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miR395 is a general component of the sulfate assimilation regulatory network in Arabidopsis

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ABSTRACT

In plants, microRNAs play an important role in many regulatory circuits, including responses to environmental cues such as nutrient limitations. One such microRNA is miR395, which is strongly up-regulated by sulfate deficiency and targets two components of the sulfate uptake and assimilation pathway. Here we show that miR395 levels are affected by treatments with metabolites regulating sulfate assimilation. The precursor of cysteine, *O*-acetylserine, which accumulates during sulfate deficiency, causes increase in miR395 accumulation. Feeding plants with cysteine, which inhibits sulfate uptake and assimilation, induces miR395 levels while buthionine sulfoximine, an inhibitor of glutathione synthesis, lowers miR395 expression. Thus, miR395 is an integral part of the regulatory network of sulfate assimilation.

1. Introduction

MicroRNAs (miRNA) are essential components in regulatory networks in plants and animals and have a plethora of functions. The first targets identified for plant miRNAs were transcription factors involved in development [1,2]. However, soon it became apparent that miRNAs are responsible for control of numerous other processes, prominent among which is nutrient uptake and assimilation [3–6]. MiR395 was amongst the first such miRNAs identified [3]. MiR395 is very strongly induced by sulfate limitation [3,7] and resembles in this respect miR399 and miR398, which are induced by phosphate and copper starvation, respectively [4,5]. In addition, a systematic survey showed that 20 miRNAs were differentially expressed upon nitrogen or phosphate deficiency [8]. MiR395 targets multiple genes from two gene families involved in sulfate uptake and assimilation. Computational methods predict four targets in *Arabidopsis thaliana*, the low affinity sulfate transporter *SULTR2;1* and three out of four members of the ATP sulfurylase (ATPS) gene family. ATPS encodes for the enzyme which catalyses the first step of sulfate assimilation, activation of sulfate to adenosine 5′phosphosulfate (APS) [3,9–12]. Three of the targets were confirmed experimentally in both shoot and roots of *A. thaliana*, while the cleavage of *ATPS3* seems to be restricted to the shoots only [9]. MiR395 is strongly induced by sulfate deficiency, and is crucial for regulation of sulfate homeostasis [10,11]. Overexpression of miR395 results in accumulation of sulfate in the leaves due to increased translocation from the roots [10,11].

The effects of miR395 on its targets follow different mechanisms. Only the *ATPS4* isoform of ATP sulfurylase undergoes the canonical regulation, where its transcript levels strongly decrease with increased miR395 accumulation [10,11]. *ATPS1* mRNA levels have been reported either to decrease slightly [3,11] or not to be affected by sulfate deficiency [10,13]. This is explained by a simultaneous increase in *ATPS1* transcription [10]. Transcript levels of *SULTR2;1* are actually higher in sulfate deficient roots than in control roots, due to a non-overlapping cell-specific expression pattern for *SULTR2;1* [9]. Nonetheless, the placement of miR395 in the sulfate deficiency regulatory network was strongly corroborated by showing that the induction of miR395 accumulation is dependent

Abbreviations: APS, adenosine-5′-phosphosulfate; ATPS, ATP sulfurylase; APR, adenosine-5′-phosphosulfate reductase; APK, APS kinase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SOT, sulfotransferase; GFP, green fluorescent protein; PSK, phytosulfokine

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on SLIM1, a central regulator of sulfate limitation response [9,10,14].

Sulfate assimilation is highly regulated according to plant demand for reduced sulfur [12]. Sulfate uptake and reduction capacity increase during sulfate starvation in response to the cysteine precursor *O*-acetylserine (OAS), or when glutathione levels are depleted by stress or by inhibition of synthesis with buthionine sulfoximine (BSO) [12,14–17]. On the other hand, they are inhibited when reduced sulfur is available, e.g., as cysteine or glutathione, or during nitrogen deficiency [18,19]. Although the physiological responses to these different conditions have been well described, little is known about molecular mechanisms and regulatory networks responsible for translating the signals. So far, the increased transcript accumulation of sulfate transporters and many (but not all) genes by sulfate deficiency has been shown to be dependent on SLIM1 [12] and the coordination of primary sulfate assimilation and glucosinolate synthesis achieved by a family of six MYB factors [20]. In addition, a central role of LONG HYPOCOTYL5 (HY5) for integration of light and metabolic signals in regulation of APS reductase has recently been revealed [21].

