The Sulfur Pathway and Diagnosis of Sulfate Depletion in Grapevine

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Abstract Sulfur is an essential nutrient to all plant species. Plants assimilate sulfur in a well-described pathway, which has been taken up by roots. Regulatory mechanism has been the subject of many research papers. However, recent studies highlighted differences between crop plants and the model plant *Arabidopsis thaliana*. Our work focuses on the identification of genes involved in the sulfur metabolism in the *Vitis vinifera* genome, and their response to sulfur deficiency and other abiotic stress endured by grapevine in the field, namely water stress. Here, we describe the identification and brief characterization of the first assimilation enzymes involved in the sulfur pathway, the enzyme responsible for sulfur activation, ATP sulfurylase (ATPS), and the two enzymes that reduce sulfate to sulfide, Adenosine 5′-phosphosulate reductase (APR) and Sulfite reductase (SiR). A reduction was observed in the number of ATPS and APR isoforms identified in *V. vinifera* genome when compared to *A. thaliana* or *Glycine max* genomes. Two ATPS isoforms were present in the *Vitis* genome, of which only *ATPS1* transcript was detected in the tested tissues, and one APR isoform, suggesting an absence of redundancy in the role of both enzymes. *ATPS1*, *APR* and *SiR* transcript level was up-regulated in response to 2 days exposure to sulfur deficiency in *V. vinifera* cell cultures, which was completely reversed by the addition of GSH to the culture medium. Apparently, oxidative stress triggered GSH has a pivotal role in the regulation of *ATPS1*, *APR* and *SiR* transcription level, since their up-regulation was observed in mRNA from field grapevine berries under water stress, which is known to induce oxidative stress.

Grapevine (*Vitis vinifera* L.) is one of the most important crops worldwide for winemaking and also for table grapes. Grapevines can be successfully grown in a range of different climates and management conditions. Global climate changes are associated with water deficit and high evapotranspiration rates that can affect berry development, yield, and wine quality (Hannah et al. 2013).

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In plants sulfur (S) is a major essential plant nutrient required for plant growth and development. Unlike animals, vascular plants use sulfate (SO_4^{2-}) taken by the root system as the primary S source for plant growth (Clarkson et al. 1993) and reduce SO_4^{2-} to sulfide (S^{2-}) in plastids of phototrophic organisms, including vascular plants (Shibagaki and Grossman 2008). Sulfide is further assimilated into the amino acids cysteine and methionine. Then most sulfate taken up by plants is incorporated into proteins (Leustek and Saito 1999; Leustek et al. 2000). Organic sulfur can be found in glutathione (GSH), the thiol tripeptide that mediates redox reactions by the interchange of dithiol-disulfide. In addition, several secondary S-metabolites have been suggested to play key roles in defense against pathogens (Hell and Kruse 2007) such as glucosinolates, which are produced mostly by members of the Brassicaceae.

Sulfate assimilation in vascular plants is accomplished in three steps catalyzed by enzymes whose codifying genes were confirmed in *Vitis vinifera* genome: *VvATPS1* and *2* (ATP sulfurylase, EC: 2.7.7.4); *VvAPSR* (APS reductase, EC: 1.8.99.2); VvSiR (sulfite reductase, EC: 1.8.7.1); *VvSERAT1-3* (Serine acetyltransferase, EC 2.2.1.30) and *VvOASTL1-13* (*O*-acetylserine (thiol) lyase, EC 2.5.1.47) (Amâncio et al. 2009).

The Bordeaux mixture, a high-S-content fungicide, has been used since the nineteenth century for the control of downy and powdery mildew in grapevine (Williams and Cooper 2004). Besides its major effect as a fungicide, it was an important source of S. Since sulfur fungicides have been substituted for organic compounds, the S supply to vineyards was reduced. Recent studies of SO_4^{2-} uptake, assimilation, and symptoms of sulfur deficiency in *Vitis* species were reported by our group (Tavares et al. 2008, 2013, 2015; Amâncio et al. 2009). In the first study sulfate uptake was correlated with the expression of sulfate transporter genes in cell systems of two *Vitis* species (*V. vinifera* and *V. rupestris*) (Tavares et al. 2008); the second investigation established a link between sulfur deficiency and phenolic compounds (Tavares et al. 2013) and the third work characterized the serine acetyl-transferase protein family revealing major differences to the best described *A. thaliana* family (Tavares et al. 2015). Altogether the results previously obtained directed our attention to the first steps of sulfate assimilation, namely sulfate activation and reduction, which were explored in different grapevine experimental systems and environmental conditions.

