Heterologous expression of a rice miR395 gene in *Nicotiana tabacum* impairs sulfate homeostasis

Ning Yuan, Shuangrong Yuan, Zhigang Li, Dayong Li, Qian Hu & Hong Luo

Sulfur participates in many important mechanisms and pathways of plant development. The most common source of sulfur in soil—SO$_4^{2-}$—is absorbed into root tissue and distributed into aerial part through vasculature system, where it is reduced into sulfite and finally sulfide within the subcellular organs such as chloroplasts and mitochondria and used for cysteine and methionine biosynthesis. MicroRNAs are involved in many regulation pathways by repressing the expression of their target genes. *MiR395* family in *Arabidopsis thaliana* has been reported to be an important regulator involved in sulfate transport and assimilation, and a high-affinity sulphate transporter and three ATP sulfurylases (ATPS) were the target genes of *AtmiR395* (*Arabidopsis thaliana miR395*). We have cloned a *miR395* gene from rice (*Oryza sativa*) and studied its function in plant nutritional response. Our results indicated that in rice, transcript level of OsamiR395 (*Oryza sativa miR395*) increased under sulfate deficiency conditions, and the two predicted target genes of *miR395* were down-regulated under the same conditions. Overexpression of OsamiR395h in tobacco impaired its sulfate homeostasis, and sulfate distribution was also slightly impacted among leaves of different ages. One sulfate transporter (SULTR) gene NtaSULTR2 was identified to be the target of *miR395* in *Nicotiana tobacum*, which belongs to low affinity sulfate transporter group. Both *miR395* and NtaSULTR2 respond to sulfate starvation in tobacco.

As a rudimental and essential element, sulfur is one of the six macronutrients required for plant growth and participates in many fundamental physiological and biochemical processes. In nature, sulfur exists in both inorganic and organic forms, and sulfate (SO$_4^{2-}$) is the most common inorganic source of sulfur plants acquire from soil. The sulfate absorption and assimilation pathway in plants is a complex system. In the very beginning, sulfate is absorbed into root tissue. Except for a small amount of sulfate stored in vacuole of root cells, the majority of them are distributed into aerial part through vasculature system. Upon transfer into subcellular organs such as chloroplasts and mitochondria in cells of aerial part, the sulfate is reduced into sulfite, then sulfide used for the synthesis of cysteine and methionine, two amino acids that play a pivotal role in sulfate assimilation pathway$^{1}$, and essential for supporting many important redox reactions in plants. The reduced form of the cysteine could function as an electron donor and its oxidized form could act as an electron acceptor.

Given the important role sulfur plays in plant growth and development, its deficiency (−S) would cause severe problems to plants, resulting in decreased plant yields and quality$^2$. To genetically improve plant sulfate uptake and utilization under −S conditions, it is essential to fully understand the functions of the genes encoding sulfate transporters and other important components involved in sulfate assimilation pathways$^3$.

Over the course of the past 20 years, essential genes involved in sulfate uptake, distribution and assimilation pathways have been identified and well-studied in different plant species. *Sis1*, *Sis2* and *Sis3* were the first sulfate transporter genes cloned from *Stylosanthes hamata* responsible for initial sulfate uptake and internal transport$^4$. In *Arabidopsis*, since the cloning of the first sulfate transporters, AST56 and AST68 two decades ago$^5$, at least 12 *Arabidopsis* sulfate transporters belonging to five different groups have been identified$^6$. These include two high-affinity sulfate transporters SULTR1;1 and SULTR1;2 responsible for uptake of sulfate from soil$^7$ and low-affinity sulfate transporters SULTR2;1 and SULTR2;2 responsible for internal transport of sulfate from root to shoot$^7$, SULTR3;5, the function partner of the SULTR2;1 that facilitates the influx of sulfate$^8$, and SULTR4;1 and SULTR4;2 involved in distribution of sulfate between *Arabidopsis* vacuoles and symplastic$^9$. The ORYaSultr1;1
and ORYsa:Sultr4;1 are the first two sulfate transporters cloned from rice in early 2000s\(^2\), followed by the identification of additional 12 sulfate transporters\(^3\).

ATP sulfurylase (ATPS) catalyzes the synthesis of the essential metabolic intermediate, adenosine 5'-phosphosulfate (APS), and this step is the branch point of the sulfate assimilation pathway followed by the synthesis subpathways of either cysteine or other sulfated compounds. ATPS has been extensively studied for the past decade because of its important role in the sulfate assimilation pathway and its functions in sulfate transport and assimilation. The targets of miRNA families sharing the same gene ancestors and regulating the same biological events. Research for the past two decades has led to the identification of 21 miRNA families including many well-studied ones such as miR156 and miR393 that seem to be highly conserved between monocots and dicots\(^4\). MiR395 is also on the list, but experimental support is still lacking.

