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ORIGINAL ARTICLE

Impact of sulfur starvation on cysteine biosynthesis in T-DNA mutants deficient for compartment-specific serine-acetyltransferase

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Abstract Sulfur plays a pivotal role in the cellular metabolism of many organisms. In plants, the uptake and assimilation of sulfate is strongly regulated at the transcriptional level. Regulatory factors are the demand of reduced sulfur in organic or non-organic form and the level of *O*-acetylserine (OAS), the carbon precursor for cysteine biosynthesis. In plants, cysteine is synthesized by action of the cysteine–synthase complex (CSC) containing serine acetyltransferase (SAT) and *O*-acetylserine-(thiol)-lyase (OASTL). Both enzymes are located in plastids, mitochondria and the cytosol. The function of the compartmentation of the CSC to regulate sulfate uptake and assimilation is still not clearly resolved. To address this question, we analyzed *Arabidopsis thaliana* mutants for the plastidic and cytosolic SAT isoenzymes under sulfur starvation conditions. In addition, subcellular metabolite analysis by non-aqueous fractionation revealed distinct changes in subcellular metabolite distribution upon short-term sulfur starvation. Metabolite and transcript analyses of SERAT1.1 and SERAT2.1 mutants [previously analyzed in Krueger et al. (Plant Cell Environ 32:349–367, 2009)] grown under sulfur starvation conditions indicate that both isoenzymes do not contribute directly to the transcriptional regulation of genes involved in sulfate uptake and assimilation. Here, we summarize the current knowledge about the regulation of cysteine biosynthesis and the contribution of the different compartments to this metabolic process. We relate hypotheses and views of the regulation of cysteine biosynthesis with our results of applying sulfur starvation to mutants impaired in compartment-specific cysteine biosynthetic enzymes.

Keywords Serine acetyltransferase · *O*-acetylserine-(thiol)-lyase · CSC complex · SAT mutants · Non-aqueous fractionation

Introduction

Sulfur is an essential macro nutrient for plant growth and development. Within plants, sulfur is mainly present as sulfate, which is taken up into cortical root and epidermal cells by a family of proton sulfate co-transporters (Lass and Ullrich-Eberius 1984; Hawkesford et al. 1993; Smith et al. 1995; Takahashi et al. 1997). Long distance transport from root to shoot occurs with the transpiration flow through the xylem (Herschbach and Rennenberg 1991). In leaves, sulfate is taken up into the cells and stored in the vacuole or transported into chloroplasts. The sulfate transporters SULTR4.1 and SULTR4.2 were shown to be located in the tonoplast membrane and function as sulfate efflux carriers (Kataoka et al. 2004). However, sulfate transporters localized in plastids, the organelles primarily important for sulfate reduction, have not been described so far

