DOI: 10.1007/s10535-015-0511-4 BIOLOGIA PLANTARUM 59 (3): 491-496, 2015

Effect of sulfate availability on root traits and microRNA395 expression in sugar beet

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Abstract

Nutritional stress is one of the main limits to sugar beet yield. This study evaluated morphological and molecular responses of sugar beet to changes in sulfate availability. Morphological characteristics of the root system and the accumulation of microRNA395 (miR395) were examined in sulfate(S)-supplemented and S-deprived seedlings under hydroponic conditions. We also investigated the functional role of miR395 in regulating the expression of *APS1* gene coding for ATP-sulfurylase in roots and leaves. The S-deprived seedlings showed a significant increase in the number of root tips, in the miR395 expression in leaves but not in roots, and in the expression of *APS1* gene. Our results indicate that miR395 may be a useful biomarker for sulfate status in sugar beet.

Additional key words: ATP-sulfurylase, *Beta vulgaris*, root morphology.

Introduction

Sulfate assimilation is strongly regulated in a demand-driven way, but the molecular basis of this regulation is almost unknown (Forieri *et al*. 2013). Transcriptomic approaches have been used recently to better understand the regulation of plant responses to sulfate starvation (Watanabe *et al*. 2012). In response to sulfate depletion, plants induce sulfate uptake, by the coordinated activation of specific sulfate transporters, and increase ATP sulfurylase (ATPS) activity (De Kok *et al*. 2012). Important regulatory players in plant nutrient uptake processes are the microRNAs that target transcription factors and down-regulate their expression by mRNA cleavage or deadenylation (Zeng *et al*. 2014). Jones-Rhoades and Bartel (2004) observed that microRNA395 (miR395) plays an important role in the regulatory pathway of sulfur metabolism in plants. During sulfate starvation in *Arabidopsis*, the expression of miR395 is significantly up-regulated together with other components of sulfate assimilation, such as the low-affinity sulfate transporter SULTR2,1 and the ATPS activity, the first steps of sulfate assimilation, encoded by *APS* genes (Liang *et al*. 2010, Jagadeeswaran *et al*. 2014). Here, the role of miR395 appears pointless (Kawashima *et al*. 2011) because miRNAs are supposed to down-regulate the expression of the target transcripts. This mismatch is partly explained by the discovery of the *SLIM-1* gene coding for a protein activated by sulfate deprivation and able to induce the expression of sulfate transporters in the root with a consequent increase of sulfate uptake. The combined action of both miR395 and *SLIM-1* is involved not only in the fine-tuning of *ATPS* transcription and ATPS activity to match the needs of plants for reduced sulfur, but also in a fine-regulation and optimization of sulfate homeostasis in plant tissues (Kawashima *et al*. 2009). Sulfate homeostasis under sulfur starvation has been studied recently and its regulation depends on different tissues and developmental phases. This complex spatial-temporal post-transcriptional mechanism (Kruszka *et al*. 2012) is not entirely clear and still prevents the understanding of the plant sulfate-starvation regulatory pathway (Yoshimoto *et al*. 2002, Buchner *et al*. 2004, Kataoka *et al*. 2004, Parmar *et al*. 2007, Barberon *et al*. 2008, Liang and Yu 2010).

Sulfur assimilation is strictly synchronized with nitrogen assimilation and plays a central role in crop productivity (Lappartient and Touraine 1996, Kopriva and Rennenberg 2004, Davidian and Kopriva 2010). The sugar beet yield and technological quality also depends on sulfate and nitrogen availability and assimilation (Steven *et al*. 2000). A better understanding of the sugar

Submitted 24 July 2014, *last revision* 2 December 2014, *accepted* 22 December 2014.
Abbreviations: ATPS - ATP sulfurylase; miR395 - microRNA395.
Acknowledgements: The authors wish to thank Dr. Ericka Havecker and Prof. David Baulcombe (the University of Cambridge, UK) for the Northern blot analysis. The work was supported by ex-60% funds from the University of Padova, Italy.
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beet responses to sulfate deprivation is needed to develop cultivars with a greater tolerance to this nutrient starvation (Saccomani *et al*. 2009). Root morphology, sulfur content, and the role of the miR395 were evaluated in leaves and roots of sugar beet sulfate starved plants or in plants grown under different sulfate concentrations in the nutrient solution. The aim of this study was to investigate the changes of the regulatory pathways of sulfate metabolism in response to different sulfate availability.