It is largely known that the experimental set up can influence the results. In a study performed to verify differences of plant sensitivity to chemical treatments the sensitivity varied whether species were treated under greenhouse or field conditions (Fletcher et al. 1990). Under water stress different responses were obtained at physiological and transcriptional levels in grapevine plants cultivated in greenhouse and field (Luisa Carvalho, personal communication). Different systems were used to tackle S metabolism in grapevine. Cell suspensions were selected as biological material to obtain a homogeneous experimental system. Studies with maize cells had reported a response to S deficiency following the same trend as intact plants, a de-repression of sulfate uptake (Clarkson et al. 1999). Also cell cultures allow S manipulation in short periods. Cell suspensions of *V. vinifera* var.

Touriga Nacional and *V. rupestris* were obtained as described in Tavares et al. 2008. Cell suspensions were sourced from liquid culture callus material and were grown in 250 ml flasks on a rotary shaker at 100 rpm, in the dark at 25 °C. After at least two weekly cycles in full sulfate (+S, 1.5 mM) sub-cultures were prepared for sulfate treatments: +S conditions (control) and sulfate depletion (−S, sulfate substituted by chloride). Leaves from *V. vinifera* Touriga Nacional subjected to abiotic stress were obtained as described in Rocheta et al. (2014) and Coito et al. (2012). Rooted cuttings were transferred to 3 l pots filled with sterilized soil and placed in the growth chamber. The following growth conditions were adjusted: light intensity 200 μmol m^{-2} s^{-1}, 16 h light and 8 h darkness, 25 °C at day/23 °C at night, and watering with nutrient solution when necessary. The potted plants were subjected to drought and sampled when the pre-dawn leaf water potential was −0.9 MPa. *V. vinifera* cv. Touriga Nacional berries were collected from plants subjected to two irrigation regimes: rainfed (non-irrigation) and full irrigation (water supplied according to evapotranspiration rates) as described in Lopes et al. (2011). Berries were collected at veraison (50% colored berries) and full maturation.

The analysis of the *Vitis vinifera* genome indicates the presence of genes of two ATPS isoforms, one APR isoform with two splicing variants and one SiR isoform. ATPS and APR from plant and algae are encoded by small multigene families. A low number of isoforms is usually found in basal land plants and green algae, the studied species disclosing one or two distinct ATPS isoforms, and a APR unique isoform (Kopriva et al. 2007). In Arabidopsis and soybean genomes, genes encoding four ATPS isoforms and three APR isoforms were identified (Anjum et al. 2015; Hatzfeld et al. 2000; Yi et al. 2010). However, similar to *Vitis vinifera*, in the *Oryza sativa* genome only two ATPS isoforms were identified (Kopriva et al. 2007) and *Selaginella moellendorffii* seems to be the only vascular plant that has a unique ATPS (Kopriva et al. 2009). A single gene encodes SiR in *Vitis vinifera* in accordance with Arabidopsis (Takahashi et al. 2011) while in tobacco and soybean two isoforms were detected (Yi et al. 2010).

In *V. vinifera* the identified genes are located on distinct chromosomes; only ATPS2 shares the same chromosome with two sulfate transporters genes. Both ATPS genes depict five exons and four introns, however ATPS2 is organized in small exons and very long introns, which increased the genomic size from 5.3 to 11 Kbp. SiR is organized in eight exons and seven introns. In contrast the APR gene has only four or five exons. Two variants were identified for the APR gene that differ in a small sequence, variant one interpreted the sequence as an intron, in comparison variant 2 incorporated the sequence in the mRNA (Table 1).

The *Vitis vinifera* sulfate activation and reduction protein sequences present similar traits to other known plant proteins such as protein length and conserved domains (Table 1). *V. vinifera* ATPS coding regions have the N-terminal leader sequences characteristic for plastid-targeting transit peptides and a conserved ATPS catalytic domain (CD00517, Marchler-Bauer et al. 2011), typical of all described plant ATPS. All four ATPS from *Arabidopsis thaliana* (Rotte and Leustek 2000) and *Glycine max* (Yi et al. 2010) have been predicted as chloroplast isoforms

Table 1 Identification of ATP sulfurylase (ATPS), APS reductase (APR) and sulfide reductase (SiR) genes in *Vitis vinifera* genome

Gene	Chr	Genomic Region[a]	mRNA[a]	Protein[a]	Localization
ATPS1	5	NW_003724020.1 5.3 Kbp	XM_002283536.3 1960 bp	XP_002283572.1 467 aa	C
ATPS2	18	NW_003724132.1 11Kbp	XM_002276957.2 2253 bp	XP_002276993.1 483 aa	C
APSr	12	NW_003724079. 3.9 Kbp	XM_002269703.3 (2) 1928 bp XM_010658907.1 (1) 1885 bp	XP_002269739.2 (2) 467 aa XP_010657209.1 (1) 498 aa	C
SiR	6	NW_003724030.1 6.4 Kbp	XM_002285362.2 2690 bp	XP_002285398.1 687 aa	C