Sequences of mature mir395 are highly conserved between model plant, Arabidopsis and crop species. The sequence of mature OsamiR395 is highly conserved while the pre-microRNA sequences are divergent. It has previously been demonstrated in Arabidopsis that mature AthmiR395 transcript accumulates under sulfur-limited conditions\(^18\). To investigate whether OsamiR395 also responds to low sulfate conditions as its counterpart in Arabidopsis, transcript level of OsamiR395 in two weeks old rice plants grown in N6 solid medium supplemented with different concentrations of sulfate was analyzed. Both northern blotting and stem-loop RT-PCR results showed that the transcripts of mature OsamiR395 accumulated under low sulfate conditions (0 and 20\(\mu\)M \(\text{SO}_4^{2-}\)), but declined significantly under sulfate-adequate conditions (1500 and 2000\(\mu\)M \(\text{SO}_4^{2-}\), Fig. 1a,b). In plant nucleus, miRNA gene is first transcribed into a long pri-miRNA, which is then processed into pre-miRNA and finally mature miRNA that is later translocated by HASTY into cytoplasm and induces the degradation of its target gene(s). To further understand whether OsamiR395 is regulated at the transcription level or post-transcription level, real-time PCR experiment was conducted to investigate the transcript level of pri-OsamiR395h in two weeks old rice plants grown in N6 solid medium supplemented with different concentrations of sulfate was analyzed. Both northern blotting and stem-loop RT-PCR results showed that the transcripts of mature OsamiR395 accumulated under low sulfate conditions (0 and 20\(\mu\)M \(\text{SO}_4^{2-}\)), but declined significantly under sulfate-adequate conditions (1500 and 2000\(\mu\)M \(\text{SO}_4^{2-}\), Fig. 1a,b).

In plant nucleus, miRNA gene is first transcribed into a long pri-miRNA, which is then processed into pre-miRNA and finally mature miRNA that is later translocated by HASTY into cytoplasm and induces the degradation of its target gene(s). To further understand whether OsamiR395 is regulated at the transcription level or post-transcription level, real-time PCR experiment was conducted to investigate the transcript level of pri-OsamiR395h in two weeks old rice plants grown in N6 solid medium supplemented with 0, 20, 1500 or 2000\(\mu\)M \(\text{SO}_4^{2-}\). Real-time PCR results showed that excess sulfate could repress the accumulation of pri-OsamiR395h transcript (Fig. 1c). Conversely, the transcription level of pri-OsamiR395h increased significantly under sulfate deficient conditions (0 and 20\(\mu\)M \(\text{SO}_4^{2-}\), Fig. 1c). Transcript levels of pri- and mature OsamiR395 exhibit the same trend under sulfate starvation stress, indicating that OsamiR395 expression is transcriptionally regulated by sulfate. Sulfate starvation stress induces the expression of pri-OsamiR395, leading to the production of more mature OsamiR395 transcripts.

Computational analysis of the rice genome sequences leads to the identification of four putative targets of OsamiR395, including one ATPS and three sulfate transporter genes, OsaSULTR2;1, OsaSULTR2 and OsaSULTR3;4 (Fig. 2a)\(^17,27\). RT-PCR results indicated that OsaATPS did not exhibit any responses in both roots and leaves under –S stress. OsaSULTR3;4 did not respond to sulfate treatment in leaves either, but was down-regulated in roots with the increasing sulfate concentrations, exhibiting similar expression pattern as OsamiR395 (Fig 2b). OsaSULTR2;1 and OsaSULTR2 genes were both down-regulated in leaves with the increasing sulfate concentrations (Fig. 2b), similar to the expression pattern of OsamiR395 in response to sulfate treatment (Fig. 1). On the contrary, they were both up-regulated in roots in response to increasing sulfate concentrations.
(Fig. 2b). It should be noted that OsaSULTR2 exhibited the highest induction under 20μM sulfate, suggesting that other regulation machineries may also participate in the regulation of the OsaSULTR2 gene under this particular condition. These results support the hypothesis that OsaSULTR2;1 and OsaSULTR2 are the putative target genes of, and regulated by OsamiR395 in rice roots. In rice leaves, however, OsamiR395-mediated transcript cleavage of the OsaSULTR2;1 and OsaSULTR2 genes may not be able to take place due to their non-overlapping tissue-specific expression. Instead, there may exist some other mechanisms regulating the expression of OsaSULTR2;1 and OsaSULTR2. This is also likely the case for OsaSULTR3;4 in roots. Similar phenomena have previously been observed in Arabidopsis.

