the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2010) **62**, 1046–1057 doi: 10.1111/j.1365-313X.2010.04216.x

MicroRNA395 mediates regulation of sulfate accumulation and allocation in *Arabidopsis thaliana*

Gang Liang[1,2], Fengxi Yang[1] and Diqiu Yu[1,*]

[1]*Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China, and*

[2]*Graduate 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 vacuolar 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

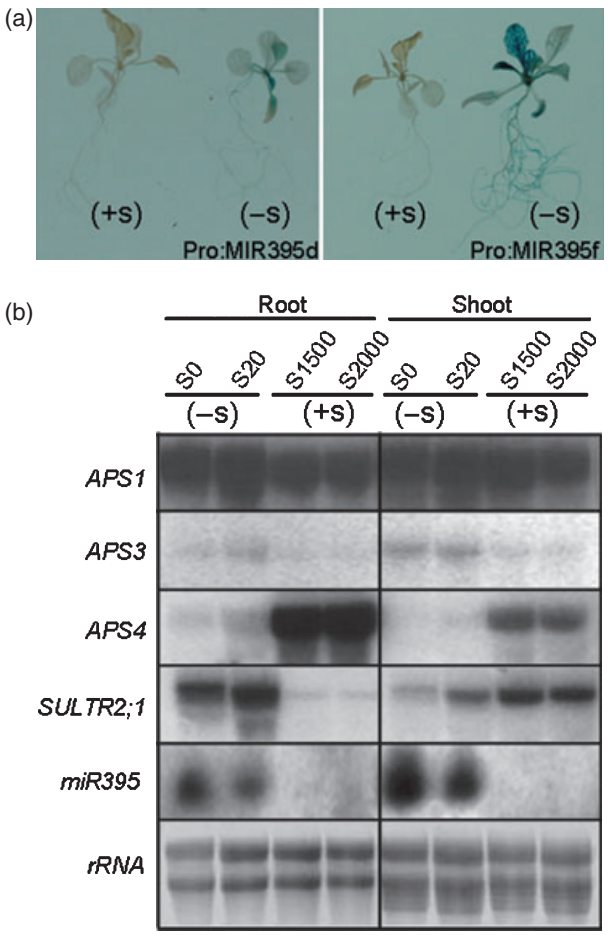

Figure 1. Expression of miR395 and its target genes.
(a) Specific expression of miR395 upon sulfate starvation. *MIR395* promoter–GUS plants were grown on MS medium with sulfate (+S, left) or MS medium without sulfate (–S, right).
(b) Northern blot analyses of miR395 and its target genes in wild-type 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.