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(Hawkesford and De Kok 2006). In chloroplasts, sulfate becomes activated through adenylation to adenosine-5′-phopsphosulfate (APS) by ATP-sulfurylase. Subsequently, APS is reduced to sulfite by APS-reductase (APR) and further reduced to sulfide by the action of sulfite reductase (SIR). The incorporation of the sulfide moiety into the β position of alanine is the final step in the synthesis of the first sulfur containing organic molecule cysteine (Kopriva et al. 2009). The carbon precursor for cysteine biosynthesis is provided by *O*-acetylserine (OAS), the activated form of serine. Cysteine biosynthesis from serine is catalyzed by serine acetyltransferase (SAT) and *O*-acetylserine-(thiol)-lyase (OASTL). SAT generates OAS which subsequently condensates with sulfide in the reaction catalyzed by OASTL to form cysteine. One important feature of the homodimeric OASTL is that it assembles with SAT into the cysteine–synthase complex (CSC), which was reported to represent a main control point for the regulation of cysteine synthesis (Kredich and Tomkins 1966; Droux et al. 1998; Wirtz and Hell 2006). Due to the presence of SAT and OASTL activity in the cytosol, mitochondria and plastids, it was assumed that cysteine can be synthesized in all three compartments (Brunold and Suter 1982; Lunn et al. 1990; Ruffet et al. 1995; Warrilow and Hawkesford 1998; Hesse et al. 1999; Howarth et al. 2003; Kawashima et al. 2005). Although remarkable progress has been made in understanding the function of the compartmentation of cysteine biosynthesis (Heeg et al. 2008; Haas et al. 2008; Watanabe et al. 2008a, b; Krueger et al. 2009), it is still not clearly resolved why the enzymes for cysteine biosynthesis are needed in all three cellular compartments. The *Arabidopsis thaliana* genome encodes nine OASTL-like isoforms located in the cytosol, plastids and mitochondria (Warrilow and Hawkesford 1998; Wirtz and Hell 2006; Heeg et al. 2008; Watanabe et al. 2008a). Although all nine members of the OASTL-like family share high sequence homology on nucleotide and amino acid level, some were shown to be rather active as β-cyano-alanine-synthases and presumably function in cyanide detoxification (Watanabe et al. 2008a). The tight association of SAT and OASTL in a bi-enzyme complex was first described by Kredich and Tomkins (1966) for *Salmonella typhimurium*. Several studies indicate that this multimeric complex also exists in different plant species (Droux et al. 1998; Bogdanova and Hell 1997; Berkowitz et al. 2002). With respect to their stoichiometric ratio, it has been demonstrated that OASTL activity exceeds SAT activity by 345-fold in plastids, 200-fold in the cytosol and 10-fold in mitochondria (Ruffet et al. 1994; Heeg et al. 2008), which indicates that SAT is exclusively present in the complex bound form. As it was shown that cysteine biosynthesis is most efficient at a ratio of SAT to OASTL of 1:350, it was assumed that the chloroplast is the main site for cysteine biosynthesis in plants (Ruffet et al. 1994; Droux et al. 1998). In vivo interaction of SAT and OASTL was recently confirmed in fluorescent resonance energy transfer (FRET) studies (Wirtz and Hell 2006). Complex formation strongly depends on concentrations of OAS and sulfide, the two substrates for cysteine biosynthesis. High OAS concentrations promote CSC dissociation, whereas sulfide stabilizes the complex (Kredich et al. 1969; Berkowitz et al. 2002; Wirtz and Hell 2006, 2007). This suggested that the CSC might play a central role for the regulation of cysteine biosynthesis depending on the sulfur status of the plant. Upon sulfur limiting conditions, accumulating OAS is believed to dissociate the CSC leading to reduced SAT activity and, consequently reduced OAS production and less acetyl-CoA consumption. Five SAT isoforms with different expression patterns and biochemical properties exist in *A. thaliana* (Kawashima et al. 2005). SERAT3.1 and SERAT3.2 are expressed at low levels and exhibit very low substrate affinities for serine and acetyl-CoA. Although both are assigned as SAT enzymes they can only partly compensate for whole SAT activity in *Arabidopsis* (Watanabe et al. 2008b). In comparison, analysis of quadruple mutants for the different SAT isoenzymes shows that cytosolic SERAT1.1, plastidic SERAT2.1 and mitochondrial SERAT2.2 alone can take over the function of the other SAT isoforms (Watanabe et al. 2008b). However, a second approach with transgenic *Arabidopsis* plants silenced for the mitochondrial SAT isoform showed dramatically reduced growth rate, OAS levels and flux into thiols, indicating a predominant function of the mitochondrial SERAT2.2 for cysteine biosynthesis in plants (Haas et al. 2008). In plants, SAT activity is modulated by feedback inhibition through cysteine, similarly as observed for enteric bacteria. In *A. thaliana*, SERAT1.1, SERAT2.2 and SERAT3.2 isoenzymes are sensitive to feedback inhibition by cysteine; however, the K_i value of these isoforms is different (Noji et al. 1998; Wirtz and Hell 2003; Kawashima et al. 2005; Wirtz and Hell 2006). Feedback regulation is a common mechanism in regulation cysteine biosynthesis. In enteric bacteria, cysteine levels decrease upon sulfur starvation, which results in activation of feedback-sensitive SAT and, in consequence, OAS levels and hence, NAS levels rise, leading to the activation of the cysteine regulon. De-repression strongly depends on activity of the tetrameric cysB-protein (Ostrowski et al. 1989; Kredich 1992). The cysB-protein acts as transcriptional activator of the cysteine regulon, whereas it has been shown to inhibit initiation of transcription on its own promoter. Binding of cysB to the promoters of the cysteine regulon and hence initiation of transcription is enhanced by binding of *N*-acetylserine (NAS), which derives from pH-dependent chemical conversion of OAS (Ostrowski and Kredich 1990; Lynch et al. 1994). A similar mechanism for