Materials and methods

Seeds of sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll) cv. Shannon (obtained from *Lion Seeds*, Maldon, UK) were surface-sterilized in 1 % (m/v) sodium hypochlorite for 10 min and germinated on filter paper moistened with distilled water in the dark at 25 °C for 48 h. For the analysis of sugar beet response to changes in sulfate availability, two $MgSO_4$ concentrations were tested: 100 mM, which represents an optimal sulfate supply, and 500 mM, which represents a high sulfate availability. Firstly, seedlings were grown in an S-deprived solution containing 200 µM $Ca(NO_3)_2$, 200 µM KNO_3, 200 µM $MgCl_2$, 40 µM KH_2PO_4, and microelements (Arnon and Hoagland 1940). On the 6th day, $MgCl_2$ was replaced by 100 µM $MgSO_4$ or 500 µM $MgSO_4$. Sixty seedlings were collected immediately and 48 h after the change of S availability. All treatments were done in triplicate and the experiment was performed five times.

Fresh leaves and roots were rinsed three times in distilled water, blotted with paper towels and ground to powder in liquid nitrogen. Sulfate was extracted in 20 cm^3 of *Millipore* water by incubation at 70 °C for 30 min. The extract was centrifuged at 20 000 *g* for 30 min, and the supernatant filtered through a 0.45-µm filter unit. Sulfate content was determined by optical emission spectroscopy with inductively-coupled plasma (*ICP-OES*, *Spectro*, Kleve, Germany).

Roots were stained with 0.1 % (m/v) toluidine blue (*Sigma-Aldrich*, Montreal, Canada) for 15 min to increase contrast. The stained root systems were placed in 3 mm deep water in a plexiglas tray and scanned (*STD 1600*, *Regent Instruments*, Quebec, Canada). The scanned images were analyzed to determine total root length, average root diameter, root surface area, and total number of root tips by the *WinRhizo* software (*Regent Instruments*).

Total RNA was extracted from 100 mg of leaf and root tissues using a *Eurogold TriFastTM* kit (*EuroClone*, Milan, Italy) following the manufacturer's recommend-ations. The RNA was quantified by absorbance at 260 nm and stored at -80 °C. The RNA quality was assessed using a small RNA kit (*Agilent Technologies*, Palo Alto, CA, USA) on *Agilent 2100 Bioanalyzer*. One microgram of total RNA was reverse transcribed using a *QuantiMir* kit (*System Biosciences*, Mountain View, CA, USA) in a total volume of 0.02 cm^3 following the manufacturer's recommendations. The cDNAs were used to analyze the expression of *miR-395* and its target gene *APS1* by real-time PCR. Real-time PCR experiments were conducted in a final volume of 0.02 cm^3 containing 0.01 cm^3 of a 2× *Power Syber Green* PCR master mix (*Life Technologies*, *Carlsbad, CA, USA* USA), 0.002 cm^3 of primers, and 0.001 cm^3 of cDNA. Real-time RT-PCR was performed on a *QuantStudio 12K Flex* real-time PCR system (*Life Technologies, Carlsbad, CA, USA*) using the following thermocycler program: 10 min pre-incubation at 95 °C followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold method was used to analyze *miR395* and *APS1* expressions. Cycle threshold change values (ΔC_T) were calculated on the basis of the *18S rRNA* internal standard. The relative expression was calculated using the ΔC_T method previously applied by Hajizadeh *et al*. (2011). For Northern blot analysis, miR395 was analyzed with radioactive probes following the protocol indicated by Havecker *et al*. (2012) and was performed in collaboration with Prof. David Baulcombe (the University of Cambridge, UK). A primer sequence used in the analysis of *miR395* expression was obtained from a previous study with *Arabidopsis thaliana* (Axtell and Bartel 2005). Primers for amplification of *APS1* and *18S* genes were designed based on sequences selected from the reference genome *RefBeet_0.9* draft assembly (http://bvseq.molgen.mpg.de). Primer sequences are reported in Table 1.