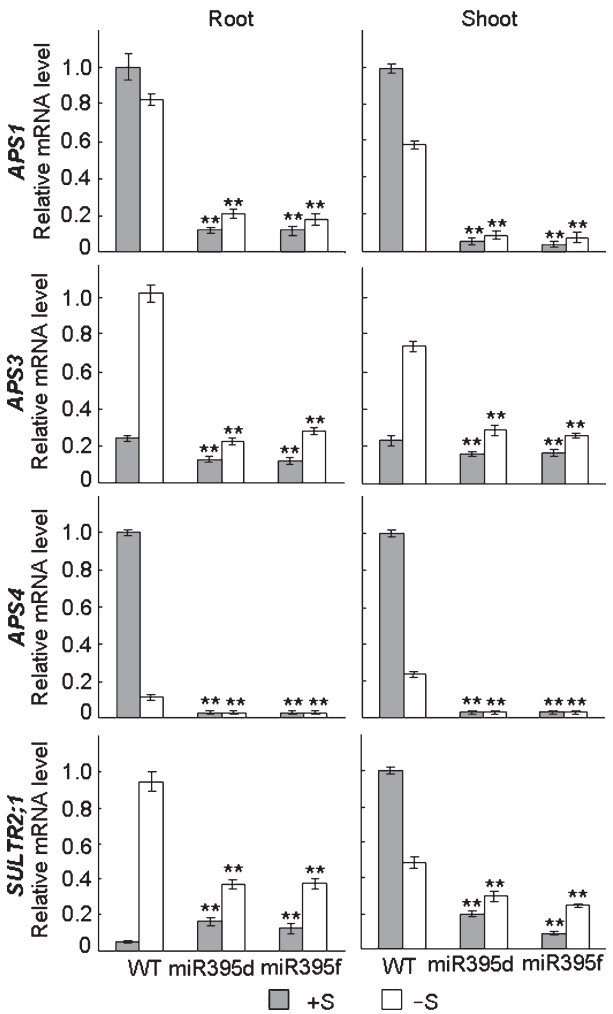

Figure 2. Expression of miR395 targets in wild-type and miR395-over-expressing plants.
Real-time quantitative RT-PCR analysis of *APS1*, *APS3, APS4* and *SULTR2;1*. Plants were grown for 10 days on MS medium with 1500 μM sulfate (+S) or MS medium without sulfate (–S). RNA was isolated from roots and shoots, respectively. The quantitative RT-PCR analysis was repeated for three biological replicates, each of whichconsisted 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 ($P < 0.01$; $n = 3$).

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

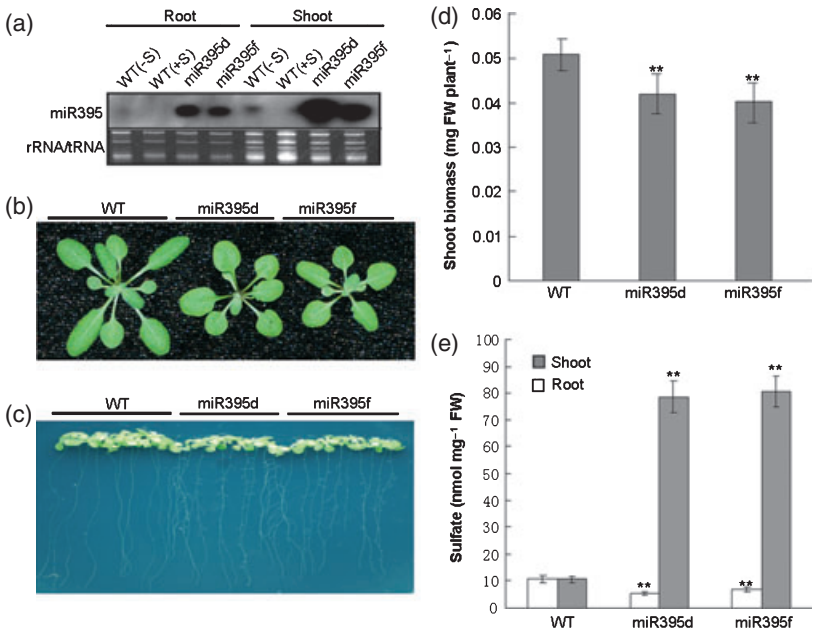

Figure 3. Phenotypes of miR395-over-expressing plants.
(a) Northern analysis of miR395-over-expressing plants.
(b) Small stature and slight chlorosis of miR395-over-expressing plants. Plants were grown for 3 weeks in soil.
(c) More lateral roots were produced by miR395-over-expressing plants. Plants 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).

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

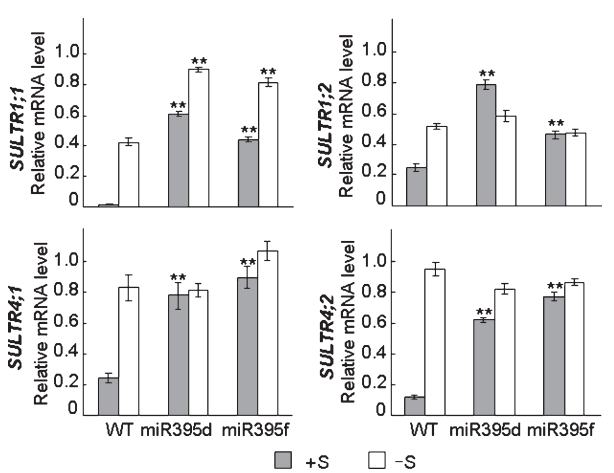

Figure 4. Expression of sulfate starvation-responsive genes in wild-type and miR395-over-expressing plants.
Real-time quantitative RT-PCR analysis of *SULTR1;1*, *SULTR1;2*, *SULTR4;1* and *SULTR4;2*. Plants were grown for 10 days on MS medium with 1500 μM sulfate (+S) or MS medium without sulfate (−S). 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.