Here we show that miR395 is an integral part of the sulfate assimilation regulatory networks beyond the sulfate deficiency response. We show that miR395 levels respond to several treatments regulating sulfate assimilation, such as feeding with OAS, BSO, and cysteine. However, the effects of such modulated miR395 levels on its targets are marginal, and transgenic manipulation of miR395 levels does not affect the general response to these treatments. We show that miR395 accumulation is dependent on SLIM1 not only during sulfate deficiency and that this accumulation is most probably triggered by reduced internal sulfate levels.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (ecotype Col-0) plants were used as WT in this study. The creation of *35S::miR395* and *MIM395* plants was described previously [10,22]. Mutants with modified contents of sulfate and glutathione were described as follows: *fou8* [23], *cad2* [24], *rax1* [25], *sultr1;2* [26]. Plants were grown for two weeks on vertical plates with GM-agarose media [27] at 20 °C under 16-h-light/8-h-dark cycles, before being transferred to fresh GM plates (control) or treatment plates (1 mM OAS, 1.25 mM BSO, 1 mM Cys, 1.25 mM BSO and 1 mM Cys) for a further three days [9,10]. Three independent pools of roots from at least 40 plants and shoots of 20 plants were homogenised and used for all subsequent measurements, and experiments were replicated independently.

2.2. RNA isolation and expression analysis

For the detection of miRNAs, total RNA was extracted from Arabidopsis shoot and root tissues and analysed as described in [10]. For expression analysis of the miR395 targets total RNA was isolated from roots by phenol/chloroform/isoamylalcohol (25:24:1) extraction and LiCl precipitation and repurified using an RNeasy Plant Mini Kit (Qiagen) including the DNAse treatment. Expression of *ATPS1-4*, *SULTR2;1*, and *APR2* genes was analysed by real-time quantitative RT-PCR (qPCR) as described in [10]. The Arabidopsis *TIP41* gene was used as a standard for *ATPS* and *SULTR2;1* measurements and the *UBC* gene was used for determination of *APR2* (for primer sequences see Table S1) [10]. Relative quantification of expression levels was performed using the comparative Ct method. At least three independent RNA preparations from independently grown plants were analysed with three technical replicates for the qPCR.

2.3. Enzyme assays

APS reductase activity was determined as the production of [^{35}S]sulfite, assayed as acid volatile radioactivity formed in the presence of [^{35}S]APS and dithioerythritol as reductant [28]. ATPS was measured as the APS and pyrophosphate-dependent formation of ATP [29]. The protein concentrations were determined according to Bradford with bovine serum albumin as a standard.

2.4. HPLC analysis of low molecular weight thiols

Thiols were determined in 20–30 mg of leaf material by a standard HPLC method as described in [28].

2.5. Determination of sulfate uptake and translocation

Sulfate uptake and translocation was measured using [^{35}S]sulfate, essentially as described in [10]. Plants were grown on vertical GM plates for 14 days, before transfer to either GM plates (control) or GM + 1 mM OAS plates (OAS) for a further 3 days. Subsequently, the plants were transferred into 24-well plates, with the roots submerged in 1 mL of nutrient solution adjusted to a sulfate concentration of 0.2 mM and supplemented with 6.7 μCi [^{35}S]sulfate (Hartmann Analytic) to specific activity of 1,860 kBq (nmol sulfate)$^{-1}$ and incubated in light for 4 h. After incubation seedlings were washed 3 times with 1 mL of cold non-radioactive nutrient solution and carefully blotted with paper tissue. Roots and shoots were separated, weighed, transferred into 1.5 mL tubes, and frozen in liquid nitrogen. The ^{35}S in plant material was quantified using scintillation counting.

3. Results

3.1. Regulation of miR395 by OAS

The effects of sulfate starvation on miR395 levels and its targets have been well characterised [9–11]. Since the sulfate assimilation pathway is also highly regulated by pathway intermediates and products, we tested whether miR395 accumulation is affected by these metabolites as well. In the first experiment, OAS clearly induced miR395 levels in the roots, but not in the shoots (Fig. 1A). The OAS effect on miR395 was however weaker than that of sulfate deficiency. The induction of miR395 was not accompanied by corresponding changes in transcript accumulation of its targets. *ATPS1* and *SULTR2;1* mRNA levels were higher in OAS treated roots than in the control roots, whereas *ATPS4* and other ATPS isoforms were not affected (Fig. 1B). Correspondingly, no difference in ATPS activity was observed between control and OAS-treated roots (Fig. 1C). The OAS treatment resulted in strong increase of cysteine and glutathione contents in the roots (Fig. 1D) and induction of *APR2* mRNA (Fig. 1B) in agreement with the expected response.