Chr chromosome number, [a]NCBI reference numbers, *C* putative chloroplastic localization predicted in TargetP site (http://www.cbs.dtu.dk/services/TargetP/)

although ATPS activity is detected in chloroplasts and cytosol in Arabidopsis (Rotte and Leustek 2000) and spinach leaves (Lunn et al. 1990). Recently, Bohrer et al. (2015) showed that in Arabidopsis leaves, ATPS2 has a dual localization, namely in cytosol and chloroplasts, suggesting that a downstream methionine in the transit peptide sequence could act as an additional initiation translation site. In addition, such methionine is not present on the transit peptide sequence of ATPS1, 3 and 4. ATPS2 seems to be a distinct ATPS isoform, which probably confers different physiological roles; this isoform is also the only ATPS from Arabidopsis that is not a target to miRNA395 post-transcriptional control (Kawashima et al. 2009). Likewise, only the ATPS2 sequence from *Vitis vinifera* has an additional methionine on the transit peptide sequence and, contrary to VvATPS1, it is not a target of Vitis miRNA395 as predicted by the psRNATARGET tool (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao 2011). In fact, the unrooted tree constructed with *Vitis*, Arabidopsis, soybean and *Populus* ATPS protein sequences (Fig. 1a) also shows a group including the ATPS2 sequences and a second group where the other sequences are not clearly separated. If a certain degree of redundancy in ATPS isoforms is observed among other plant species (Kopriva et al. 2009), apparently in the *Vitis vinifera* genome such redundancy is reduced or absent. A unique APR isoform was identified in the *Vitis* genome, with two variants that share 94% sequence homology. Sulfate reduction occurs exclusively in chloroplasts and, similarly to all plant species *Vitis* APR has a N-terminal transit peptide for plastid-targeting and a conserved multidomain consisting of an reductase domain and a C-terminal thioredoxin-like domain (CD02993, Marchler-Bauer et al. 2011).

Arabidopsis (Kopriva et al. 2009) and soybean (Yi et al. 2010) have three APR isoforms, apparently some level of redundancy exists, since Arabidopsis plants without functional APR1 or APR2 do not show obvious differences when compared with the wild type, however the disruption of *APR2* leads to an 80% decrease in APR activity and an accumulation of sulfate indicating that APR2 is a major

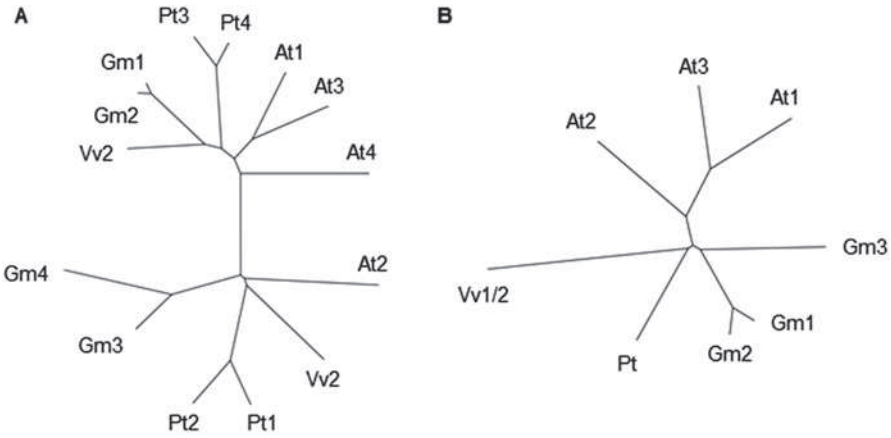

Fig. 1 Phylogenetic analysis of ATPS sulfurylase (ATPS, **a**) and APS reductase (APR; **b**) protein sequences. Unrooted tree constructed using the T-Cofee and PHYLIP programs, PRODIST and NEIGHBOR (http://tcoffee.crg.cat/ and http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome, respectively). At *Arabidopsis thaliana*, Gm *Glycine max*,, Pt *Populus trichocarpa*, Vv *Vitis vinifera*

isoform in Arabidopsis (Loudet et al. 2007). The identification of APR in the *Vitis* genome shows a lack of redundancy and the phylogenetic analysis of the unique *Vitis* APR together with APR protein sequences from Arabidopsis, soybean and *Populus* shows that the *Vitis* protein is closer to Arabidopsis and soybean APR2. The APR protein sequences from the selected species seem to group better inside each species, which could be determined by species-specific evolution of APR (Fig. 1b). Together with APR, SiR is strictly plastidic. *Vitis* SiR contains the siroheme and a [4Fe-4S] cluster typical of plant SiR proteins and the transit peptide for chloroplast targeting (Table 1).