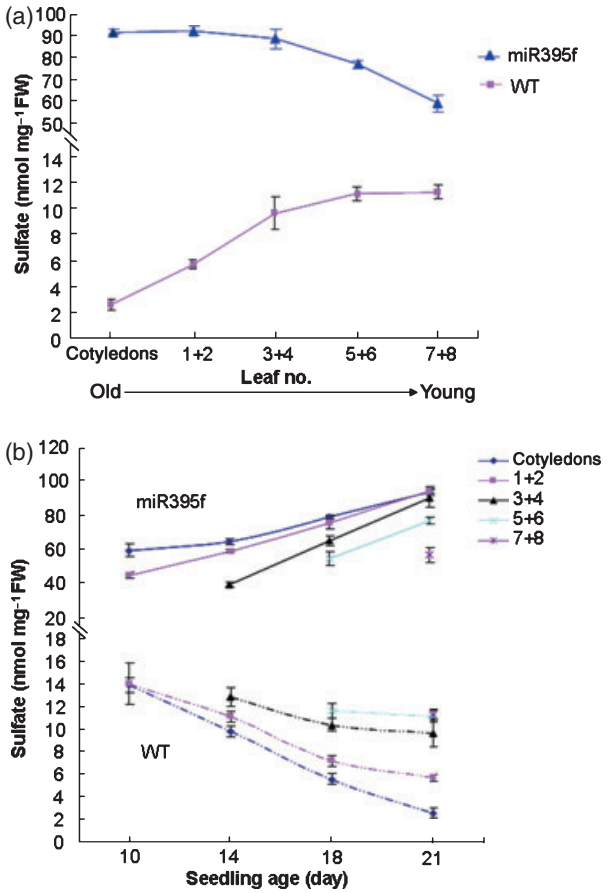

Figure 5. Distribution of sulfate between leaves.
(a) Sulfate content of two proximal leaves. miR395-over-expressing plants were defective in remobilization of sulfate between leaves.
(b) Changes in sulfate concentration in the leaves of wild-type plants (dotted lines) or miR395f-over-expressing plants (solid lines). Individual leaves (cotyledons, 1st and 2nd leaves, 3rd and 4th leaves, 5th and 6th leaves, and 7th and 8th leaves) were collected at the indicated times, starting with 10-day-old seedlings.
(a,b) Two proximal leaves were collected as one sample. Error bars represent SD (n = 3).