To test whether mis-expression of miR395 affects the response of sulfate uptake and assimilation to OAS, plants constitutively expressing the two forms of miR395, miR395c and miR395e [10], and plants where the action of miR395 was inhibited by a target mimic [10,22], were analysed alongside the *slim1–1* mutants and WT Col-0 (Fig. 2). OAS treatment resulted in increased sulfate uptake in all genotypes. As observed previously [10,11], over-expression of miR395 resulted in higher translocation of sulfate from roots to the shoots at control conditions, but this was not affected by OAS in all genotypes except *MIM395* (Fig. 2B).

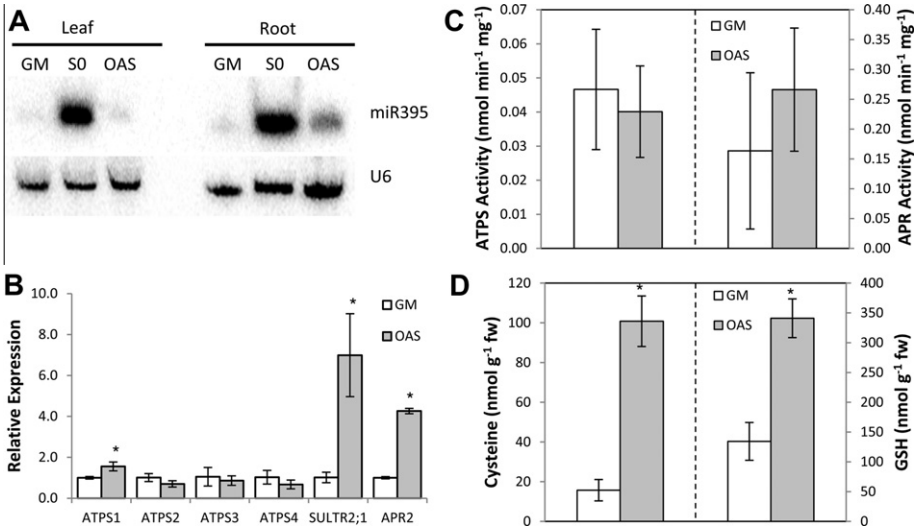

Fig. 1. Analysis of miR395 and its targets in Arabidopsis roots following OAS feeding. Col-0 seedlings were grown for two weeks on agar plates with GM growth media (GM) before transfer for four days on standard GM, GM without a sulfur source (S0), or GM supplemented with 1 mM *O*-acetylserine (OAS). (A) Northern blot analysis of miR395. The blot was exposed for one day. (B) Quantitative RT-PCR of 4 *ATPS* genes, *SULTR2;1* and *APR2*. The mRNA levels were compared to *TIP41* or *UBC* (*APR2*), and levels in plants grown on GM are set to 1. (C) ATPS and APR enzyme activities. (D) Cysteine and glutathione levels. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk show significant (Student's *t*-test; $P \leqslant 0.05$) difference between OAS treated and control plants.