the regulation of sulfur uptake and assimilation has been postulated for plants with the feedback-sensitive cytosolic SERAT1.1 (Saito 2000; Liu et al. 2006). Although no functional homolog of a transcriptional activator has been identified in plants so far, regulation of gene expression through the pathway intermediates OAS, sulfide and glutathione is well known for plants (Neuenschwander et al. 1991; Vauclare et al. 2002; Hirai et al. 2003; Nikiforova et al. 2003; Buchner et al. 2004; Maruyama-Nakashita et al. 2004a, b, 2006; Hopkins et al. 2005; Durenkamp et al. 2007). Externally applied OAS induces expression of APR and sulfate transporters, whereas sulfide and glutathione negatively regulate the expression of these genes (Neuenschwander et al. 1991; Vauclare et al. 2002; Buchner et al. 2004; Maruyama-Nakashita et al. 2004a, 2006). Recently, the transcription factor sulfur limitation responses less mutant 1 (SLIM1) was identified by EMS mutagenesis of plants expressing green fluorescent protein (GFP) under the control of the SULTR1.2 promoter (Maruyama-Nakashita et al. 2006). Sulfate uptake and plant growth were significantly reduced in the slim1 mutant under sulfur starvation conditions compared to the wild type. As SLIM1 expression is constitutive and not altered upon sulfur starvation, regulation of transcription mediated by SLIM1 might be controlled at the posttranslational level (Maruyama-Nakashita et al. 2006). With respect to post-translational regulation of sulfate uptake and assimilation, it was recently shown that small deletions in the C-terminal region of the sulfate transporter anti-sigma (STAS) domain results in loss of transport capacity for sulfate (Rouached et al. 2005). Moreover, the STAS domain appears to interact with OASTL (Rouached et al. 2005; Shibagaki and Grossman 2007). Thus, this domain might represent a new site for the regulation of sulfate uptake. Adjustment of sulfate uptake and assimilation with cysteine biosynthesis is important as cysteine serves as precursor for many sulfur-containing compounds essential for cell metabolism and survival like methionine and its derivatives, essential vitamins, co-factors such as thiamine, iron–sulfur proteins and glutathione (Beinert 2000; Leustek et al. 2000; Marquet 2001; Mendel and Hansch 2002; Wittstock and Halkier 2002; Hesse and Höfgen 2003; Meyer 2008). Glutathione is a highly abundant non-protein thiol present in many organisms (Noctor et al. 2002). In plants, glutathione plays an important role in the detoxification of reactive-oxygen-species (ROS), which are produced in response to many biotic and abiotic stresses as part of the glutathione-ascorbate cycle (May et al. 1998; Noctor and Foyer 1998; Ruiz and Blumwald 2002; Xiang et al. 2001; Meyer 2008). Glutathione is synthesized in two sequential ATP-consuming reactions catalyzed by γ-glutamylcysteine-synthetase (GSH1) and glutathione synthetase (GSH2; Hell and Bergmann 1990; May et al. 1998; Noctor et al. 2002). In plants, γ-glutamylcysteine synthesis by GSH1 takes place exclusively in the plastids and represents the rate limiting step in glutathione biosynthesis (Wachter et al. 2005; Hothorn et al. 2006). Moreover, GSH1 activity is tightly redox-controlled by glutathione and feedback inhibited by γ-glutamylcysteine (Cobbett et al. 1998; Jez and Cahoon 2004; Hothorn et al. 2006; Pasternak et al. 2008). The K_m values for cysteine (1.6 mM) and glutamate (9.1 mM) of *A. thaliana* GSH1 enzymes are relatively high compared to *Nicotiana tabacum* (0.19 mM cysteine, 10.4 mM glutamate) and *Brassica napus* (0.12 mM cysteine, 8.5 mM glutamate; Hell and Bergmann 1990; Jez and Cahoon 2004; Hothorn et al. 2006). In contrast, the K_m value of the *A. thaliana* GSH2 enzyme, which exhibits cytosolic and plastidic localization (Wachter et al. 2005), has been determined to be 0.038 mM for γ-glutamylcysteine, 0.057 mM for ATP and 1.51 mM for glycine (Jez and Cahoon 2004). Despite GSH2 appears to be dually targeted, multiple transcript analysis and determination of enzyme activity of GSH2 in purified chloroplasts indicate that GSH2 is predominantly localized in the cytosol (Hell and Bergmann 1990; Wachter et al. 2005; Pasternak et al. 2008). Uptake studies using isolated wheat chloroplast indicate that glutathione is taken up into chloroplasts by a low and high affinity uptake system (Noctor et al. 2002).

Although substantial knowledge about cysteine and glutathione biosynthesis in plants has been compiled over the last decade (Nikiforova et al. 2006; Wirtz and Hell 2006; Heeg et al. 2008; Haas et al. 2008; Meyer 2008; Pasternak et al. 2008; Watanabe et al. 2008a, b; Krueger et al. 2009) the understanding of its regulation is far from being complete.