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

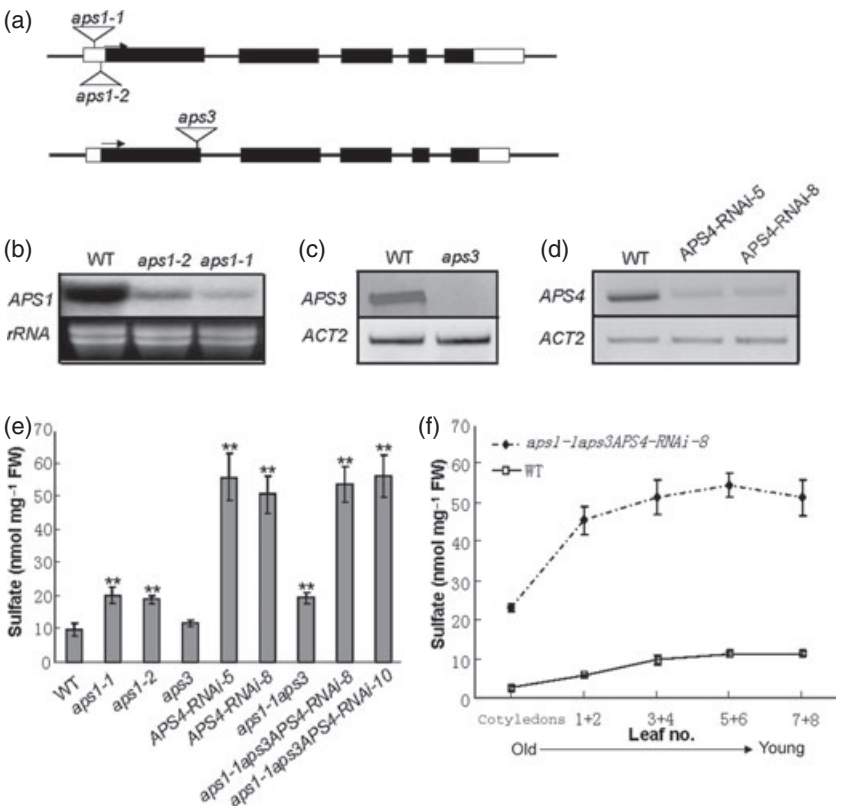

Figure 6. Phenotypes caused by loss of function of APS.
(a) Location of T-DNA in the *APS1* and *APS3* genes. The thick bars and lines indicate the exons and introns, respectively. White bars indicate the 5′ and 3′ UTRs. Triangles indicate the location of the T-DNA.
(b) Northern blot confirmation of *aps1-1* and aps*1-2* homogenous mutants.
(c) Confirmation of the *aps3* homogenous mutant.
(d) Confirmation of *APS4-RNAi* transgenic plants.
(e) Sulfate concentration of shoots from 21-day-old plants grown in soil. Error bars represent SD. Student's *t* test indicated that the values indicated by two asterisks were significantly different from the wild-type value ($P < 0.01$; $n = 10$).
(f) Phenotypes of *aps1-1 sultr2;1 APS4RNAi* plants. Sulfate content of two proximal leaves from 21-day-old plants grown in soil. *aps1-1* and *aps3* mutants were used for crossing. *35S:APS4RNAi* was then transformed into the double mutant *aps1-1 aps3*. Error bars represent SD ($n = 3$).

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

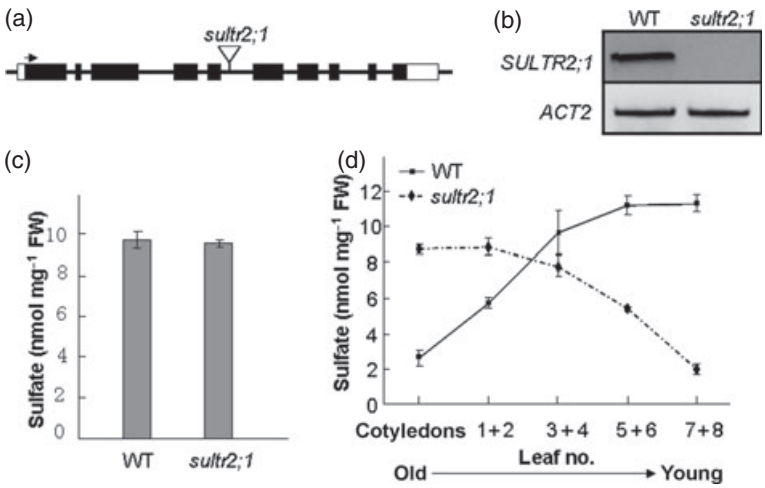

Figure 7. Phenotypes of the *SULTR2;1* T-DNA insertion line.
(a) Location of the T-DNA in the *SULTR2;1* gene. The thick bars and lines indicate the exons and introns, respectively. White bars indicate the 5′ and 3′ UTRs. The arrow indicates the start codon. The triangle indicates the location of the T-DNA.
(b) Confirmation of the *sultr2;1* knockout mutant.
(c) Sulfate concentration in the shoots of plants grown for 21 days in soil. *sultr2;1* mutant plants accumulate normal sulfate levels. Error bars indicate SD (n = 10).
(d) Distribution of sulfate between the leaves of the *sultr2;1* mutant. Error bars represent SD (n = 3).

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 *SULTR2;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 suggests 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 *SULTR2;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

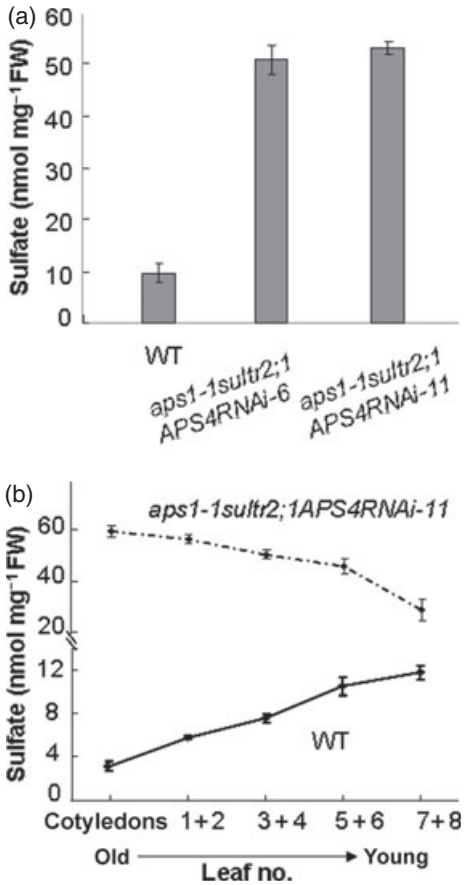

Figure 8. Phenotypes of *aps1-1 sultr2;1 APS4RNAi* plants.
(a) Sulfate concentration of shoots from 21-day-old plants grown in soil. Error bars represent SD (n = 3).
(b) Sulfate concentration of two proximal leaves from 21-day-old plants. *aps1-1 sultr2;1 APS4RNAi* plants are defective in the remobilization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