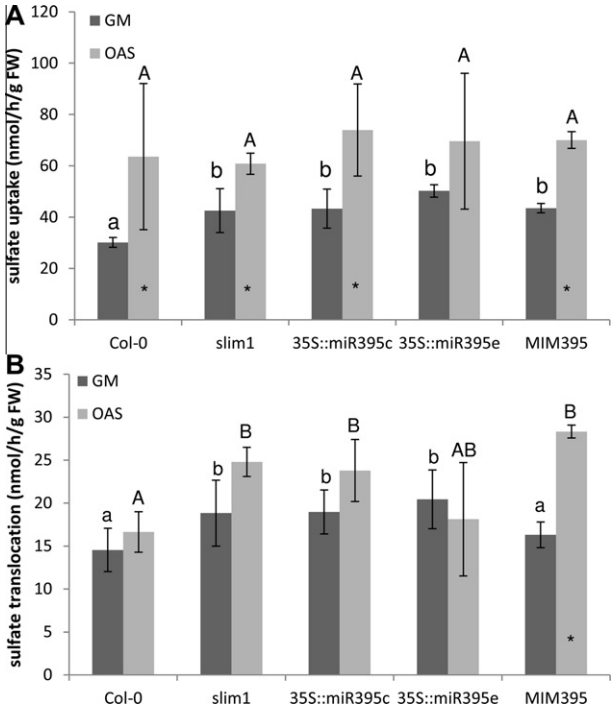

Fig. 2. Analysis of miR395 and its targets in Arabidopsis roots following OAS feeding. Arabidopsis Col-0, *slim1*, two lines overexpressing miR395, *35S::miR395c* and *35S::miR395e*, and plants with reduced accumulation of miR395 by target mimicry (*MIM395*) were grown on agar plates with GM growth media for two weeks and then transferred to either GM or GM supplemented with 1 mM *O*-acetylserine (OAS). (A) Sulfate uptake and (B). translocation rate of sulfate from roots to the shoots was determined. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk show significant (Student's *t*-test; $P \leqslant 0.05$) difference between OAS treated and control plants, different letters indicate significant differences between treatments.

3.2. Regulation of miR395 by BSO and cysteine

Corresponding to the model of demand-driven regulation, the inhibition of glutathione synthesis by BSO induces the sulfate assimilation pathway [17,30]. BSO treatment caused a reduction in miR395 accumulation in the roots (Fig. 3A). However, the mRNA levels of its targets as well as other ATPS isoforms also decreased (Fig. 3B). Only *APR2* transcript levels were increased as expected. Correspondingly, APR activity was increased in BSO-treated roots compared to controls whereas ATPS activity was not affected (Fig. 3C). The correct function of the treatment was confirmed by low levels of glutathione in BSO-treated roots (Fig. 3D). Cysteine levels were surprisingly not affected in the BSO-treated roots, however, they were highly increased in the leaves (data not shown), confirming the known effect of BSO on thiol levels in plants [12].

On the other hand, cysteine, which inhibits sulfate uptake and assimilation, caused an increase in miR395 levels in both roots and leaves. The induction of miR395 was abolished when BSO was added simultaneously with the cysteine treatment to prevent the metabolisation of the cysteine to glutathione (Fig. 4A). The treatments and the altered expression of miR395 had little effect on the target transcripts that were largely unaffected, as well as mRNAs for other ATPS isoforms (Fig. 4B). In agreement with the qPCR results, ATPS activity did not differ in roots treated with Cys or Cys and BSO and control roots. On the other hand, APR activity was reduced by Cys treatment but this increase was reversed by addition of BSO together with Cys (Fig. 4C). Thiol levels corresponded to the expectations, feeding Cys increased both Cys and glutathione in the roots while the latter was prevented by addition of BSO (Fig. 4D).

3.3. Dissection of miR395 regulation

A large part of the response of plants to sulfate starvation, including the induction of miR395, is controlled by SLIM1 [9,10,14]. Therefore we tested whether the induction of miR395 by the other treatments is also under SLIM1 control. After OAS treatment miR395 was increased in roots of Col-0 but not of *slim1–1* mutant line, where the levels were higher under control conditions (Fig. 5A). Thus, miR395 is an integral part of sulfate assimilation regulatory network and acts downstream of the SLIM1 regulator. Consistently over many experiments, in control conditions miR395 accumulated to higher levels in *slim1–1* than in Col-0. A major difference between the two genotypes is lower

