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Reciprocal regulation among miR395, *APS* and *SULTR2;1* in *Arabidopsis thaliana*

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Sulfur element plays a pivotal role in plant growth and development. Recently, we have demonstrated that miR395 is crucial for the sulfate homeostasis through regulating the sulfate uptake, transport and assimilation in *Arabidopsis thaliana*. miR395 controls the sulfate concentration in the shoot by targeting three ATP sulfurylase genes (*APS*), which encode the first enzymes catalyzing sulfate activation in sulfur assimilation pathway. Furthermore, miR395 also regulates the transport of sulfate between leaves. Under sulfate starvation conditions, upregulated miR395 represses the expression of *SULTR2;1*, which then confined the transport of sulfate from mature to young leaves. Of note, transcript expression analysis suggested that, unlike *APS1* and *APS4* mRNA, *APS3* and shoot *SULTR2;1* is in accordance with miR395 in response to sulfate deprivation. We proposed that the differential regulation of targets by miR395 may be required for adaptation to the sulfate deficiency environment. In addition, our results revealed that there is reciprocal regulation between *SULTR2;1* and *APS* genes through miR395.

Key words: sulfate, miR395, *APS1*, *APS3*, *APS4*, *SULTR2;1*, sulfate transport, sulfate assimilation

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MicroRNAs (miRNAs) are a class of noncoding small RNAs, which post-transcriptionally regulate target mRNAs by cleavage or/and translation repression.[1,2] Several plant miRNAs have been identified to be involved in nutrients response, such as nitrogen, phosphor or sulfur.[3,4] Recent research suggested that miR395 is inducible by sulfate deprivation, and it targets two families of genes, *ATP Sulfurylases* and *SULTR2;1*, both of which function in sulfate metabolism pathway.[5-7] Our latest research revealed how miR395 functions in sulfate metabolism by regulating its target genes.[5] In miR395 overexpressing transgenic plant that exhibits sulfur deficiency symptoms, three *APS* genes (*APS1, APS3, APS4*) are repressed, which suppresses the activation of sulfate and then results in the over-accumulation of sulfate in the shoot. Meanwhile, the *SULTR2;1* transcripts are down-regulated, which then disrupts the transport of sulfate from mature leaves to young. Additionally, *SULTR1;1* and *SULTR1;2* are significantly upregulated, which contribute to the influx of sulfate from soil. In spite of the sulfate over-accumulation in the shoot, the root sulfate content is lower than that of wild-type plants, which can be attributed to the induced *SULTR4;1* and *SULTR4;2*, the products of which serve for the efflux of sulfate from root vacuoles. These evidences suggest that miR395 regulates the homeostasis of sulfate in *Arabidopsis thaliana*.

Although all target genes are repressed in the miR395 overexpressing transgenic plants, they display varied responses to sulfate starvation in wild-type plants.[5] Under sulfate depletion, *SULTR2;1* is induced in roots and repressed in shoots, respectively. Kawashima et al.[6] revealed that the spatially overlapping expression of miR395 and *SULTR2;1* led to the negative correlation between them in shoots, and the different tissue-specific expression allowed the positive correlation between them in roots. As expected, *APS1* and *APS4* transcripts decreased in