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 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, *SULTR1;1*, *SULTR1;2*, *SULTR4;1* and *SULTR4;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

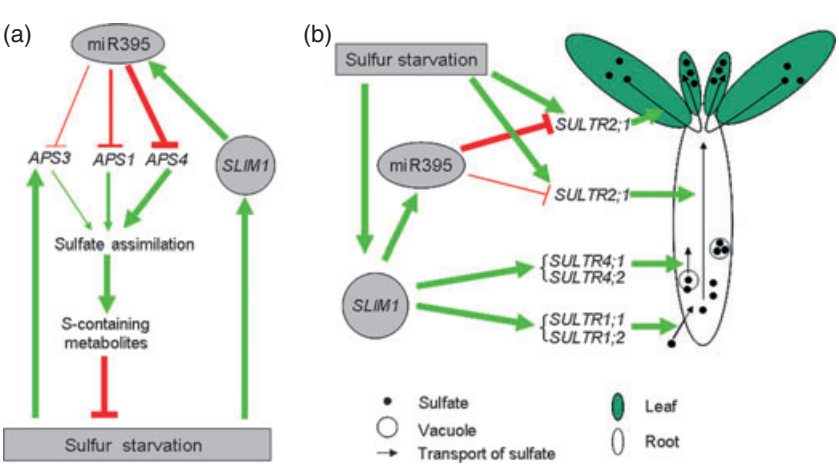

Figure 9. Models for plant sulfate signaling involving miR395.
(a) Regulation of *APS* genes by miR395.
(b) Regulation of sulfate transport by miR395.
(a,b) Expression of *APS* genes and *SULTR2;1* is regulated by miR395 induced by sulfur starvation, dependent on *SLIM1*. *APS3* and *SULTR2;1* are dually regulatedby sulfate starvation. The expression of sulfate starvation-responsive genes (*SULTR1;1*, *SULTR1;2*, *SULTR4;1* and *SULTR4;2*) is regulated by *SLIM*. Green lines indicate positive regulation; red lines indicate negative regulation. Thin, middle-sized and thick lines indicate slight, moderate and strong effects, respectively.

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 transcription 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 *SUITR4;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 $(NH_4^+)_2SO_4$. 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, Pre-miR395d-R, Pre-miR395f-L and Pre-miR395f-R (Table S2). The PCR products of the precursor sequence were cloned into the pUCM-T vector (Fermentas, http://www.fermentas.com) and confirmed by sequencing. The miR395 precursors 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 pHANNIBAL 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/vectors.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 mediim 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-indoyl-β-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 (Tababai and Bremner, 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 F_2 seeds were screened by PCR at one locus, and plants homozygous for the insertion at this locus were then screened at the second locus. *35S: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 [α-^{32}P]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 oligonucleotides 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 (http://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 genees 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_046518), *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) (2006AA02Z129) and Science Foundation of Yunnan Province (2004C0051M).

SUPPORTING INFORMATION

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

Figure S1. Expression of miR167 during sulfate starvation.
Figure S2. Mature miR395 sequences.
Figure S3. Characterization of *APS4-RNAi* plants.
Figure S4. Molecular characterization of multiple mutants.
Figure S5. Predicted miR395 target genes in various species.
Table S1. Conservation of miR395 and its target genes in various species.
Table S2. Sequences of primers used in this study.
Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Achard, P., Herr, A., Baulcomb, D.C. and Harberd, N.P. (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131**, 3357–3365.

Adai, A., Johnson, C., Mlotshwa, S., Archer-Evans, S., Manocha, V., Vance, V. and Sundaresan, V. (2005) Computational prediction of miRNAs in *Arabidopsis thaliana*. *Genome Res.* **15**, 78–91.

Adiputra, I.G.K. and Anderson, J.W. (1992) Distribution and redistribution of sulfur taken up from nutrient solution during vegetative growth in barley. *Physiol. Plant.* **85**, 453–460.