The presence of transcripts encoding enzymes involved in sulfate activation and reduction, ATPS1 and 2, APR and SiR, were examined in different grapevine tissues by RT-PCR (Fig. 2). *ATPS2* was the only gene not detected in RNA isolated from the sampled tissues, namely leaves (young and mature) and roots of potted plants, berries collected in the field and cells from cell culture (Fig. 2b, in berries). *V. vinifera* ATPS2 protein sequence was most similar to the protein sequences of *Populus*, ATPS1 and ATPS2, and AtAPS2 (Fig. 1a) recently proven to have dual subcellular chloroplastic/cytosolic localization, although a physiological role of the cytosolic isoform remains unknown (Bohrer et al. 2015). It has been speculated that cytosolic ATPS may be linked to cytosolic APS kinase in providing PAPS for the secondary metabolism (Rotte and Leustek 2000), namely in the production of glucosinolates. *V. vinifera* invests a great deal of resources in secondary metabolites, such as phenolic compounds, and its genome is enriched in genes devoted to secondary metabolism (Velasco et al. 2007). *V. vinifera* synthesizes no glucosinolates, which might be the reason why *ATPS2* transcripts were not detected in different plant tissues. RNA isolated from berries at *veraison* showed a very

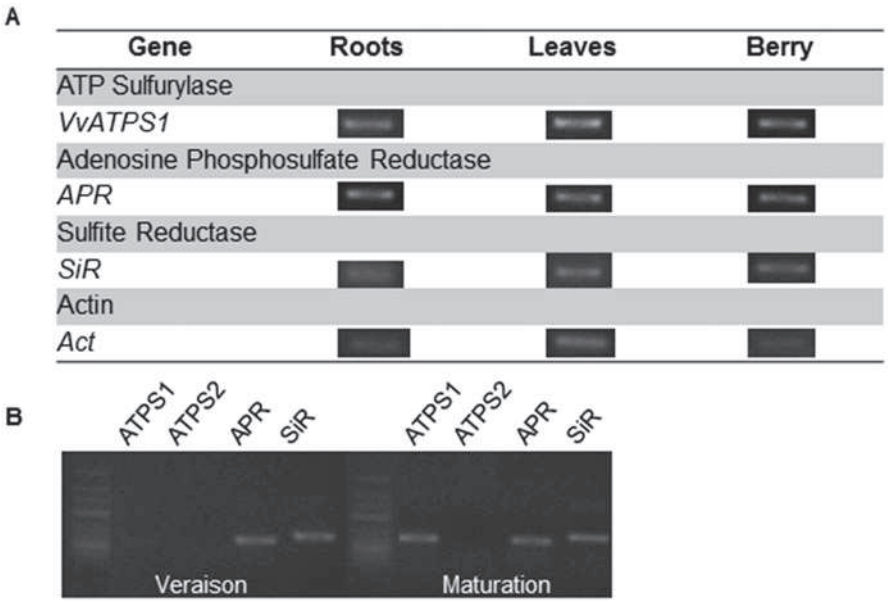

Fig. 2 (**a**) mRNA detection by RT-PCR in different *Vitis vinifera* tissues with specific primers design to ATP sulfurylase1 (ATPS1), APS reductase (APR) and sulfide reductase (SiR) sequences identified in Table 1. (**b**) detection of *ATPS1* and *2*, *APR* and *SiR* transcripts in grapevine berries collected at veraison and full maturation. RNA extraction and RT-PCR were performed according with standard molecular biology techniques

weak signal in RT-PCR using *ATPS1* primers, contrary to mature berries RNA which produced a strong signal indicating presence of the gene (Fig. 2b). Interestingly, a group three sulfate transporter was up-regulated at berry maturation (Guillaumie et al. 2011) and in seeds, transcripts for sulfur metabolism genes, namely group three sulfate transporters, were over-represented when compared with pulp transcripts (Grimplet et al. 2007). Together these results suggest a stimulus of sulfur metabolism at berry maturation, in particularly in seeds.

The transcript levels of *VvATPS1*, *VvAPR* and *VvSiR* analyzed by qPCR responded equally to S depletion in cell cultures; all genes were up-regulated in cells after 2 days in an S deficient medium (Fig. 3a, b and c), and an increase in mRNA level was observed in *VvAPR* (Fig. 3b). S deficiency is known to be responsible for increasing the transcript levels of group one sulfate transporters and *APR* in plant species, including *Vitis vinifera* (Tavares et al. 2008), the reason why APR activity and sulfate transport are considered to exert the highest control over the S metabolic pathway (Vauclare et al. 2002). Nevertheless ATPS up-regulation was also observed in several plant species, namely *Arabidopsis thaliana* and *Zea mays* (reviewed by Anjum et al. 2015). The addition of sulfate and GSH to the S deficient medium completely reverses the up-regulation of *VvATPS1*, *VvAPR* and *VvSiR* transcripts. Interestingly, in *Vitis* cells the level of GSH as measured by HPLC (Tavares et al. 2015) was the first S compound to