To confirm the results of semi-quantitative RT-PCR, real-time RT-PCR was conducted to determine the expression levels of OsamiR395 and its putative targets in rice under −S condition (N6 medium without sulfate) and +S condition (regular N6 medium). Real-time PCR results were consistent with that of the semi-quantitative RT-PCR. In both leaves and roots, pri- and mature OsamiR395 were up-regulated under −S condition (Fig. 2c). Among the four putative target genes, only OsaSULTR2;1 and OsaSULTR2 were significantly down-regulated in rice roots under −S condition, exhibiting opposite trend of expression to OsamiR395 (Fig. 2d), in agreement with the results obtained by semi-quantitative RT-PCR and supporting the notion that OsaSULTR2;1 and OsaSULTR2 are the putative targets of OsamiR395 in rice roots.

**Expression of the OsamiR395 and its target genes is spatiotemporally regulated.** Besides the response of OsamiR395 and its targets to sulfate starvation stress, we also investigated the expression patterns of
OsamiR395 and its target genes in different developmental stages and tissues. To this end, we particularly focused on the primary miRNA level for one of the rice OsamiR395 genes, OsamiR395h and the expression of its putative target genes in both roots and leaves at different developmental stages under normal growth conditions. The RT-PCR results showed that the expression of pri-OsamiR395h was strongly induced only in the roots of the four weeks old plants, but otherwise remained very low in both roots and leaves in any other developmental stages (Fig. 3).

The expression of the ATPS again was quite stable in both tissues throughout the rice development, but an elevated expression level in roots was observed compared to that in leaves (Fig. 3). The expression levels of the three sulfate transporter genes were variable, but none of them was inversely correlated with that of the OsamiR395h (Fig. 3).

**Heterologous expression of pri-OsamiR395h in Nicotiana tabacum.** To further study the role OsamiR395 plays in sulfate transportation and distribution, we generated a chimeric DNA construct containing
the pri-OsamiR395h sequence driven by the CaMV35S promoter (Fig. 4a). This construct was then introduced into tobacco (Nicotiana tabacum) to produce a total of 10 independent transgenic events. RT-PCR analysis suggested rice pri-OsamiR395h was successfully expressed in tobacco (Fig. 4b), and small RNA northern blotting result suggested rice pri-OsamiR395h was successfully processed into mature miRNA (Fig. 4c). The detection of tobacco endogenous mature NtamiR395 in northern blotting indicated that mature NtamiR395 shares a highly conserved sequence with its rice homolog. Three independent transgenic events were selected for further analysis.

|                | 2 weeks | 4 weeks | 8 weeks |
|----------------|---------|---------|---------|
| Leaf           |         |         |         |
| Root           |         |         |         |
| Leaf           |         |         |         |
| Root           |         |         |         |

Figure 4. Heterologous expression of pri-OsamiR395h in Nicotiana tabacum. (a) The Schematic diagram of rice pri-OsamiR395h overexpression construct. Rice pri-OsamiR395h sequence containing stem-loop structure of OsamiR395h was cloned from rice genomic DNA and put under the control of the CaMV35S promoter. The hptII gene driven by CaMV35S promoter was used as selectable maker. The pre-OsamiR395h sequence was underlined. (b) RT-PCR analysis of pri-OsamiR395h expression in wild type and three transgenic tobacco lines. Total RNA samples were prepared from two weeks old wild type and transgenic tobacco plants grown in MS medium. Ntal25 was used as reference gene. (c) Small RNA northern blotting analysis of mature miR395 transcripts in wild type and three transgenic tobacco lines. Total RNA samples were prepared from two weeks old wild type and transgenic tobacco plants grown in MS medium. rRNA was used as loading control. WT: wild type plant. OE: overexpression line.

Figure 3. Expression level of pri-OsamiR395h and its target genes in rice leaf and root tissues at different developmental stages. Total RNA samples were prepared from leaf and root tissues of rice harvested at indicated time points and used for RT-PCR analysis. OsaSIZ1 was used as a reference gene. Experiment was repeated three times.
Overexpression of the rice pri-\textit{OsamiR395h} impairs sulfate homeostasis and leads to retarded plant growth in transgenic tobacco. It has previously been shown that overexpression of \textit{AthmiR395} in \textit{Arabidopsis} impairs its sulfate distribution and assimilation\textsuperscript{19}. To evaluate the impact of the \textit{OsamiR395} in tobacco sulfate metabolism and plant development, we first measured the total sulfur contents in transgenic tobacco plants and wild type (WT) controls. Not surprisingly, the total leaf sulfur content of all the transgenic lines was 2.16 to 2.50 times higher than that in WT controls. On the contrary, the root sulfur content in transgenic lines was 32\% to 42\% less than that in WT controls (Fig. 5a).