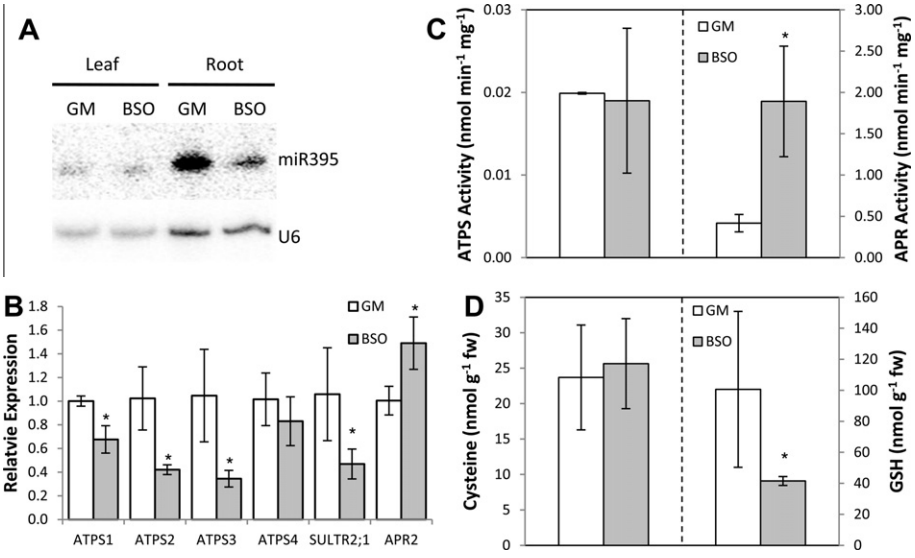

Fig. 3. Analysis of miR395 and its targets in Arabidopsis roots following BSO treatment. Col-0 seedlings were grown for two weeks on agar plates with GM growth media (GM) before transfer for four days on either standard GM or GM supplemented with 1.25 mM buthionine sulfoximine (BSO). (A) Northern blot analysis of miR395. The blot was exposed for four days. (B) Quantitative RT-PCR of 4 *ATPS* genes, *SULTR2;1* and *APR2*. The mRNA levels were compared to *TIP41* or *UBC* (*APR2*), and levels in plants grown on GM are set to 1. (C) ATPS and APR enzyme activities. (D) Cysteine and glutathione levels. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk show significant (Student's *t*-test; $P \leqslant 0.05$) difference between BSO treated and control plants.

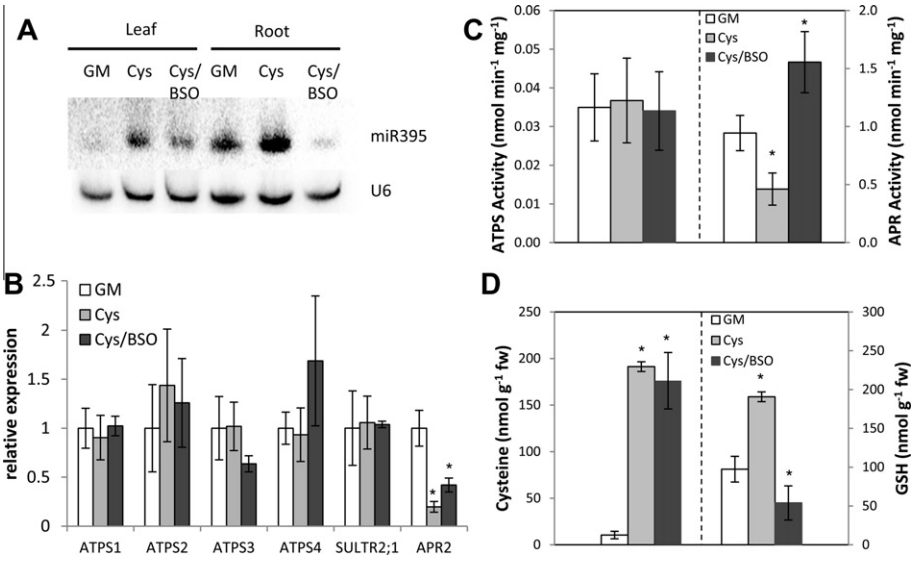

Fig. 4. Analysis of miR395 and its targets following cysteine treatment. Col-0 seedlings were grown on agar plates with GM growth media (GM) for two weeks, before transfer for four days on GM, GM supplemented with 1 mM cysteine, or GM with 1 mM Cys and 1.25 mM BSO. (A) Northern blot analysis of miR395. The blot was exposed for four days. (B) Quantitative RT-PCR of 4 *ATPS* genes, *SULTR2;1* and *APR2*. The mRNA levels were compared to *TIP41* or *UBC* (*APR2*), and levels in plants grown on GM are set to 1. (C) ATPS and APR enzyme activities. (D) Cysteine and glutathione levels. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk show significant (Student's *t*-test; $P \leqslant 0.05$) difference between treated and control plants.

sulfate content in *slim1–1* even under normal S supply [10]. Therefore, we compared miR395 levels in several other mutants with altered contents of sulfur containing compounds: the *fou8* mutant which was recently shown to possess lower levels of sulfate and glutathione [23], *cad2* and *rax1*, two allelic mutants in a gene for γ-glutamylcysteine synthetase, the first enzyme in glutathione synthesis, possessing low glutathione levels [24,25], and *sultr1;2* deficient in the high affinity sulfate transporter [26] and therefore having low sulfate content. The miR395 levels were significantly increased in roots and shoots of the *fou8* and *sultr1;2* mutants with low sulfate levels but unaffected in *cad2* and *rax1* (Fig. 5B).