Table 1. Sequences of designed primers used in the molecular analysis.

Name	Sequence (5'→3')
miR395a	GAGTTCCCCCAAACACTTCAG
APS1 forward	AGAAGAAGATCAAAACATGGCTTC
APS1 reverse	TTCTCTCTTTTTCTCTCTCCTCAAA
18S forward	GTAACCCGTTGAACCCCATT
18S reverse	CCATCCAATCGGTAGTAGCG

The data are expressed as mean values ± standard errors. Analysis of variance (one-way *ANOVA*) for morpho-physiological variables was performed using the software *Statistica 10.0* (*StatSoft*, Tulsa, OK, USA). Means were compared using the Fisher's protected LSD test at $\alpha < 0.05$.

Results

The root sulfate content of seedlings grown at 500 μM sulfate for 48 h was not significantly different from that of seedlings grown at 100 μM sulfate, whereas the leaf sulfate content of seedlings grown at 500 μM sulfate was significantly higher ($P < 0.01$) than that at 100 μM sulfate (Fig. 1). The -S(48) seedlings displayed a significantly higher (115 %, $P < 0.01$) number of root tips than the S(0) seedlings (Table 1).

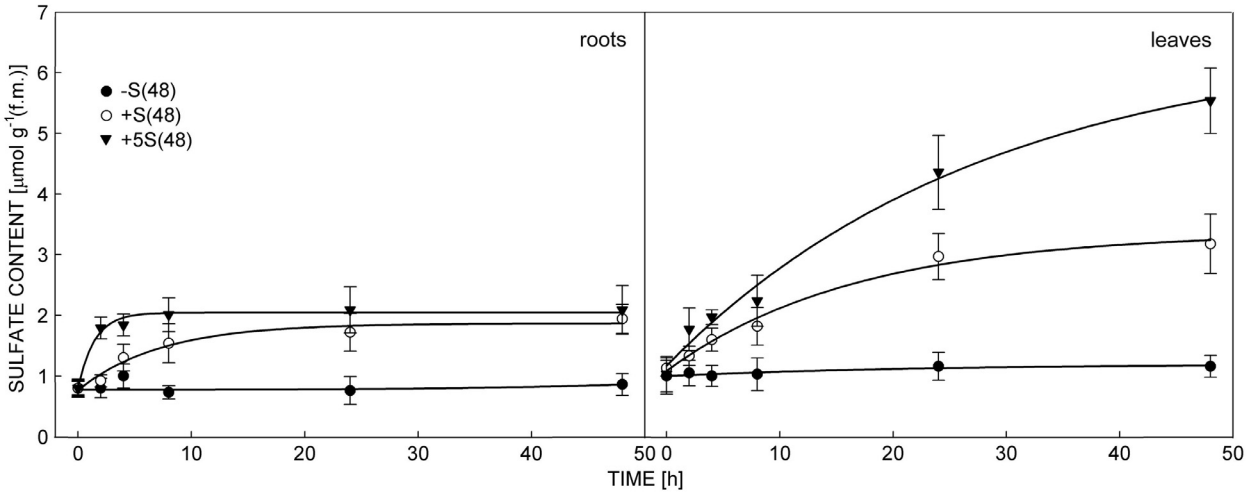

Fig. 1. The sulfate content in roots and leaves of seedlings S-deprived for 6 d and then maintained in an S-deprived [-S(48)] solution or in a solution containing 100 μM sulfate [+S(48)] or 500 μM sulfate [+5S(48)] for 48 h. Means ± SE, $n = 60$.

Table 2. The total root length, root surface area, average root diameter, and number of root tips of seedlings S-deprived for 6 d [-S(0)] and then maintained in an S-deprived solution [-S(48)] or in a solution containing 100 μM sulfate [+S(48)] or 500 μM sulfate [+5S(48)] for 48 h. Means followed by a different letter in the same column are significantly different at $P \leq 0.01$ ($n = 60$).