Next, we determined the sulfate-S (sulfate-sulfur) concentration in WT and transgenic plants. Again, the difference in sulfate-S concentrations between transgenics and WT controls was similar to that of the total sulfur contents. In transgenic leaf tissues, the sulfate-S concentration was 1.35 to 1.96 times higher than that in WT leaves, whereas in roots, transgenics had 38\% to 57\% less sulfate than WT controls (Fig. 5b). This result indicated that the high-level of miR395 accumulation in transgenic plants impacts the uptake and transportation of sulfur and sulfate.

Figure 5. Overexpression of pri-\textit{OsamiR395h} impacts tobacco sulfate transportation and distribution. (a) Statistical analysis of total sulfur in leaf and root tissues. Samples were harvested from four weeks old wild type and three transgenic tobacco lines. Data are presented as means of three biological replicates contains mixed samples from five biological replications, error bars represent SD (n = 3). (b) Statistical analysis of sulfate-S concentrations in leaf and root tissues. Samples were harvested from four weeks old wild type plants and three transgenic tobacco lines. Data are presented as means of fifteen biological replicates, error bars represent SD (n = 15). The statistically significant difference between groups was determined by one-way ANOVA (F(df\text{between}, df\text{within}) = F \text{ ration}, p = p-value, where df = degrees of freedom). Means not sharing the same letter are statistically significantly different (P < 0.05). (c) Statistical analysis of sulfate concentration in tobacco leaves of different ages. Leaves of 12 weeks old wild type and three transgenic tobacco lines were harvested in the positions as indicated in the figure. Data shown are an average of three biological replicates, error bars represent SD (n = 3). DW: dry weight. FW: fresh weight. WT: wild type.
resulting in interrupted sulfate assimilation pathway and consequently retardation in plant growth because of the
shortage of cysteine and other sulfate metabolic products.

Identification of miR395 target gene in tobacco. To understand how the excess miR395 impacts tobacco sulfate homeostasis at the molecular level, we sought to identify putative new target genes of miR395 using two approaches. We first used the DNA sequences of the Arabidopsis SULTR2;1 and ATPS genes to blast against the Nicotiana tabacum EST sequences. All the DNA sequences with high similarity (identity of more than 70%) were used to do alignment with complementary sequence of the mature OsamiR395h. The following criteria were used to determine the predicted target sequences with minor modifications: (1) No more than four mismatches between OsamiR395h and its predicted target genes; (2) No more than two constitutive mismatches
between OsamiR395h and its predicted target genes; (3) No mismatches between position 10 and 11; (4) No gaps between OsamiR395h and its predicted target genes. Besides, we also designed primers based on the AthmiR395 target genes (AthSULTR2;1 and AthATPS1, 3, 4) to amplify and identify the putative homologous genes in tobacco.

Using these approaches, we identified a novel gene named NtaSULTR2 to be a putative target of OsamiR395h (Fig. 7). Semi-quantitative RT-PCR analysis revealed that NtaSULTR2 was significantly down-regulated in transgenic tobacco (Fig. 7a). We cloned the full-length cDNA sequence of NtaSULTR2 using RACE (Rapid Amplification of cDNA Ends) method, and identified the target site of miR395 at between 135 bp to 156 bp. The target site was compared with the complementary sequence of mature OsamiR395h and NtamiR395. Asterisks indicate the identical sequences. (c) Phylogenetic analysis of NtaSULTR2 protein. Protein sequences of NtaSULTR2 and 16 sulfate transporters of rice and Arabidopsis were used to establish phylogenetic tree with MEGA6. In this phylogenetic tree, NtaSULTR2 protein is classified into the second group of sulfate transporter subfamily together with AthSULTR2;1, AthSULTR2;2 and OsaSULTR2;1.

Sulfate regulates tobacco NtamiR395 and NtaSULTR2. To confirm that NtaSULTR2 is the target of miR395 in tobacco, we investigated the expression level of both NtaSULTR2 and mature NtamiR395 under different sulfate concentrations.

In leaf tissues, the transcription of the mature NtamiR395 was gradually up-regulated, contrary to the gradually reduced sulfate concentration. However, NtaSULTR2 did not exhibit an opposite, but a similar expression pattern to NtamiR395 with its lowest transcript level being under 1500 μM (NH$_4$)$_2$SO$_4$ (Fig. 8a).