As these experiments revealed a correlation between miR395 levels and sulfate content, we tested whether sulfate levels can explain the regulation of miR395 by OAS, BSO, and cysteine. Only cysteine treatment and combined Cys/BSO resulted in an increase of sulfate content in the roots, in roots of plants treated with OAS or BSO sulfate levels were unaffected (Fig. 6). Thus, the correlation between sulfate levels and miR395 accumulation is disrupted during these treatments.

4. Discussion

4.1. Regulation of miR395 accumulation

MicroRNAs often accumulate in response to changes in nutrient availability [3–8]. Among the nutrition-responsive miRNAs the

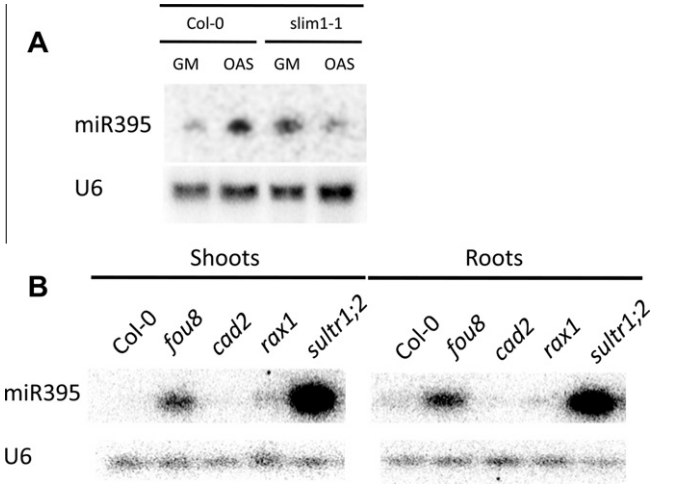

Fig. 5. Regulation of miR395. The levels of miR395 were determined by northern blotting. (A) Col-0 and *slim1-1* seedlings were grown on agar plates with GM growth media (GM) for two weeks, before transfer for four days on GM or GM supplemented with 1 mM OAS. Total RNA was isolated from the roots. (B) The various Arabidopsis mutants were grown for 2 weeks on GM agar plates. Total RNA was isolated from the roots and leaves. As controls, the blots were stripped and rehybridised with probe against U6 RNA.

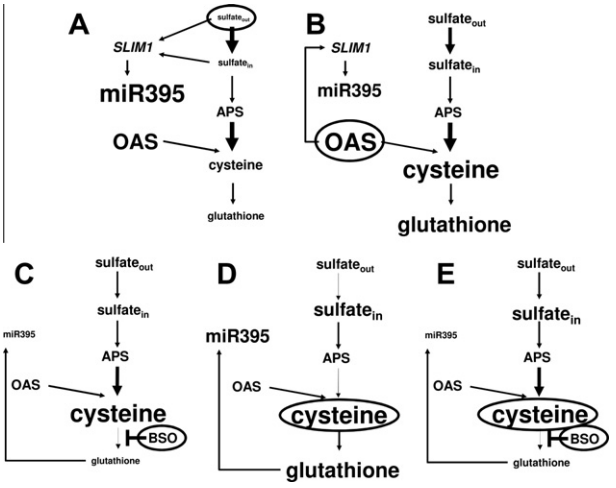

Fig. 7. Schematic representation of regulation of sulfate assimilation and miR395 accumulation by (A) sulfur deficiency; (B) treatment with OAS; (C) inhibition of glutathione synthesis by BSO; (D) treatment with cysteine; and (E) simultaneous treatment with cysteine and BSO. The size of the letters represent the changes in metabolite or miR395 accumulation, the thickness of arrows shows increase or decrease in sulfate uptake, flux through primary assimilation and rate of glutathione synthesis. The compounds used for the treatments are circled.

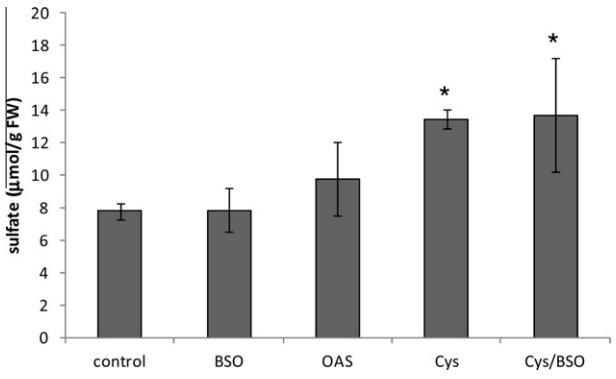

Fig. 6. Sulfate levels in roots of treated plants. Col-0 seedlings were grown on agar plates with GM growth media (GM) for two weeks, before transfer for four days on GM, GM supplemented with 1.25 mM BSO, 1 mM OAS, 1 mM cysteine, or 1 mM Cys and 1.25 mM BSO. Sulfate contents in the roots were determined by HPLC. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk show significant (Student's *t*-test; $P \leqslant 0.05$) difference between treated and control plants.