Application of nutrient stress conditions in combination with transcript, enzyme and metabolite analysis of T-DNA insertion mutants for SERAT1.1, SERAT2.1 and the respective double mutant should give additional insight into regulation and homeostasis of cysteine biosynthesis and related biosynthetic processes.

Materials and methods

Plant cultivation

Sterile *Arabidopsis* seeds were transferred to agar plates, stratified for one night at 4°C and grown on agar plates (Murashige and Skoog 1962) for 10 days. Ten-day-old seedlings were carefully transferred to autoclaved pipette-tip boxes (Eppendorf, Hamburg, Germany) filled with 0.5 l Hoagland medium (Arnon and Hoagland 1939) and grown in the greenhouse (16/8 h light/dark cycle, 140 µmol m^{-2} s^{-1} photon flux density, 50% humidity,

21°C). To adapt seedlings to lower humidity, the boxes were covered with a transparent cover for 5 days. From 5 days onwards, the cover was completely removed. The hydroponic cultures were grown for additional 3 weeks before starvation experiments were conducted.

Enzyme assays

Soluble protein extracts were prepared using 100 mg frozen *Arabidopsis* leaf or root material or fractions of lyophilized powder from non-aqueous gradients and 500 µL extraction buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES)/KOH, pH 7.4, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycoltetraacetic acid (EGTA), 0.1% (v/v) Triton-X (Sigma-Aldrich, Munich, Germany), 10% (v/v) glycerol, 5 mM 1,4-bis-sulphanylbutane-2,3-diol (DTT), 2 mM benzamidine, 2 mM ε-aminocapronic acid, 0.5 mM phenylmethane sulphonylfluoride (PMSF) and 1 g L^{-1} polyvinylpolypyrrolidone (PVPP)] modified from Geigenberger and Stitt (1993). After centrifugation, the supernatant was desalted and total protein quantified according to Bradford (1976). SAT (EC 2.3.1.30) activity was assayed via high-performance liquid chromatography (HPLC) by the determination of OAS content (Lindroth and Mopper 1979; Kim et al. 1997; Krueger et al. 2009). OASTL (EC 2.5.1.47; OASTL) activity was determined by measuring cysteine formation using the method described by Gaitonde (1967). UDP-glucose-pyrophosphorylase (EC 2.7.7.9; UGPase) activity was determined according to Zrenner et al. (1993) and NADP-glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.13; GAPDH) activity was determined according to Stitt et al. (1983).

Metabolite analysis

Individual soluble thiols were determined as the sum of their reduced and oxidized forms. 50 mg of freshly ground frozen plant material or material from NAF was added to 10 mg polyvinylpolypyrrolidone (PVPP; previously washed with 0.1 N HCl) and 1 mL 0.1 N HCl. The samples were shaken for 60 min at room temperature and centrifuged (15 min at 13,000*g*; 4°C). After centrifugation, the supernatants were frozen at −20°C until reduction/derivatization. Prior to derivatization with mBrB (3-bromomethyl-5-ethyl-2,6-dimethyl-pyrazolo[1,2-α]pyrazol-1,7-dione, Calbiochem), thiols were reduced by incubation with 10 mM DTT for 40 min at room temperature. HPLC was conducted with a Hypersil ODS C18 column. Samples were eluted with increasing concentrations of methanol in an acetic acid/methanol mixture. Column eluent was monitored by fluorescence detection (λex 380/λem 480). Sulfide was determined following a modified protocol from Vetter et al. (1989), Völkel and Grieshaber (1992) and Wohlgemuth et al. (2000). Twenty milligrams of freshly ground frozen plant tissue was extracted for 30 min in the dark with 160 µL extraction buffer [10 µL 3-bromomethyl-5-ethyl-2,6-dimethyl-pyrazolo(1,2-a)pyrazol-1,7-dione (Calbiochem, Darmstadt, Germany); 100 µL 160 mM HEPES/16 mM EDTA, pH 8.0; 50 µL acetonitrile] and was stabilized with 100 µL 65 mM methanesulphonic acid. The extracts were centrifuged for 15 min at 14,000*g* and 4°C. The supernatant was diluted one to four with solvent solution (88% of 0.25% acetic acid, 12% methanol) and analyzed with a Merck (Darmstadt, Germany)/Hitachi (Tokyo, Japan) HPLC system [AS-2000 autosampler; L-6200 intelligent pump; F-1050 fluorescence detector; RP select B (5 mM), LiChrospher 60, LiChroCART 125-4 chromatography column; D-7000 HPLC System Manager Version 2.1].The column eluent was monitored by fluorescence detection (lex 380/lem 480). OAS was determined following a modified protocol from Kim et al. (1997) described in Krueger et al. (2009). For organic ion analysis, 20 mg of freshly ground frozen plant material or material from NAF was homogenized in 200 mL 0.1 mM HCl. Samples were centrifuged for 5 min at 14,000*g* and 4°C. The supernatant was transferred to Ultrafree MC 5000 MC NMWL Filter Unit (Millipore, Schwalbach, Germany) and was centrifuged for 90 min at 5,000*g* and 4°C. After filtration, 20 mL of the diluted sample was analyzed by HPLC with conductivity detection facilitating a Dionex ICS-2000 system (Dionex, Idstein, Germany). Ions were eluted in a KOH gradient.