In root tissues, the situation was different. The transcript level of the mature NtamiR395 increased in response to sulfate depletion, similar to that observed in leaves, whereas NtaSULTR2 exhibited a roughly opposite, but more complex expression pattern (Fig. 8b). Compared to sulfate depletion conditions with 0 μM (NH$_4$)$_2$SO$_4$ supply, NtaSULTR2 was up-regulated under both 20 μM and 2000 μM (NH$_4$)$_2$SO$_4$, but down-regulated under 1500 μM (NH$_4$)$_2$SO$_4$. The results indicate that NtaSULTR2 might be regulated by NtamiR395 in roots but not in leaf tissues. These results correspond to the previous studies in Arabidopsis and rice showing that the expression level of AthSULTR2 is opposite to that of AthmiR395 in some, but not all plant tissues most likely due to the fact...
that the spatial expression pattern of AthmiR395 does not overlap with that of AthSULTR2;18,19,30, which could probably also explain the similar observation in tobacco from this study.

MiR395 mediates the cleavage of NtSULTR2 mRNA. To further confirm that NtSULTR2 is the true target of miR395, we conducted RLM-RACE (T4 RNA Ligase Mediated Rapid Amplification of cDNA Ends) to verify that NtSULTR2 transcripts are cleaved by miR395. We used RNA from the miR395-overexpressing transgenic tobacco plants to facilitate the detection of cleaved NtSULTR2 mRNA.

We used the forward primer ASP (Adapter Specific Primer) and the reverse primer GSP (Gene Specific Primer) to conduct the first round PCR after the adapter-linked first strand cDNA ends were generated. The RNA adapter has a length of 44 bp, and the reverse GSP is localized 545 bp downstream of the predicted miR395 target site in the NtSULTR2 mRNA, so the product of the first round PCR should have a length of about 589 bp. As shown in Fig. 9, the first round PCR with transgenic tobacco cDNA indeed generated a clear band of about 600 bp.

A second round PCR was then conducted using the first round PCR product as template and a new set of primers to confirm the authenticity of the PCR product. The forward primer NASP (Nest Adapter Specific Primer) is localized on the adapter from 14 bp to 44 bp, and the reverse primer NGSP (Nest Gene Specific Primer) is localized 463 bp downstream of the predicted miR395 target site in the NtSULTR2 mRNA, so the product of the second round PCR should be about 589 bp. As shown in Fig. 9, the second round PCR indeed generated a clear main band of about 500 bp as expected. Cloning and sequencing of the PCR product further confirmed the predicted miR395 cleavage site in the NtSULTR2 mRNA.

Discussion

Previous studies on Arabidopsis miR395 have indicated its involvement in sulfate starvation response by repressing the expression of genes in sulfate transportation and assimilation pathways.

Under −S condition, the accumulation of AthmiR395 is enhanced under low internal sulfate levels, and correlated to GSH pool, indicating that the regulation of AthmiR395 is mediated by internal sulfate level and redox
signaling in *Arabidopsis*22,31. The increased *AthmiR395* then represses the expression of *AthATPS1, AthATPS3, AthATPS4* and *AthSULTR2;1*19,22. Further study in *Arabidopsis* revealed a whole picture of how *AthmiR395* is involved in plant response to sulfate starvation. When sulfate supply is limited, the induced *AthmiR395* mediates the degradation of *ATPS* mRNA leading to the accumulation of sulfate in leaf tissues as a result of decelerated sulfate assimilation19. At the same time, the cleavage of *AthSULTR2;1* mRNA in shoots by *AthmiR395* results in blocked sulfate transport into new leaves from old ones 19. Furthermore, the impaired sulfate homeostasis and reduced sulfate assimilation impact seed germination under ABA-treated condition32.

MiR395 is highly conserved across species, which strongly suggests that its function in regulating plant response to nutrition, particularly sulfate supply could also be conserved during evolution. Our results in rice indicate that indeed, the transcript of mature *OsamiR395* increases under −S condition, and this change in expression might be regulated at the transcription level (Fig. 1). Computational prediction led to the identification of four putative target genes of *OsamiR395* in rice. We confirmed that *OsaSULTR2;1* and *OsaSULTR2* are regulated by *OsamiR395* in roots suggesting that they may be the *OsamiR395* target genes.