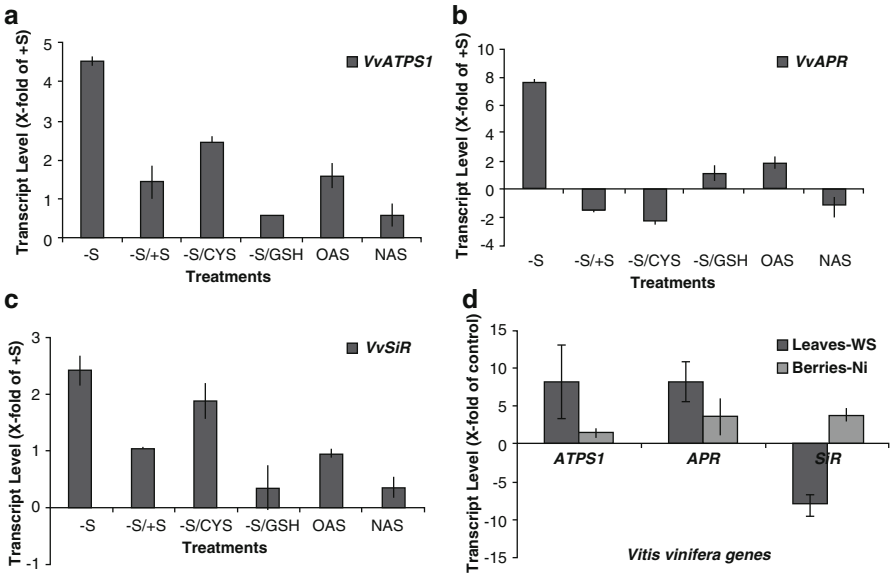

Fig. 3 Relative expression of ATP sulfurylase1 (*ATPS1*), APS reductase (*APR*) and Sulfide reductase (*SiR*) transcripts in grapevine cell culture (**a**, **b** and **c**) and leaves and berries (**d**). (**a**, **b** and **c**). Cells collected after 2 days in sulfur deficiency (−S) and sulfur sufficient (+S) medium, after which sulfur (S), cysteine (cys) and GSH were added to the –S medium and *O*-acetylserine (OAS) and *N*-acetylserine (NAS) to +S. Relative expression was compared to +S cells using RT-qPCR. (**d**) Water stress grapevine leaves and non-irrigated berries relative transcript level of *ATPS1*, *APR* and *SiR* was compared to control leaves and full irrigated berries by RT-qPCR

significantly decrease in cells after 1 day in S deficient medium. Cysteine added to cells in an S deficient medium also triggered a reversion of the up-regulation observed in the transcript level of *VvAPR* (Fig. 3b); apparently cysteine had a direct effect on the *VvAPR* transcription though we only detected a decrease in the amount of cysteine inside the cells 5 days after S limitation (Tavares et al. 2015). OAS is considered to act as a positive regulator of sulfate transporters, and commonly has similar effects under conditions of S deficiency (Takahashi et al. 2011). Consequently OAS added to *Vitis vinifera* cell culture showed the same up-regulation effect as S deficiency, although the magnitude was not so drastic (Fig. 3a, b and c).

In leaves from potted plants and berries collected from field plants, both grown under conditions of water stress the *VvATPS* and *VvAPR* transcript level increased. In contrast, *VvSiR* was down-regulated in leaves and up-regulated in berries (Fig. 3d). A change in ATPS activity in response to oxidative stress was reported by Kopriva et al. (2007). Similar results were obtained under conditions of abiotic stress (reviewed in Anjum et al. 2015). A high demand for GSH, an important S-compound in the response to oxidative stress, occurs under water stress. This may unfold an up-regulation effect in the mRNA of the first enzymes of sulfur assimilation. Likewise, the serine acetyltransferase (SAT) mRNA level was up-regulated under water stress conditions in *Vitis vinifera* leaves (Tavares et al. 2015).

As *VvSERAT2;1* was up-regulated in leaves of plants under water stress, this result prompted us to test the genes for the first enzymes of the S assimilation pathway (ATPS, APR and SiR) in leaves and berries of plants under water deficit. Although the patterns of transcripts expression were distinctly different between the two plant organs, the results suggest that some enzymes involved in sulfate metabolism are regulated by S-status and by environmental conditions, e.g. water deficit. Our study showed that major characteristics of ATPS, APR and SiR are very well conserved among plant species. However, it would be interesting to highlight differences between these enzymes in order to obtain a complete overview of S assimilation in different plant species.