miR395 and miR399 are the most studied as they are involved in regulation of homeostasis of two essential macronutrients, sulfate and phosphate [4,9–11]. The roles of these miRNAs in plant response to the respective nutrient deficiency have been well established [9–11,31]. The experiments described here, however, point to a much more general involvement of miR395 in sulfate assimilation regulatory circuits.

MiR395 was extensively investigated for its function in sulfate deficiency response [9–11]. It is induced by sulfate deficiency and through its action on target transcripts of *ATPS1*, *ATPS4*, and *SULTR2;1* it causes increased translocation of sulfate from the roots to the shoots. This is achieved by simultaneous restriction of SULTR2;1 expression to xylem and reduction of flux through sulfate assimilation in the roots [9–11]. Little is known about the molecular mechanisms of the control of sulfate starvation response, apart from the involvement of the SLIM1 transcription factor [14]. MiR395 induction by sulfate deficiency is also SLIM1-dependent [9]. This induction is, however, not necessarily connected to low levels of external sulfate in the traditional sulfate starvation experiments [9–14]. We show here that in mutants characterised by low sulfate accumulation, *fou8* and *sultr1;2* [23,26], miR395 levels are elevated even at sufficient sulfate supply (Fig. 5B). It seems therefore, that the trigger of miR395 accumulation is linked to internal sulfate levels and not external sulfate availability (Fig. 5B), as shown recently for regulation of expression of protein coding genes responding to sulfate starvation [23].

Since several genes induced by sulfate deficiency have been shown to be regulated directly by the cysteine precursor OAS [32], it was of interest to test if miR395 shares the same regulation. OAS treatment triggers a response similar to sulfate deficiency: increased sulfate uptake, APR activity and flux through the pathway [16,21] (Figs. 2 and 7). Also similar to sulfate starvation, OAS increased levels of miR395 (Figs. 1 and 7). Thus, miR395 regulation is analogous to regulation of many other genes affected by sulfate starvation and OAS, and seems to be part of the same transcriptional network [13,32]. Indeed, the regulation of miR395 expression by OAS was eliminated in the *slim1* mutant (Fig. 5A) in analogy to SLIM1-dependency of miR395 induction in sulfate deficient plants [9]. It appears, therefore, that response to OAS treatment shares the same regulatory circuit as sulfate starvation, including SLIM1.

This result has a wider implication, as it shows that SLIM1 also participates in other regulatory networks than sulfate starvation. While the *SLIM1* gene has been found in a genetic screen for reduced response to sulfate limitation [14], the *slim1* mutant differed from Col-0 in sulfate uptake and translocation even at normal sulfate supply (Fig. 2). MiR395 levels are higher in the *slim1* mutant than in Col-0 (Fig. 5a; [10]) and together with the increase in sulfate translocation (Fig. 2; [10]) this can be explained by lower sulfate content in the mutant. Clearly SLIM1 is not the only transcription factor responsible for regulation of sulfate starvation response as, e.g., APS reductase regulation is SLIM1-independent [14]. The mechanism of action for SLIM1 is not clear, as its transcript levels are not affected by sulfate starvation and its binding to the corresponding promoters has never been demonstrated. The higher sulfate uptake in *slim1* on normal sulfate supply is actually consistent with the higher mRNA levels for *SULTR1;1* at control conditions determined by Maruyama-Nakashita et al. [14],

suggesting that SLIM1 may possess a dual function as activator at low S supply and repressor during a normal sulfur status.

The accumulation of miR395 in the *fou8* and *sultr1;2* mutants and in OAS treated plants, however, was much lower than in sulfate starved plants (Figs. 1, 5B). While sulfate limitation results in OAS accumulation, OAS treatment did not affect sulfate levels (Fig. 6) and the miR395 induction was only moderate (Fig. 1). Therefore, it seems that OAS is not the only signal of sulfate deficiency. While OAS is capable of inducing miR395 expression by itself, in the sulfate starvation response its role is probably only in amplification of the signal.