Knowledge about the functions of rice sulfate transporters is limited. Phylogenetic analysis grouped the fourteen rice sulfate transporters together with their *Arabidopsis* counterparts11, suggesting that they may share similar function. *OsaSULTR2;1* and *OsaSULTR2* may be responsible for the root-to-shoot sulfate transportation and distribution of sulfate between leaves of different ages. Our results (Fig. 2b–d) showed that the expression patterns of rice sulfate transporter genes were different from their *Arabidopsis* homologs, both *OsaSULTR2;1* and *OsaSULTR2* were reduced in leaves with the increasing sulfate concentrations. We speculate that the two sulfate transporter genes and *miR395* may be differentially expressed in different leaf tissues and thus, *OsaSULTR2;1* and *OsaSULTR2* may not be subjected to *miR395* regulation. Instead, other regulatory machineries may participate in the control of their expression in response to sulfate levels. It is likely that when rice plants are subjected to sulfate starvation, there is a need for the two sulfate transporters to be active, driving the transportation of sulfate from old leaves to younger ones to ensure plant growth and development. However, with abundant sulfate supply in the environment, there is no need for sulfate distribution to young leaves, and therefore the expression of both *OsaSULTR2;1* and *OsaSULTR2* declines.

The miRNA-mediated gene regulation mechanism emerged about 425 million years ago, which is at a very early stage of plant phylogeny prior to the divergence of monocot and dicot plants33. This suggests that monocot
and dicot plants should have a similar miRNA-mediated gene regulation mechanism, and some highly conserved miRNA families regulating the same biological process have evolved from the same gene ancestors. Indeed, research data in the past twenty years indicate that 21 miRNA families, such as miR156 and miR399, are conserved in sequence across monocots and dicots. More specifically, Zhang et al. found that 9 miRNA families are highly conserved, 10 miRNA families are moderately conserved and 16 miRNA families including miR395 are lowly conserved across plant species. In a later work, miR395 family was identified in the common ancestor of all embryophytes. Besides the miRNA sequences, the genes involved in miRNA and siRNA biogenesis pathways are also conserved across species. In plants, Dicer-like (DCL) is a key protein in the miRNA genesis pathway. DCLs interacting with HYPERHASTIC LEAVES1 (HYL1) and C2H2-zinc finger protein SERRATE (SE) in D-bodies cleaves the pri-miRNA from the base to yield a pre-miRNA with stem-loop structure, and this pre-miRNA is sliced again to yield mature miRNA. Phylogenetic analysis indicated that divergence of DCL1 gene associated with miRNA production from other DCLs could be traced to the time before the emergence of moss Physcomitrella patens, indicating that DCLs may have the same origin and are conserved across vascular plants.

Based on previous findings, we hypothesize that miRNA biogenesis pathway in dicots could accept pri-miRNAs from monocots, and process it into mature miRNA with function. To verify our hypothesis, the full-length DNA sequence of pri-OsamiR395h was cloned from rice genome. The expression cassette of the CaMV35S-controlled rice pri-OsamiR395h was then prepared and introduced into tobacco genome. By performing small molecule northern blotting, we observed high transcript level of miR395 in transgenic tobacco under normal condition, indicating that rice pri-OsamiR395h could be successfully expressed and processed into mature miR395 in tobacco (Fig. 4). At the same time, we also observed low level of endogenous mature miR395 in WT tobacco, confirming that tobacco mature miR395 is highly conserved with its rice homolog. All of the three transgenic tobacco lines exhibited impaired sulfate homeostasis and distribution (Fig. 5). Furthermore, transgenic plant had retarded growth phenotype (Fig. 6). All the facts suggest that mature OsamiR395 functions in transgenic tobacco.

Data obtained from this research revealed that the sulfate-S contents in transgenic tobacco are higher in leaf tissue, but lower in root tissue than those in WT controls. An even more significant difference in total sulfur content was observed between WT controls and OsamiR395h overexpression plants (Fig. 5a,b). Besides, we also observed that sulfate distribution between leaves of different ages is impaired in transgenic tobacco plants (Fig. 5c).

To reveal the molecular mechanism underlying miR395-mediated plant sulfate metabolism, we studied genes impacted by excessive dose of miR395 in transgenic tobacco, and identified a novel sulfate transporter gene NtaSULTR2 belonging to the second group of sulfate transporter genes (Fig. 7). Based on the results of real-time PCR and RML-RACE, we verified that NtaSULTR2 is the target gene of miR395 (Figs 8 and 9). We believe that the repression of NtaSULTR2 gene in transgenic tobacco plants partially impaired the sulfate homeostasis. In Arabidopsis shoot tissue, sulfate transporter AthSULTR2;1 is localized in both xylem and phloem, particularly in phloem parenchyma cells surrounding sieve and companion cells, and involved in distribution of sulfur between leaves of different ages. Although no ATP5 gene have been identified and cloned in tobacco, we believe that there must be one or more ATP5 gene(s) repressed in transgenic tobacco, causing interrupted sulfate assimilation. The interruption of the sulfate assimilation pathway would cause a shortage in cysteine and other sulfate metabolic products, resulting in retarded plant growth and triggering plant sulfate starvation signaling, which would promote sulfate absorption and transport into leaf tissue, and consequently a much more sulfur accumulation in leaves of transgenics than in that of WT controls (Fig. 5a,b).