NAF

The method described here is based on the method described by Krueger et al. (2009) for the determination of subcellular metabolite levels in *A. thaliana* leaves. Four gradients were made from biologically independent plant material of plants grown under control conditions and one gradient for the respective starvation condition. For all analyzed metabolites and enzymes in the fractions of the gradients, the recovery rate was determined. UGPase activity as cytosolic marker was determined according to Zrenner et al. (1993). GAPDH activity as chloroplast marker was determined according to Stitt et al. (1983). According to Winter et al. (1994), nitrate was used as vacuolar marker. Nitrate concentration was analyzed by ion-exchange chromatography (IEC). Marker measurements from three technical replicates of each gradient were averaged (Riens et al. 1991). The compartmental distribution was estimated according to Riens et al. (1991) using a C version of the compartment calculation program Bestfit (Steinhauser et al., unpublished data).

RNA extraction and RT-PCR

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and was digested with TURBO DNA-free (Ambion, Huntingdon, UK). Absence of genomic DNA contamination was confirmed by PCR on the RNA sample before cDNA synthesis. PCR reactions contained 1 μM Aktin Primer, *Taq* polymerase and buffer (Finnzymes, Espoo, Finland) and 5 mM deoxyribonucleotides. After 5 min of initial denaturation at 92°C, 30 cycles of 30 s at 92°C, 30 s at 60°C and 2 min at 74°C were performed. Amplicons were made visible on ethidium bromide-stained agarose gels. Total RNA (5 μg) was subjected to cDNA synthesis using oligo(dT) primer and Superscript III (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. The RT-PCR of SULTR4.2 sulfate transporter (At3g12520), SULTR2.1 sulfate transporter (At5g10180), SULTR1.2 sulfate transporter (At1g78000), APR3 APS-reductase 3 (At4g21990) and ACTIN (AT3G18780) cDNA was conducted in three repetitions with cDNA from biologically independent plant material of wild type, *csat*, *psat* and *dmsat* plants. Quality of cDNA was assessed by quantitative PCR using primers amplifying sequences located at the 3′- or 5′-end of glyceraldehyde-3-phosphate dehydrogenase (At1g113440) mRNA, respectively (GAPDH five prime end 5′-TCTCG ATCTCAATTTCGCAAAA-3′; 5′-CGAAACCGTTGATT CCGATTC-3′ and three prime end 5′-TTGGTGACAAC AGGTCAAGCA-3′; 5′-AAACTTGTCGCTCAATGCAA TC-3′). Only cDNA where the ratio between the relative amount of 3′ and 5′ amplicon was below 1.5 was used for further analysis. PCR was conducted in an optical well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR® Green (Applied Biosystems) was used to monitor product formation. PCR conditions were as described by Czechowski et al. (2004) with minor modifications. Reactions contained 5 μL two-times SYBR® Green Master Mix reagent (Applied Biosystems), 1 μL of 1:5 diluted cDNA and 200 nM of each gene-specific primer. Total reaction volume was 10 μL. The thermal profile used was 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. SDS 2.0 software (Applied Biosystems) was used for data analysis. Primer sequences used for the amplification of sulfur starvation marker genes: SULTR4.2, 5′-ACCACAGTGTGCTTTAGCAGCAAT-3′; 5′-TCTCTTGTCCACACGCCACAGA-3′, APR3, 5′-GGA AGAGATCCTCCGTGAAAGC-3′; 5′-CTGTAACCTCAG AAGCAACAATGGA-3′, SULTR1.2, 5′-TCACCCTGTG GACGGAAGTC-3′; 5′-GTTTCATCGGAACATGTCCAC C-3′, SULTR2.1, 5′-ATTGTTGCTCTAACCGAGGCGAT T-3′; 5′-TGTACCCTTTTATTCCGGCGAACG-3′, Actin, 5′-CTCAAAGACCAGCTCTTCCATC-3′; 5′-GCCTTTG ATCTTGAGAGCTTAG-3′.