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Impact of Sulfate Deprivation and H_2S Exposure on the Metabolites of the Activated Methyl Cycle in Chinese Cabbage

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Abstract The activated methyl cycle is a central metabolic pathway used to generate (and recycle) several important sulfur-containing metabolites including methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) and enable methylation. We have developed a precise and sensitive method for the simultaneous measurement of several sulfur metabolites based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and ^{34}S-metabolic labeling of sulfur-containing metabolites including glutathione and the metabolites of the activated methyl cycle. Sulfate deprivation resulted in a decreased biomass production and content of glutathione, methionine, SAH of both shoot and root, and SAM of the root of Chinese cabbage. Foliarly absorbed H_2S may able to replace sulfate taken up by the root as sulfur source for growth and an atmospheric concentration of 0.2 μl l^{-1} alleviated the decrease in the content of sulfur metabolites. The SAM content of the shoot was hardly affected upon sulfate-deprivation, resulting an increase in the SAM/SAH ratio, indicating a potential higher methylation capacity under this condition.

Seedlings of Brassicacea are characterized by their high growth rate (up to 0.4 g g^{-1} day^{-1}) and high sulfur demand; the sulfate uptake rate of some species may exceed 40 μmol g^{-1} fresh weight root day^{-1} (Shahbaz et al. 2010; Stuiver et al. 2014; Aghajanzadeh et al. 2016). The uptake of sulfate by the root is adjusted to the sulfur demand for growth, even at an external sulfate concentrations close to the K_m value

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of the high affinity sulfate transporters (approx. 5 μM; Koralewska et al. 2007, 2012). The interaction between atmospheric H_2S and pedospheric sulfate nutrition of plants has been extensively studied during the last three decades (De Kok 1990; De Kok et al. 2000, 2002, 2007). Foliarly absorbed H_2S was directly metabolized into cysteine and subsequently into other organic sulfur compounds, and exposure resulted in an increase in the content of water-soluble non-protein thiol content (*viz*. cysteine and glutathione) of the shoot (De Kok 1990; De Kok et al. 2000, 2002, 2007). H_2S exposure hardly affected the total sulfur and sulfate contents of *Brassica*, even not at relatively high atmospheric concentrations (De Kok et al. 2000), but resulted in a down-regulation of the uptake of sulfate by the root and reduction in the shoot (De Kok et al. 2000, 2002, 2007; Koralewska et al. 2008; Shahbaz et al. 2014; Aghajanzadeh et al. 2014, 2016). Upon sulfate-deprivation, the assimilated foliarly absorbed H_2S may replace sulfate as a sulfur source for growth of *Brassica* (De Kok et al. 2000, 2002, 2007; Buchner et al. 2004; Koralewska et al. 2008; Shahbaz et al. 2014; Aghajanzadeh et al. 2016). H_2S exposure hardly affected the up-regulated expression and activity of the high affinity sulfate transporters in sulfate-deprived *Brassica* (Koralewska et al. 2008; Shahbaz et al. 2014), and the decrease in shoot to root biomass partitioning upon sulfate deprivation remained largely unaffected upon H_2S exposure (Koralewska et al. 2008; Shahbaz et al. 2014; Aghajanzadeh et al. 2016). There is apparently a rather poor shoot to root signaling in Brassica of the regulation of both the sulfate transporters in the root and shoot to root biomass partitioning, indicating that both are determined by the sulfate concentration in the root environment rather than by the sulfur status of the plant itself.

Methionine is an essential metabolite in plants and all-living organisms (Ravanel et al. 1998). Apart from a role as a protein constituent, methionine is the precursor of *S*-adenosyl-L-methionine (SAM), the primary biological methyl-group donor (Roje 2006). The highly reactive methylated sulfur of SAM is used by a broad range of methyltransferases (Poel et al. 2013). A by-product of SAM-dependent transmethylation, *S*-adenosylhomocysteine (SAH) is released, which is recycled to methionine via homocysteine through the activated methyl cycle (Bürstenbinder and Sauter 2012). SAH strongly inhibited methyltransferase through competition with the substrate SAM (Barbes et al. 1990; Moffatt and Weretilnyk 2001). The ratio of cellular SAH and SAM is indicative for the methylation capacity of the cell (Fulnecek et al. 2011; Poel et al. 2013). In the current study the impact of sulfate deprivation and H_2S exposure on the content of the metabolites involved in the active methyl cycle was studied in Chinese cabbage.