Allen, E., Xie, Z., Gustafson, A.M., Sung, G.H., Spatafora, J.W. and Carrington, J.C. (2004) Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*. *Nat. Genet.* **36**, 1282–1290.

Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. *Cell*, **121**, 207–221.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C. and Chiou, T.J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol.* **141**, 1000–1011.

Awazuhara, M., Fujiwara, T., Hayashi, H., Watanabe-Takahashi, A., Takahashi, H. and Saito, K. (2005) The function of SULTR2;1 sulfate transporter during seed development in *Arabidopsis thaliana*. *Physiol. Plant.* **125**, 95–105.

Axtell, M.J. and Bartel, D.P. (2005) Antiquity of microRNAs and their targets in land plants. *Plant Cell*, **17**, 1658–1673.

Bari, R., Datt-Pantm, B., Stitt, M. and Scheible, W.R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* **141**, 988–999.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281–297.

Bartel, B. and Bartel, D.P. (2003) MicroRNAs, at the root of plant development? *Plant Physiol.* **132**, 709–717.

Brodersen, P., Sakvarelidze-Achard, L., Brunun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L. and Voinnent, O. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science*, **320**, 1185–1190.

Buchner, P., Takahashi, H. and Hawkesford, M.J. (2004) Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. *J. Exp. Bot.* **55**, 1765–1773.

Carrington, J.C. and Ambros, V. (2003) Role of microRNAs in plant and animal development. *Science*, **301**, 336–338.

Chen, X. (2004) A microRNA as a translational repressor of *APETALA2* in Arabidopsis flower development. *Science*, **303**, 2022–2025.

Chiou, T.J. (2007) The role of microRNAs in sensing nutrient stress. *Plant Cell Environ.* **30**, 323–332.

Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F. and Su, C.L. (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell*, **18**, 412–421.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Law, T.F., Grant, S.R., Dangl, J.L. and Carrington, J.C. (2007) High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE*, **2**, e219.

Floyd, S.K. and Bowman, J.L. (2004) Gene regulation: ancient microRNA target sequences in plants. *Nature*, **428**, 485–486.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K. and Zhu, J.K. (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* **15**, 2038–2043.

Gandikota, M., Birkenbil, R.P., Hohmann, S., Cardon, G.H., Saedler, H. and Huijser, P. (2007) The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings. *Plant J.* **49**, 683–693.

Griffiths-Jones, S. (2004) The microRNA registry. *Nucleic Acids Res.* **32**, 109–111.

Guddeti, S., Zhang, D.C., Li, A.L., Leseberg, C.H., Kang, H., Li, X.G., Zhai, W.X., Johns, M.A. and Mao, L. (2005) Molecular evolution of the rice miR395 gene family. *Cell Res.* **15**, 631–638.

Hartmann, T., Mult, S., Suter, M., Rennenberg, H. and Herschbach, C. (2000) Leaf age-dependent differences in sulphur assimilation and allocation in poplar (*Populus tremula* × P. alba) leaves. *J. Exp. Bot.* **51**, 1077–1088.

Hatzfeld, Y., Lee, S., Lee, M., Leustek, T. and Saito, K. (2000) Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidopsis thaliana*. *Gene*, **248**, 51–58.

Hawkesford, M.J. and De Kok, L.J. (2006) Managing sulfur metabolism in plants. *Plant Cell Environ.* **29**, 382–395.

Hell, R. (1997) Molecular physiology of plant sulfur metabolism. *Planta*, **202**, 138–148.

Himelblau, E. and Amasino, R.M. (2001) Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. *J. Plant Physiol.* **158**, 1317–1323.

Hsieh, L.C., Lin, S.I., Shih, A.C.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H. and Chiou, T.J. (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. *Plant Physiol.* **151**, 2120–2132.

Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress induced miRNA. *Mol. Cell*, **14**, 787–799.

Kataoka, T., Hayashi, N., Yamaya, T. and Takahashi, H. (2004a) Root-to-shoot transport of sulfate in *Arabidopsis*. Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. *Plant Physiol.* **136**, 4198–4204.

Kataoka, T., Watanabe-Takahashi, A., Hayashi, N., Ohnishi, M., Mimura, T., Buchner, P., Hawkesford, M.J., Yamaya, T. and Takahash, H. (2004b) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. *Plant Cell*, **16**, 2693–2704.