Results

OAS concentration increases and cysteine and sulfide levels decrease upon sulfur starvation

OAS, sulfide and cysteine concentrations were determined in leaf tissue of plants grown for 5 days under sulfur starvation conditions and compared to concentrations in leaves of plants grown under control conditions. Under control conditions, the total sulfide content was with 13.1 pmol mg^{-1} FW similar to the total cysteine concentration (17.4 pmol mg^{-1} FW) (Fig. 1). OAS had, with 2.5 pmol mg^{-1} (FW), a very low steady state concentration. After sulfur starvation conditions were applied to hydroponic cultures for 5 days, OAS levels increased significantly to 8 pmol mg^{-1} (FW), whereas sulfide and cysteine concentrations decreased to 7 pmol mg^{-1} (FW) and 12 pmol mg^{-1} (FW), respectively.

SAT and OASTL activities are not significantly altered upon sulfur starvation

In order to understand the regulation of cysteine biosynthesis and the impact of the subcellular compartments, we applied sulfur starvation to SAT mutants missing the cytosolic (At5g56760; *csat*), plastidic (At1g55920; *psat*) or both (*dmsat*) SAT isoenzymes. Compared to wild-type, SAT activity was slightly reduced in *csat* and *dmsat*

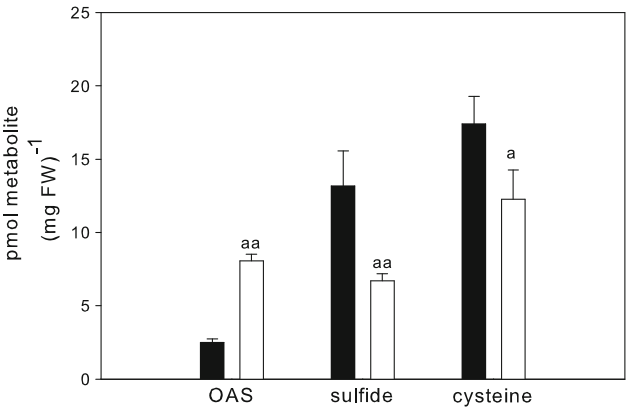

Fig. 1 Determination of *O*-acetylserine (OAS), sulfide and cysteine concentrations in *Arabidopsis thaliana* leaf material. Total tissue content of OAS, sulfide and cysteine of wild type plants grown in hydroponic culture and transferred for 5 days to media with (*black bars*) or without (*white bars*) sulfate. Data are presented in pmol mg^{-1} (FW) as mean values ± SD of three biologically independent replicates collected from at least ten plants per replicate