Seeds of Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr. cv. Kasumi F1; Nickerson Zwaan, Made, The Netherlands) were germinated in vermiculite in a climate controlled room. Day and night temperatures were 22 °C and 18 °C (±1 °C), respectively, relative humidity of 60–70% and a 14-h photoperiod at a photon fluence rate of 300 ± 20 μmol m^{-2} s^{-1} (400–700 nm) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Ten-day-old seedlings were transferred to an aerated 25% Hoagland nutrient solution (pH 5.9) with 0.5 mM sulfate (+S, sulfate sufficient) or 0 mM sulfate (−S, sulfate deprived; all sulfate salts replaced by chloride salts) in 13 l stainless

steel containers (ten sets of plants per container, three plants per set). Plants were exposed to 0.2 μl l^{-1} H_2S in 150 l cylindrical stainless steel cabinets (0.6 m diameter) with a poly(methylmethacrylate) top. The lids of the containers and the plant sets were sealed in order to prevent absorption of atmospheric H_2S by the nutrient solution. Day and night temperatures in the fumigation cabinets were 22 and 19 °C (± 2 °C), respectively, and relative humidity was 40–50%. The photoperiod was 14 h at a photon fluence rate of 300 ± 20 μmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. The air exchange was 40 l min^{-1}, whereas a ventilator stirred the air inside the cabinets continuously. Pressurized H_2S diluted with N_2 (1 ml l^{-1}) was injected into the incoming air stream and the concentration in the cabinet was adjusted to the desired level using electronic mass flow controllers (ASM, Bilthoven, The Netherlands). The air exchange was 40 l min^{-1}, and a ventilator stirred the air inside the cabinets continuously. The H_2S concentration in the cabinets was monitored by an SO_2 analyzer (model 9850) equipped with a H_2S converter (model 8770; Monitor Labs, Measurement Controls Corporation, Englewood, CO, USA). Plants were harvested after H_2S exposure. Roots were separated from shoots, weighed, and were frozen immediately in liquid N_2 and stored at −80 °C until further use. For determination of the sulfur metabolites, plant tissue was freeze-dried at −60 °C for 48–72 h. Freeze-dried plant tissues were ground to powder with liquid nitrogen in a mortar with pestle. The sulfur metabolites were extracted as described in (Chang et al. 2013). For isotope dilution mass spectrometry analysis, the ^{34}S-labeled *Arabidopsis thaliana* tissue were extracted and added to the calibration standards, QC samples, and plant samples in a fixed ratio (Chang et al. 2013). Chromatographic separations of sulfur metabolites were performed on a Thermo Accela LC system using a Thermo Scientific Hypersil Gold aQ C18 (1.9 μm, 2.1 mm × 10 cm). Separations were performed under isocratic condition at a flow rate of 0.25 ml min^{-1}. The mobile phase was composed of 0.1% formic acid in water. ESI-MS/MS analysis was performed on a Thermo Scientific TSQ Quantum Ultra. Metabolites were detected in positive ionization mode using multiple reaction monitoring scanning mode. The spray voltage was set to 3.5 kV, the ion-transfer capillary temperature was set to 280 °C, the sheath gas pressure was set to 50 (arbitrary units), and the auxiliary gas pressure was set to 15 (arbitrary units). Collision energy set at 35% was used for each metabolite (Table 1).

An 11-day exposure of Chinese cabbage to sulfate-deprived condition resulted in a decreased plant biomass production (Table 2). The shoot growth was relatively more affected than the root growth resulting in a decrease in shoot/root ratio. Exposure of sulfate-sufficient plants to 0.2 μl l^{-1} H_2S hardly affected plant biomass production, but it alleviated the reduction in biomass production of sulfate-deprived plants. The latter demonstrated that similar to previous observations that at an atmospheric H_2S concentration ≥ 0.2 μl l^{-1} the foliarly absorbed sulfide fully could replace sulfate taken up by the sulfur source for growth (Koralewska et al. 2008; Shahbaz et al. 2014; Aghajanzadeh et al. 2014, 2016). H_2S exposure of sulfate-sufficient plants resulted in a slight increase in the total glutathione (expressed as GSH) content of the shoot and not of the root (Table 3). Sulfate

Table 1 Mass spectrometry parameters with transition pairs in MRM mode and normalized collision energy (%)

Compound name	Precursorion	Production	Collision energy
Methionine	150	56	15
Methionine S34	152	106	15
GSH	308	162	20
GSH S34	310	181	20
SAH	385	134	20
SAH S34	387	136	20
SAM	399	250	20
SAM S34	401	250	20
^{32}S GSSG	613	355	25
^{34}S GSSG	617	359	25

Table 2 Impact of H_2S and sulfate deprivation on biomass production of shoots and roots of Chinese cabbage

