsequently digested using appropriate restriction enzymes and cloned into the pJS131B vector containing a GUS gene. The fused promoter–GUS genes were cloned into the pOCA28 vector containing a kanamycin resistance gene. The genomic sequence containing the MIR395d or MIR395f foldback loop was used as a synthetic precursor sequence. The sequences were amplified from *Arabidopsis thaliana* genomic DNA by PCR using primers Pre-mir395d-L and Pre-mir395f-R, and confirmed by sequencing. The MIR395 promoters were sub-cloned into the pOCA30 vector containing the CaMV 35S promoter. Silencing of the APS4 gene was performed by expressing double-stranded RNA (Figure S3a). A partial APS4 cDNA fragment was amplified using primers APS4-RNAi-L and APS4-RNAi-R (Table S2), and recombined into the pPHANNIBAL vector (http://www.cambia.org/daisy/cambia/585.html), which contains an 800 bp intron. The whole DNA fragment containing the CaMV 35S promoter was sub-cloned into the pCAMBIA 1300 vector (http://www.pi.csiro.au/RNAi/vec-tors.htm) containing a hygromycin resistance gene. All plasmids were then transformed into *Agrobacterium tumefaciens* strain GV3101. Arabidopsis transformation was performed by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected using 50 μg ml⁻¹ kanamycin or 20 μg ml⁻¹ hygromycin.

**Histochemical GUS staining**

Transgenic Arabidopsis were grown on medium containing 1500 μM sulfate (+S) or 0 μM sulfate (−S) for 2 weeks. Whole seedlings were immersed immediately in 1.5 ml staining solution containing 0.5 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-gluc, Sigma, http://www.sigmaaldrich.com) in 0.1 M sodium phosphate buffer (pH 7.3) in a microfuge tube. The reaction was performed in the dark at 37°C until a blue indigo color appeared. After the reaction, seedlings were rinsed in 0.1 M sodium phosphate buffer (pH 7.3). The samples were then rinsed twice (each time for 1 h) in 70% ethanol to remove chlorophylls.

**Determination of sulfate content**

Plant samples were harvested at the times specified in the figure legends. Extraction of sulfate from shoots and roots was performed as described by Awazuhara et al. (2005) with a minor modification. Approximately 20 mg of fresh plant samples were immersed in 1 ml of 0.1 M HCl for 2 h at room temperature. After centrifugation
at 12 000 g, the supernatant was recovered and used for the determination of sulfate content using the turbidimetric method (Tabatabai and Bremer, 1970). Appropriate calibration curves were constructed using potassium sulfate as a standard.

Screening and crossing of T-DNA insertion mutants

Putative T-DNA insertion mutants of Arabidopsis were obtained from the Arabidopsis Biological Resource Center. All T-DNA lines were in the Col background. Homozygous mutants were identified using primer ko-1 and two appropriate gene-specific primers (Table S2).

The single mutants aps1-1, aps3 and sultr2;1 were used for crossing to generate the double mutants aps1-1 aps3 and aps1-1 sultr2;1. Segregating F2 seeds were screened by PCR at one locus, and plants homozygous for the insertion at this locus were then screened at the second locus. 3SS:APS4-RNAi was transformed into aps1-1 aps3 and aps1-1 sultr2;1 mutants to generate triply repressed mutants.

Analysis of gene expression

Total RNA was isolated from plant tissues using TRIzol reagent (Invitrogen, http://www.invitrogen.com/). For high-molecular-weight RNA gel-blot analysis, 20 μg of total RNA was separated on a 1.5% agarose gel and transferred to Hybond N+ membranes (Millipore Corporation, http://www.millipore.com). Probes were labeled with [γ-32P]dATP using the Klenow fragment. Low-molecular-weight RNAs were separated by electrophoresis on denaturing 15% polyacrylamide gels, and miRNA gel-blot hybridizations were performed as described previously (Lu et al., 2005). DNA oligonucleotide templates complementary to miR395a or miR167a were end-labeled using T4 polynucleotide kinase and used for hybridizations.

For cDNA production, 0.5 μg of total RNA was reverse transcribed using oligo(dT)18 primer (Fermentas) in a 20 μl reaction mixture with RevertAid M-MuLV reverse transcriptase (Fermentas). After heat inactivation, a 1 μl aliquot was used for real-time quantitative RT-PCR. All quantitative RT-PCR analyses were performed using a Lightcycler FastStart DNA Master SYBR Green I kit on a Roche LightCycler real-time PCR machine (www.roche.com), according to the manufacturer’s instructions. ACT2 (AT3G18780) was used as a control in quantitative RT-PCR.

Accession numbers

The Arabidopsis Genome Initiative/GenBank accession numbers for the genes referred to are shown in parentheses: APS1 (AT3G22890), APS3 (AT4G14680), APS4 (AT5G43780), SULTR2;1 (AT5G10180), SULTR1;1 (AT4G08620), SULTR1;2 (AT1G78000), SULTR4;1 (AT5G13550) and SULTR4;2 (AT3G12520). The T-DNA insertion mutants used were aps1-1 (SALK_002130), aps1-2 (SALK_064518), aps3 (SAIL_659_D09) and sultr2;1 (SALK_109907).

ACKNOWLEDGEMENTS

We thank the Arabidopsis Biological Resource Center for the pools of T-DNA insertion mutants. This research was supported by the Science Foundation of Ministry of Agriculture of the People’s Republic of China (2009ZX08009-066B), the National Natural Science Foundation of China (90408022), the National High Technology Research and Development Program of China (863 Program) (2006AA0227129) and Science Foundation of Yunnan Province (2004C0511M).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:
miRNA395 and sulfate homeostasis