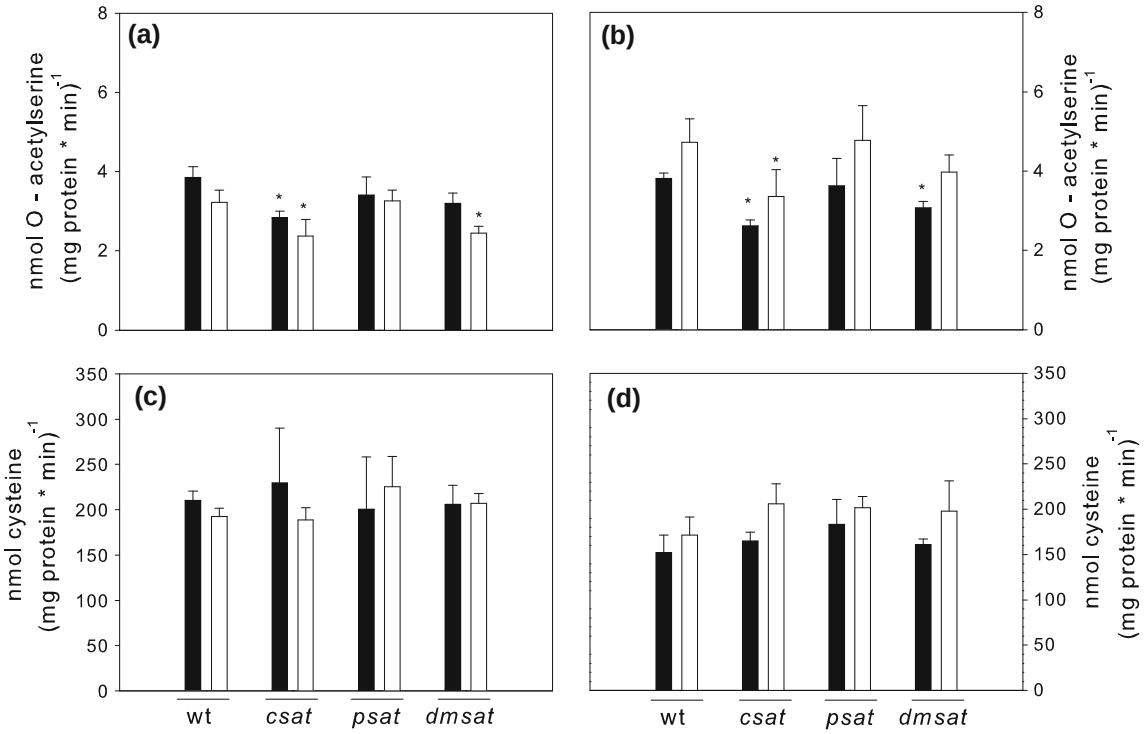

Fig. 2 SAT and OASTL activity in mutant and wild type plants grown in hydroponic culture and transferred for 5 days to media with (*black bars*) or without (*white bars*) sulfate. SAT (**a**, **b**) and OASTL (**c**, **d**) activities were analyzed in leaves (**a**, **c**) and roots (**b**, **d**) of *sat* mutant lines and wild type. Mean values and SD of four biological replicates collected from at least ten plants per replicate for each mutant and the wild type are presented. *Asterisks* mark significant differences between wild type and mutant ($P < 0.05$)

mutants independent of presence or absence of sulfur in the culture medium (Fig. 2a, b) However, upon starvation, no significant differences in SAT activity were observed in leaves and roots of wild type and mutant plants compared to the non-starved control (Fig. 2a, b). OASTL activity was not altered upon sulfur starvation or compared to wild type (Fig. 2c, d).

Cysteine biosynthesis is reduced in wild type and mutant plants under sulfur starvation conditions

OAS accumulates upon sulfide-limiting conditions. However, *csat, psat and dmsat* mutants never accumulated OAS to the same extent as wild type (Fig. 3a, b). Due to sulfur starvation, cysteine levels decreased significantly in leaves of wild type, *psat* and *dmsat* mutant plants in comparison to the non-starved control (Fig. 3c, d). In roots, only wild type and the *psat* mutant exhibited reduced cysteine levels in comparison to the non-starved control. Moreover, sulfur starvation abolished the differences between mutants and wild type. The analysis of downstream products of cysteine biosynthesis revealed a dramatic decrease in glutathione content upon sulfur starvation (Fig. 3g, h). However, none of the mutants was affected more severely than the wild type. γ-glutamylcysteine levels decreased in wild type and *psat* mutants upon sulfur starvation; nevertheless, they did not further decrease in *csat* and *dmsat* plants, those mutants, which already exhibited reduced γ-glutamylcysteine levels versus wild type under non-starved conditions (Fig. 3e, f). Compared to the wild type, no differences in γ-glutamylcysteine content were observed in either of the mutants upon sulfur starvation. To assess the extent of starvation, sulfate content was determined (Fig. 4a, b). Under non-starved conditions leaf sulfate content was reduced in *csat* ($P = 0.011$) and *dmsat* ($P = 0.061$) mutants while it remained at wild type levels for the *psat* mutant. In contrast, sulfate levels in roots remained unchanged in all three mutants compared to wild type. Upon 5 days of starvation, sulfate content was reduced to approximately 20% of the non-starved control. None of the mutants, however, displayed reduced sulfate levels compared to wild type.

Knock out of specific SAT isoenzymes does not affect the expression of genes involved in sulfate uptake and reduction

OAS is thought to be a regulator of gene expression in plants (Hirai et al. 2003; Nikiforova et al. 2003; Hopkins et al. 2005). As SAT T-DNA insertion mutants exhibited reduced OAS levels compared to wild type upon sulfur