	+Sulfate		−Sulfate	
	0 μl l^{-1} H_2S	0.2 μl l^{-1} H_2S	0 μl l^{-1} H_2S	0.2 μl l^{-1} H_2S
Shoot (g FW)	3.98 ± 0.38b	5.39 ± 1.91b	1.12 ± 0.04a	4.69 ± 0.66b
Root (g FW)	0.78 ± 0.05b	1.05 ± 0.26b	0.33 ± 0.04a	1.10 ± 0.08c
Shoot/root ratio	5.13 ± 0.27b	5.29 ± 1.75ba	3.46 ± 0.44a	4.27 ± 0.27a

Ten-day-old seedlings were grown on a 25% Hoagland solution at 0 and 0.5 mM sulfate (+S) and exposed to 0.2 μl l^{-1} H_2S for 11 days. The initial fresh biomass of the shoot and root of Chinese cabbage was 0.171 ± 0.001 g and 0.057 ± 0.001 g, respectively. Data on plant yield (g FW) and shoot/root ratio represent the mean of three measurements with three plants in each (±SD) Means with different letters are significant different at $p < 0.01$ (unpaired Student's t-test)

deprivation resulted in a strong decrease in the glutathione content of both shoot and root but this decrease was largely alleviated upon H_2S exposure, though its content remained lower than that of the sulfate-sufficient plants. Sulfate deprivation also resulted in a substantial decrease in the methionine and SAH content of both shoot and root and SAM content of the root (Table 3). However, the SAM content of the shoot was hardly affected upon sulfate deprivation, resulting in a fourfold increase in the SAM/SAH ratio (Table 3). The SAM/SAH ratio in plant tissue has often been used as a reporter of the methylation capacity ("methylation index"; Groth et al. 2016). In this view the increase in SAM/SAH ratio in the shoot upon sulfate deprivation would indicate an increased methylation capacity. Exposure of sulfate-deprived plants to H_2S alleviated the decrease in SAM and SAH content of the shoots, which were comparable to that of shoots of sulfate-sufficient plants (Table 3). However, the methionine content was slightly lower than that of sulfate-sufficient plants. The contents of methionine, SAM and SAH sulfur of the roots were decreased upon sulfate deprivation, whereas the content of these sulfur metabolites in the root of sulfate-deprived H_2S-exposed plants was quite similar to that of sulfate-sufficient plants. The activated methyl cycle is a central metabolic

Table 3 Impact of H_2S and sulfate deprivation on the content of glutathione and metabolites of activated methyl cycle in shoots and roots of Chinese cabbage

	+Sulfate		−Sulfate	
	0 μl l^{-1} H_2S	0.2 μl l^{-1} H_2S	0 μl l^{-1} H_2S	0.2 μl l^{-1} H_2S
Shoot				
Glutathione	5.67 ± 0.22c	6.69 ± 0.42d	0.18 ± 0.01a	3.87 ± 0.16b
Methionine	0.224 ± 0.004c	0.260 ± 0.039c	0.038 ± 0.006a	0.179 ± 0.032b
SAM	0.123 ± 0.008ab	0.134 ± 0.011b	0.106 ± 0.011a	0.113 ± 0.007a
SAH	0.015 ± 0.001b	0.014 ± 0.001b	0.003 ± 0.001a	0.012 ± 0.001b
SAM/SAH ratio	8.2 ± 1.1a	9.6 ± 1.5a	35.3 ± 15.4b	9.4 ± 1.4a
Root				
Glutathione	4.62 ± 0.15c	5.17 ± 0.54c	0.55 ± 0.07a	2.79 ± 0.10b
Methionine	0.136 ± 0.014b	0.135 ± 0.024b	0.024 ± 0.004a	0.121 ± 0.004b
SAM	0.207 ± 0.002b	0.210 ± 0.024bc	0.076 ± 0.016a	0.231 ± 0.002c
SAH	0.019 ± 0.002bc	0.017 ± 0.002b	0.006 ± 0.003a	0.021 ± 0.002c
SAM/SAH ratio	10.9 ± 1.3a	12.3 ± 2.9a	12.3 ± 8.6a	11.0 ± 1.1a

For experimental details, see legends of Table 2. Data on metabolite content is expressed as μmol g^{-1} dry weight (freeze-dried plant material) and represent mean of three measurements with three plants in each (± SD). Means with different letters are significant different at $p < 0.01$ (unpaired Student's t-test)

pathway responsible for the methylation of cellular components and the recycling of sulfur-containing metabolites. The methylation of essential biological molecules, e.g. nucleic acids, hormones, lipids, proteins, is of crucial importance for many key biochemical processes (Chiang et al. 1996). Apparently the foliarly absorbed sulfide by sulfate-deprived Chinese cabbage upon exposure to an atmospheric level of 0.2 μl l^{-1} H_2S was sufficient for reduced sulfur requirement of the plant to support growth and to maintain the levels of the metabolites involved in the activated methyl cycle in both the shoot and the root.

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