Treatment	Total root length [cm plant^{-1}]	Root surface area [cm^2 plant^{-1}]	Root diameter [mm plant^{-1}]	Number of root tips [plant^{-1}]
-S(0)	12.5 b	1.25 b	0.32 a	50 c
-S(48)	14.5 b	1.30 b	0.30 a	78 b
+S(48)	29.7 a	2.45 a	0.27 a	124 a
+5S(48)	29.4 a	2.40 a	0.27 a	125 a

The expression of *miR395* was not significantly different between the roots of plants grown for 48 h in 100 μM sulfate (+S) or 500 μM sulfate (+5S). The expression of *miR395* in leaves significantly increased ($P < 0.01$) in the S-deprived seedlings compared to those grown in the presence of sulfate (Fig. 2). In order to confirm the above results, the presence of miR395 was determined by Northern blot analysis in the sugar beet leaves of plants grown under the different S availability. As expected, the transcription of *miR395* was reduced at a higher S availability (Fig. 3).

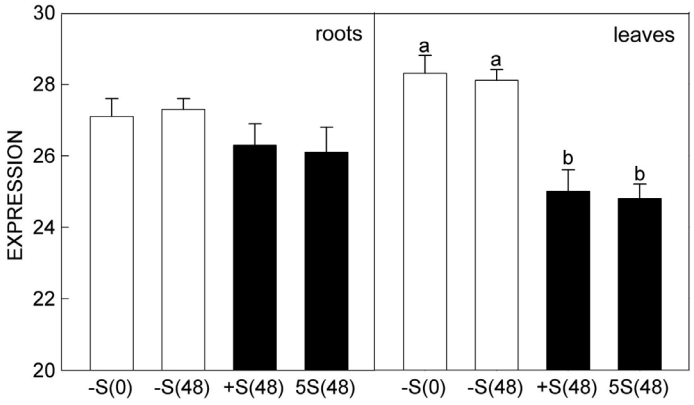

Fig. 2. The expression of miR395 in roots and leaves of seedlings S-deprived for 6 d [-S(0)] and then maintained in an S-deprived solution [-S(48)] or in a solution containing 100 μM sulfate [+S(48)] or 500 μM sulfate [+5S(48)] for 48 h. Means ± SE, $n = 60$. Means followed by a different letter are significantly different at $P < 0.01$.

The seedlings grown in complete nutrient solutions containing 100 μM sulfate (+S) and 500 μM sulfate (+5S) showed higher values than the S-deprived seedlings -S(0) and -S(48) for all parameters evaluated except for the root diameter.

The expression of *APS1* gene in roots was not significantly different in the S-supplemented or S-deprived seedlings, whereas the expression of *APS1* gene in leaves significantly increased ($P < 0.01$) in the S-deprived seedlings (Fig. 4).

Discussion

A better understanding of morpho-physiological and molecular responses of sugar beet to sulfate deprivation can lead to improved plant tolerance to nutritional stresses (Giehl *et al*. 2014). Sugar beet shows a rapid response to changes in sulfate availability. The sulfate content of roots and leaves increased according to the sulfate concentration applied in the hydroponic solution. In the S-supplemented seedlings, 48 h of treatment was needed to achieve a plateau of sulfate accumulation in roots but not in leaves (Fig. 1).

The modification of root architecture is consistent in response to sulfate availability and is an efficient strategy allowing plant adaptation to nutritional stress (Chen *et al*. 2014). Root morphogenetic responses, such as decreased or increased root elongation and abnormal root branching, can be correlated with changes of auxin synthesis (Potters *et al*. 2007). This root plasticity offers a promising way of improving mineral use efficiency (Lynch *et al*. 2014). The adaptive strategy of roots to sulfate deprivation is based mainly on a significant increase in the number of root tips that have a key role in nutritional stress perception and nutrient acquisition (Baluska *et al*. 2004). Previous studies on sugar beet (Saccomani *et al*. 2009) and *Arabidopsis thaliana* (Zhao *et al*. 2014) reported that sulfate deprivation results in an increased root/shoot ratio mainly due to root tips and fine roots production.