**Materials and Methods**

**Plant materials and growth conditions.** To investigate the expression levels of OsamiR395 and its targets in rice under different sulfate concentrations, rice seeds were surface sterilized and grown in N6 medium under 16h light/8h dark at 28°C. Sulfate salts of the N6 medium were replaced with chloride salts and supplemented with 0, 20, 1500 or 2000 μM NH₄SO₄. Sterilized rice seeds were also grown in regular N6 medium (+S) and N6 medium without SO₄⁺ (−S) under 16h light/8h dark at 28°C. Two weeks old plants were harvested for RNA isolation.

To investigate the expression patterns of OsamiR395 and its targets in different developmental stages and tissues of rice, rice seeds were grown in soil in a greenhouse. Root and leaf samples were collected two, four and eight weeks after germination.

To investigate the expression levels of pri-OsamiR395h, mature miR395 and NtaSULTR2 in tobacco, tobacco seeds were surface sterilized and grown in MS medium under 16h light/8h dark at 22°C. To prepare MS mediums with different sulfate concentrations, sulfate salts of the MS medium were replaced with chloride salts and supplemented with 0, 20, 1500 or 2000 μM NH₄SO₄. Two weeks old and four weeks old plants were harvested for RNA isolation.

To measure total sulfate content and sulfate-S concentration in tobacco, and to determine the growth rate of tobacco, tobacco were grown in soil in a greenhouse. Four weeks old and 12 weeks old plants were collected for analysis.

**Genomic DNA and total RNA isolation, and cDNA synthesis.** Plant genomic DNA was isolated following previously described method.

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Total RNA was isolated from 100 mg plant samples with Trizol reagent (Ambion, USA), and the genomic DNA is removed by using RNase-free DNase I (Invitrogen, USA). 2 μg total RNA was used to synthesize first strand cDNA with SuperScript III Reverse Transcriptase (Invitrogen, USA) according to manufacturer’s instructions. The first strand cDNA was used for semi-quantitative RT-PCR and regular real-time PCR.

To determine the transcript level of mature miR395, the first-strand cDNA used for stem-loop real-time PCR was synthesized following the regular SuperScript III Reverse Transcriptase (Invitrogen, USA) mediated method, except that the oligo (dT)20 was replaced with miR395 specific reverse transcription primer. Primers were all listed in Supplementary Table S1.

Semi-quantitative RT-PCR, stem-loop and regular real-time PCR. To conduct semi-quantitative RT-PCR, first-strand cDNA samples were diluted to 0.25 times based on the concentration of the first-strand cDNA samples. The loading volume of the cDNA samples was adjusted basing on the transcript level of a reference gene.

To conduct stem-loop and regular real-time PCR, first-strand cDNA samples were diluted to 0.025 to 0.005 times based on the concentration of the first-strand cDNA samples. Both stem-loop and regular real-time PCR were performed using SYBR Green Supermix (Bio-Rad, USA) following manufacturer’s instructions, and IQ5 real-time detection system (Bio-Rad USA) was used to detect and analyze the real-time PCR result.

Stem-loop and regular real-time PCR results were determined by using \( \Delta \Delta \text{Ct} \) method. \( \Delta \text{Ct} \) was defined as \( \text{Ct}_{\text{test}} - \text{Ct}_{\text{ref}} \), in which \( \text{Ct}_{\text{test}} \) stands for threshold cycle of one gene after treatment, and \( \text{Ct}_{\text{ref}} \) stands for threshold cycle of one gene before treatment. \( \Delta \Delta \text{Ct} \) was defined as \( \Delta \text{Ct}_{\text{ref}} - \Delta \text{Ct}_{\text{target}} \), in which \( \Delta \text{Ct}_{\text{ref}} \) stands for \( \Delta \text{Ct} \) of the endogenous gene used as a reference, and \( \Delta \text{Ct}_{\text{target}} \) stands for \( \Delta \text{Ct} \) of target gene. Finally, related expression ratio was calculated as \( 2^{\Delta \Delta \text{Ct}} \).

Primers used for semi-quantitative RT-PCR, stem-loop real-time PCR and regular real-time PCR were all listed in Supplementary Table 1.