An intriguing feature of the miR395 induction by sulfate starvation, is that the resultant down-regulation of its targets *ATPS1* and *ATPS4* is counteracting the induction of the key enzyme of sulfate reduction, APS reductase [10]. The same is true for OAS, because OAS also induces APS reductase [16,19,21]. The regulation of the sulfate assimilation pathway by OAS is part of the demand-driven regulatory circuit [12,16]. Accordingly, the pathway is repressed in the presence of reduced sulfur and induced when demand for reduced sulfur is high. The classical example of the latter is the induction caused by depletion of glutathione due to inhibition of its synthesis by BSO [17,18,30]. As mRNA for APS reductase is increased in BSO treated plants, to achieve co-ordinated regulation of the pathway miR395 was expected to be down-regulated. Indeed, BSO affected miR395 levels negatively, in accordance with the demand-driven principle (Figs. 3 and 7). Cysteine treatment, on the other hand, results in down-regulation of APS reductase mRNA levels [18] and the simultaneous up-regulation of miR395 (Figs. 4 and 7), presumably to coordinate the regulation of ATPS with the rest of the pathway. Cysteine itself, however, is not the signalling metabolite. Simultaneous treatment with cysteine and BSO to inhibit cysteine metabolisation to glutathione revealed that when the γ-glutamylcysteine synthetase is inhibited, the increase of miR395 accumulation does not take place. This is similar to the regulation of APS reductase by cysteine and glutathione [17,18]. Could glutathione be the general signalling metabolite for regulation of miR395? Measurements of S-containing compounds revealed that while sulfate levels were diminished in sulfate starved plants and increased in cysteine and cysteine/BSO treatments, cysteine and glutathione levels were affected in all treatments. When compared with the pattern of miR395 expression, the miR395 levels were correlated with glutathione content, but not sulfate or cysteine, with the exception of sulfate starvation (Fig. 7). However, as it was demonstrated that regulation of sulfate assimilation by cadmium stress is not linearly dependent on glutathione alone [33], contribution of cysteine and γ-glutamylcysteine, particularly to maintaining the redox state of the cells, cannot be excluded. These results thus indicate that regulation of miR395 is complex and linked to at least two distinct regulatory circuits, one SLIM1-dependent during sulfate starvation and one linked to glutathione or more generally thiol levels and/or redox state.

4.2. Effects of miR395 regulation on the sulfate assimilation pathway

Our results show clearly that miR395 is regulated by treatments affecting demand for reduced sulfur. However, its function in these regulatory circuits is far from clear. MiR395 causes increased translocation of sulfate from roots to leaves during sulfate starvation or when constitutively over-expressed, despite relatively small effects on the activity of its targets [9–11]. Also in the experiments described here, despite significant changes in miR395 levels, the mRNA levels of its targets were hardly affected or even regulated in an opposing manner (Figs. 1, 3 and 4). Neither the increased miR395 levels in roots of OAS- or Cys- treated plants nor the decreased levels in BSO-treated roots affected ATPS activity. The same results were obtained in shoots, where miR395 levels changed according to the demand-driven regulation but the targets were not affected. At the same time, the *APR2* mRNA levels and APS reductase activity were regulated exactly as expected. It seems therefore that the changes in miR395 levels, apart from sulfate starvation response, have only little effect on the regulation of the sulfate assimilation pathway. Indeed, while during sulfate starvation over-expression or inactivation of miR395 by target mimicry have strong effects on sulfate uptake and translocation to the roots [10,11], the regulation of these processes by OAS were not affected in corresponding genotypes (Fig. 2). However, the changes in miR395 levels in OAS, BSO, and cysteine treated plants are much lower than those in sulfate starved plants so the effects can be expected to be much weaker. Also, whole roots were analysed, so that subtle regulation of miR395 targets in specific cells and tissues might have been masked. In addition, miR395 is encoded by six genes, which are regulated differently by sulfate starvation [9]. The regulation by pathway intermediates is also likely to differ among the individual genes adding additional complexity. Thus, the function of the changing levels of miR395 during OAS, cysteine, and BSO treatments remains to be discovered.

In conclusion, we have shown that miR395 is integral part of the regulatory network of sulfate assimilation beyond the sulfate starvation response. We also demonstrated the function of the SLIM1 transcription factor in regulation of OAS response. Our data suggest that miR395 is induced by decreases in internal sulfate levels but is correlated to glutathione levels in the presence of sufficient sulfate. Since a direct regulation of the miR395 targets was not observed, it is obvious that the regulation of sulfate assimilation pathway is more complex than anticipated.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet. 2012.06.044.

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