The miR395 could be considered as sulfate starvation signal in sugar beet. Analogously, miR395 is induced in *Arabidopsis* and sorghum (Zhang *et al*. 2011, Jagadeeswaran *et al*. 2014) but not in switch grass (Matts *et al*. 2010). These findings reveal that the miR395 regulation greatly differs among plant species. During sulfate deprivation, the magnitude of miR395 induction was higher in leaves than in roots similarly as in *Arabidopsis* (Jones-Rhoades and Bartel 2004, Kawashima *et al*. 2009). In S-deprived *Brassica* seedlings, the induction of miR395 was higher in the phloem than in the root, stem, or leaf parenchyma, indicating that miR395 is translocatable and might be an information-transmitting molecule (Buhtz *et al*. 2010). The mechanism of miRNA395 action might be the inhibition of the expression of its target gene. However, our results show that the miR395 increase caused an increased expression of the *APS1* gene in the S-deprived

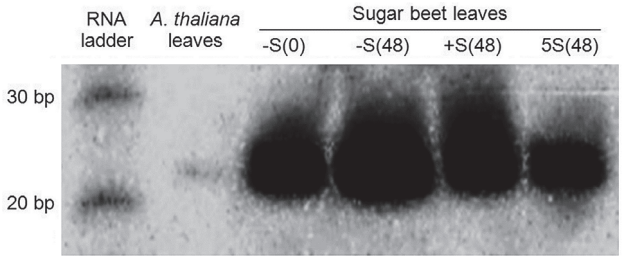

Fig. 3. Experimental validation of miR395 by Northern blot analysis using RNA extracted from seedlings S-deprived for 6 d [-S(0)] and then maintained in an S-deprived solution [-S(48)] or in a solution containing 100 μM sulfate [+S(48)] or 500 μM sulfate [+5S(48)] for 48 h. *Arabidopsis thaliana* Col-0 was used as control for comparison with sugar beet plants.

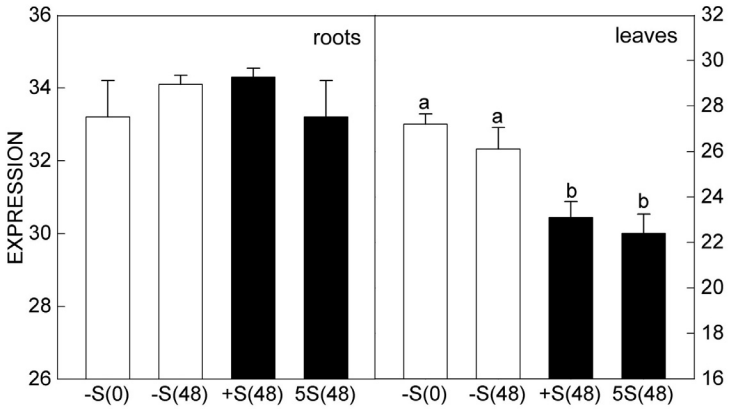

Fig. 4. The *APS1* expression in the roots and leaves of seedlings S-deprived for 6 d [-S(0)] and then maintained in an S-deprived solution [-S(48)] or in a solution containing 100 μM sulfate [+S(48)] or 500 μM sulfate [+5S(48)] for 48 h. Means ± SE, $n = 60$. Means followed by a different letter are significantly different at $P < 0.01$.

sugar beet seedlings. This agrees with Kawashima *et al*. (2009), who showed a high expression of both the *miR395* and *APS1* genes after sulfate shortage. Nevertheless, it is inconsistent with Jones-Rhoades and Bartel (2004), who stated that the shortage of sulfate leads to an increase in the expression of *miR395* and a decrease in the expression of *APS1* gene. This suggests a complex role of the miRNA395 in the response to sulfate limitation, with a possible involvement of other small RNAs in the same pathway.

In conclusion, sugar beet responses to changes in sulfate availability involve the modification of root morphology and the expression of *miRNA395*. A further analysis of the expression profiles of NA395 and its target genes will improve the understanding of the regulatory mechanisms involved in sugar beet adaption to sulfate availability.

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