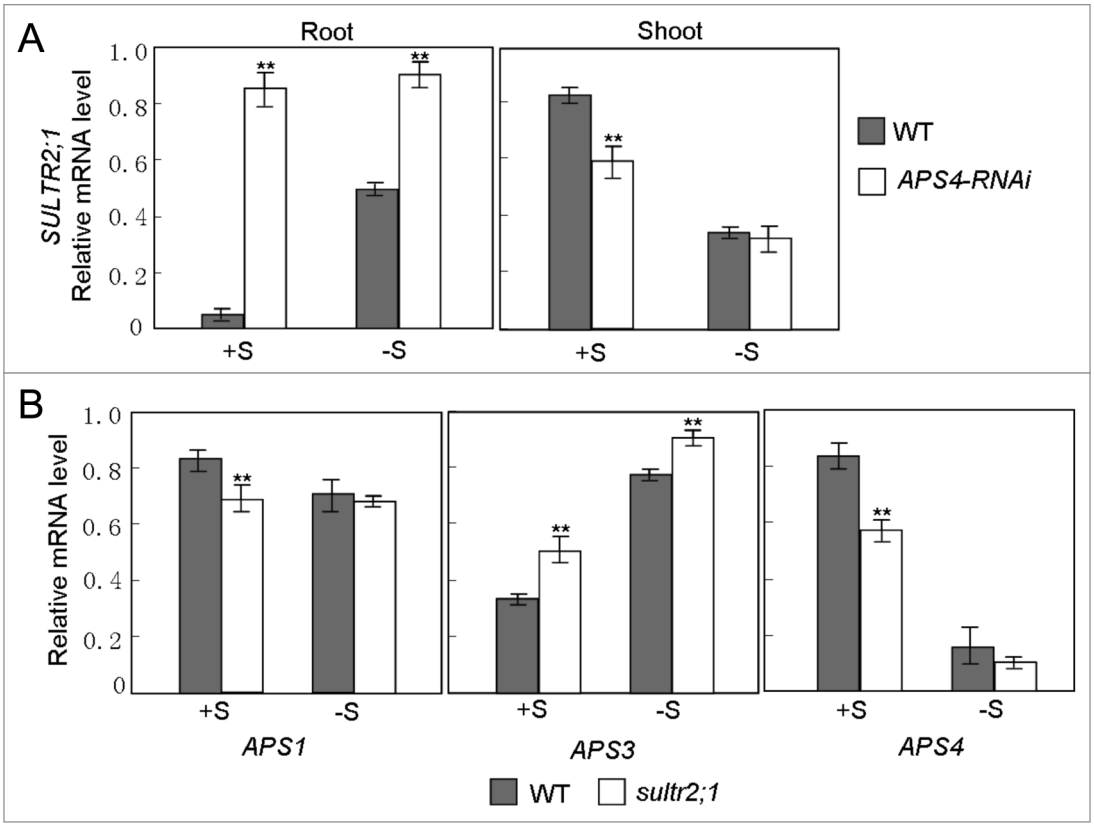

Figure 1. Real-time PCR analysis of miR395 target genes. (A) The expression of *SULTR2;1* in the root and shoot of *APS4-RNAi* plants. (B) The expression of *APS1*, *APS3* and *APS4* in the shoot of *sultr2;1* mutant plants. (A and B) Plants were grown for 10 days on MS medium with 1,500 lM sulfate (+S) or MS medium without sulfate (-S). RNA was isolated from roots and shoots, respectively. 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 ($p < 0.01$; $n = 3$).

response to sulfate deficiency. The expression of *APS3* gene was induced by sulfate starvation, although its repressor miR395 was upregulated. We suggested that *APS3* might avoid the cleavage by miR395 in a manner similar to *SULTR2;1* and serve to activate sulfate for assimilation under sulfate starvation condition when *APS1* and *APS4* were repressed. Therefore, to further investigate the accurate tissue-specific expression of these *APS* genes and their specific functions is required for understanding the mechanism underling sulfate assimilation.

In addition to the regulation of target genes by miR395, we also found that the lack of *APS4* gene results in the enhanced expression of miR395.[5] This indicates that miR395 and *APS4* are connected by a negative feedback loop. Our previous results also showed that *APS1* and *APS3* were suppressed in the *APS4*-silienced plants.[5] Depending on these evidences, we proposed that *APS4* regulated the expression of *APS1* and *APS3* by miR395. To investigate whether *SULTR2;1* was also affected by *APS4*, we detected *SULTR2;1* transcript levels in the root and shoot of *APS4*-silienced plants (**Fig. 1A**). As expected, *SULTR2;1* was downregulated in the shoot, which can be attributed to the induction of miR395. However, *SULTR2;1* was significantly induced in the root, suggesting that it responded to the sulfate starvation resulting from the lack of *APS4*. This is consistent with our previous conclusion that *SULTR2;1* is dually regulated by sulfate deficiency. According to these evidences, we draw the conclusion that *APS4* genes affected the expression of *SULTR2;1*. It is necessary to make sure whether *SULTR2;1* can affect the expression of *APS* genes. We analyzed the expression of the three *APS* genes in the shoot of *sultr2;1* mutant (**Fig. 1B**). Under sulfate sufficiency conditions, *APS1* and *APS4*, but not *APS3*, were downregulated in *sultr2;1* mutant, which was similar to their expression patterns in wild type under sulfate deficiency, although the fold-changes were not equal totally. Unlike wild-type plants, the sulfate concentration of young leaves was lower than that of mature leaves in *sultr2;1* mutants. We proposed that the sulfate deficiency of young leaves accounted for the expression patterns of *APS* genes in *sultr2;1* mutant. In conclusion, there are cross-talks between sulfate transport and assimilation pathways by miR395 targeting *SULTR2;1* and *APS* genes.

Recently, several miRNAs have been identified that they are involved in the feedback regulation of their target.[8-10] Our results suggested that miR395 targets can feedback regulate the expression of miR395, which then controls the expression of other target genes. The complicate regulation of sulfate metabolism pathway may enable plants to survive in sulfate fluctuation environment.

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