Small molecule Northern blotting. Small molecule northern blotting was performed following the method previously described with minor modification43. 10 μg total RNA denatured at 95 °C was separated in 12.5% urea-polyacrylamide gel and transferred to Hybond-N+ nylon membrane (Amersham, USA) in a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). To prepare radiolabeled probe for detecting mature miR395, DNA oligonucleotide GAGTTCCCCCAAACACTTCAC was synthesized (http://www.idtdna.com/site) and labeled with γ-[32P]-ATP by using T4 polynucleotide kinase. RNA membrane was then hybridized with radiolabeled probe and detected on a phosphorimaging screen.

Plasmid construction, bacterial strains and plant transformation. The predicted pri- OsamiR395h was amplified from rice genomic DNA and cloned at downstream of CaMV35S (Cauliflower Mosaic Virus 35S) promoter of binary vector pZH01, resulting in CaMV35S/OsamiR395h-CaMV35S/hygromycin42. This chimeric gene expression construct was then mobilized into Agrobacterium tumefaciens strain LBA4404 by electroporation for tobacco transformation. The Escherichia coli strain used in this experiment was DH5α.

The primers used for plasmid construction were all listed in Supplementary Table 1.

Determination of total sulfur content and sulfate-sulfur concentration. For determination of total sulfur, plant samples were collected and dried for 48 h at 80 °C. Total sulfur contents in dry samples were determined as previously described44. Sulfate-S concentration was determined following a previous method with minor modification43. sulfate-S concentration was determined following a previous method with minor modification.

Rapid amplification of cDNA ends. To obtain 5′ cDNA end and 3′ cDNA end of NtaSULTR2, total RNA was extracted from 100 mg two weeks old WT tobacco with Trizol reagent (Ambion, USA) and treated with RNase-free DNase I (Invitrogen, USA) to remove genomic DNA. 1 μg total RNA was then used to amplify 5′ end and 3′ end cDNA of NtaSULTR2 with SMARTer RACE 5′/3′ commercial kit (Clontech, USA) following the manufacturer’s instruction. Then, the 5′ end and 3′ end cDNA fragments were sequenced. Sequence information was used to design primers for cloning of full-length NtaSULTR2 cDNA.

The primers used for RACE and for cloning of full length NtaSULTR2 cDNA were all listed in Supplementary Table S1.

T4-RNA ligase mediated amplification of 5′ cDNA ends. To verify miR395 cleavage site within NtaSULTR2, T4-RNA ligase mediated amplification of 5′ cDNA ends was conducted following a previously described method45. Briefly, total RNA was isolated from 100 mg plant sample using Trizol reagent (Ambion, USA), followed by purification of RNA with RNasy mini kit (Qiagen, Germany). RNA adapter was ligated to the purified RNA by using T4 RNA ligase (New England Biolabs, USA). Based on the fact that miRNA-mediated mRNA cleavage will generate 5′-monophosphate ends on the 3′ end cleavage product of the target mRNAs, it is possible to ligate RNA oligonucleotide adapter to the 5′ terminus of the 3′ end cleavage product by using T4 RNA ligase, whereas such RNA oligonucleotide adapter would not be ligated to mRNAs with conventional 5′ cap45. Adapter-linked RNA was then used to synthesize first strand cDNA with SuperScript II Reverse Transcriptase (Invitrogen, USA), followed by amplification of 5′ ends using the forward primer ASP and the reverse primer GSP. The product from the first round PCR was then used as template for the second round PCR with the forward nest primer NASP and the reverse nest primer NGSP. PCR product was cloned for sequencing.

The primer sequences used for RML-RACE were all listed in Supplementary Table 1.
Phylogenetic analysis of sulfate transporters. Phylogenetic tree of NiaSULTR2 and other sulfate transporter genes in rice and Arabidopsis inferred using the Neighbor-Joining method\(^4\). The optimal tree with the sum of branch length = 3.89795523 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method\(^4\) and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 347 positions in the final dataset. Evolutionary analyses were conducted in MEGA6\(^4\). WT: wild type plant. OE: overexpression line.

Statistical analysis. Student's t test was used to test the difference between the means from two groups. 

\(P < 0.05\) was considered to be statistically significant and marked as *\(P < 0.01\) was considered to be statistically highly significant and marked as **. 

One-way ANOVA (F(df\text{between}, df\text{within}) = F \text{ ratio}, \(p = p\text{-value, where } df = \text{degrees of freedom}\) with post hoc comparisons using the Tukey HSD test was used to determine the statistically significant difference between the means from three or more groups. Means not sharing the same letter are statistically significantly different (\(P < 0.05\)).

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