Kawashima, C.G., Yoshimoto, N., Maruyama-Nakashita, A., Tsuchiya, Y.N., Saito, K., Takahashi, H. and Dalmay, T. (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *Plant J.* **57**, 313–321.

Lanet, E., Delannoy, E., Sormani, R., Floris, M., Brodersen, P., Crete, P., Voinnet, O. and Robaglia, C. (2009) Biochemical evidence for translational repression by Arabidopsis microRNAs. *Plant Cell*, **21**, 1762–1768.

Lappartient, A.G., Vidmar, J.J., Leustek, T., Glass, A.D.M. and Touraine, B. (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. *Plant J.* **18**, 89–95.

Leustek, T. and Saito, K. (1999) Sulfate transport and assimilation in plant. *Plant Physiol.* **116**, 1307–1313.

Llave, C., Xie, Z., Kasschau, K.D. and Carrington, J.C. (2002) Cleavage of *Scarecrow-like* mRNA targets directed by a class of Arabidopsis miRNA. *Science*, **297**, 2053–2056.

Lopez-Bucio, J., Cruz-Ramirez, A. and Herrera-Estrella, L. (2003) The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* **6**, 280–287.

Loudet, O., Saliba-Colombani, V., Camilleri, C., Calenge, F., Gaudon, V., Kurihara, Y. and Watanabe, Y. (2004) Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc. Natl Acad. Sci. USA*, **101**, 12753–12758.

Loudet, O., Saliba-Colombani, V., Camilleri, C., Calenge, F., Gaudon, V., Koprivova, A., North, K.A., Kopriva, S. and Daniel-Vedele, F. (2007) Natural variation for sulfate content in *Arabidopsis thaliana* is highly controlled by APR2. *Nat. Genet.* **39**, 896–900.

Lu, S., Sun, Y.-H., Shi, R., Clark, C., Li, L. and Chiang, V.L. (2005) Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. *Plant Cell*, **17**, 1378–1386.

Maher, C., Stein, L. and Ware, D. (2006) Evolution of Arabidopsis microRNA families through duplication events. *Genome Res.* **16**, 510–519.

Maruyama-Nakashita, A., Nakamura, Y., Yamaya, T. and Takahashi, H. (2004) Regulation of high-affinity sulfate transporters in plants: towards systematic analysis of sulfur signaling and regulation. *J. Exp. Bot.* **55**, 1843–1849.

Maruyama-Nakashita, A., Nakamura, Y., Tohge, T., Saito, K. and Takahashi, H. (2006) Arabidopsis *SLIM1* is a central transcriptional regulator of plant sulfur response and metabolism. *Plant Cell*, **18**, 3235–3251.

Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H. and Hoefgen, R. (2003) Transcriptome analysis of sulphur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. *Plant J.* **33**, 633–650.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B. and Bartel, D.P. (2002) MicroRNAs in plants. *Genes Dev.* **16**, 1616–1626.

Rotte, C. and Leustek, T. (2000) Differential subcellular localization and expression of ATP sulfurylase and 5′–adenylylsulfate reductase during ontogenesis of Arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. *Plant Physiol.* **124**, 715–724.

Saito, K. (2004) Sulfur assimilatory metabolism. The long and smelling road. *Plant Physiol.* **136**, 2443–2450.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M. and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. *Dev. Cell*, **8**, 517–527.

Smith, I.K. and Lang, A.L. (1988) Translocation of sulfate in soybean (*Glycine max L.* Merr). *Plant Physiol.* **86**, 798–802.

Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell*, **16**, 2001–2019.

Sunkar, R., Girke, T., Jain, P.K. and Zhu, J.K. (2005) Cloning and characterization of microRNAs from rice. *Plant Cell*, **17**, 1397–1411.

Tababai, M.A. and Bremner, J.M. (1970) A simple turbidimetric method of determining total sulfur in plant material. *Agron. J.* **62**, 805–806.

Takahashi, H., Watanabe-Takahashi, A., Smith, F.W., Blake-Kalff, M., Hawkesford, M.J. and Saito, K. (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. *Plant J.* **23**, 171–182.

Yoshimoto, N., Takahashi, H., Smith, F.W., Yamaya, T. and Saito, K. (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. *Plant J.* **29**, 465–473.

Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T. and Takahashi, H. (2003) Phloem-localizing sulfate transporter, *SULTR*1;3 mediates re-distribution of sulfur from source to sink organs in *Arabidopsis*. *Plant Physiol.* **131**, 1511–1517.