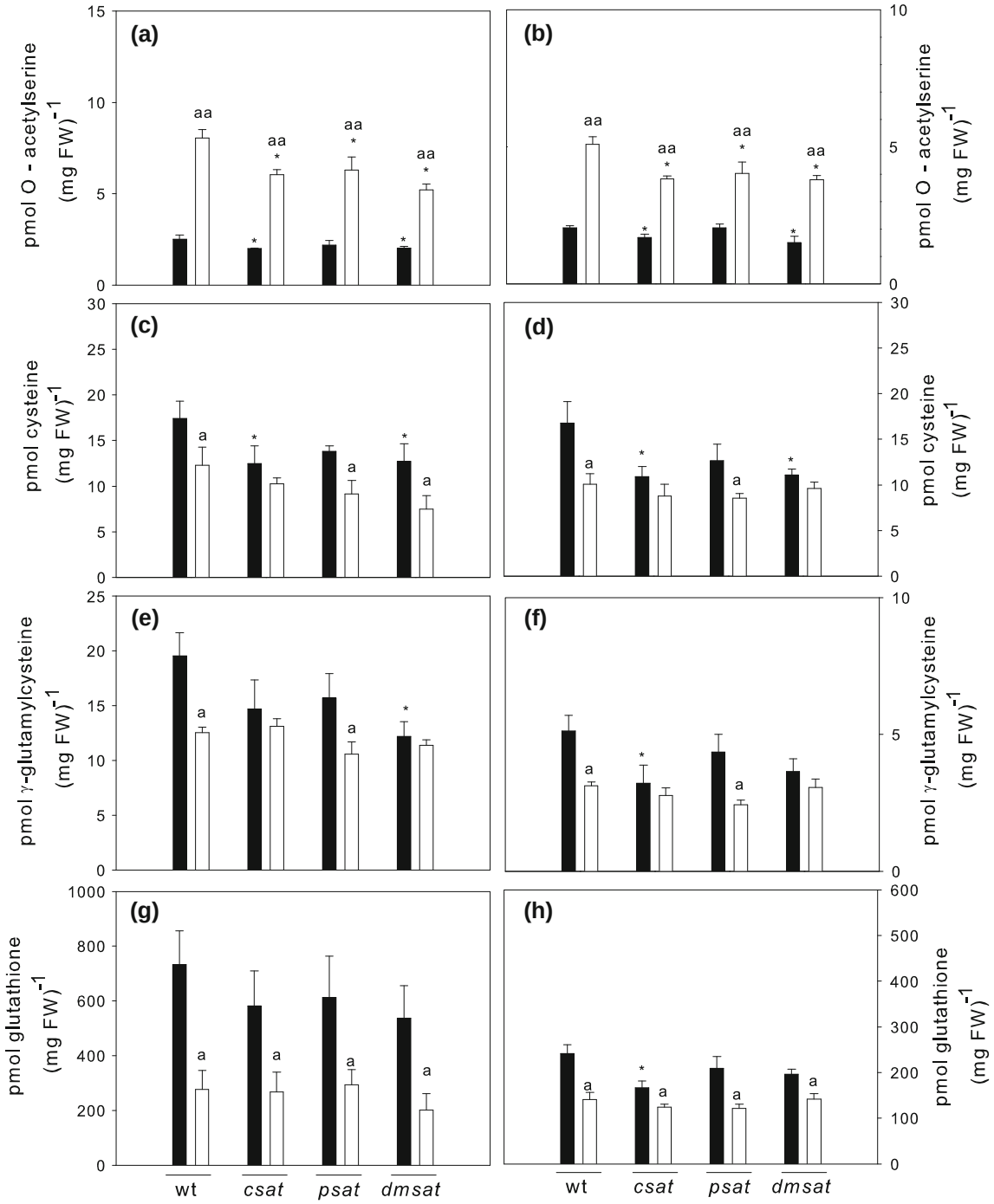

Fig. 3 Content of metabolites related to cysteine biosynthesis. OAS (**a**, **b**), cysteine (**c**, **d**), γ-glutamylcysteine (**e**, **f**) and glutathione (**g**, **h**) content in leaf (**a**, **c**, **e**, **g**) and root (**b**, **d**, **e**, **h**) tissue of mutants and wild type plants grown for 5 days under sulfur starvation conditions. Mean values and SD of three to four biological replicates collected from at least ten plants per replicate for each mutant and the wild type are presented. *Letters* indicate differences between starved plants and non-starved controls ($^{a}P < 0.05$, $^{aa}P < 0.001$), whereas *asterisks* mark significant differences between wild type and mutant ($P < 0.05$)

starvation (Fig. 3), expression of APS-reductase (APR) and several sulfate transporters was investigated upon sulfur starvation. Under non-starved conditions, expression of these genes was not modulated in the SAT mutants compared to wild type (Krueger et al. 2009). In contrast, transcripts of the sulfur starvation responsive APS-reductase 3 and the sulfate transporters SULTR4.1 and SULTR1.2 were significantly induced in leaves and roots, whereas SULTR2.1 was induced in roots and repressed in leaves after 5 days of starvation (Fig. 5). The expression

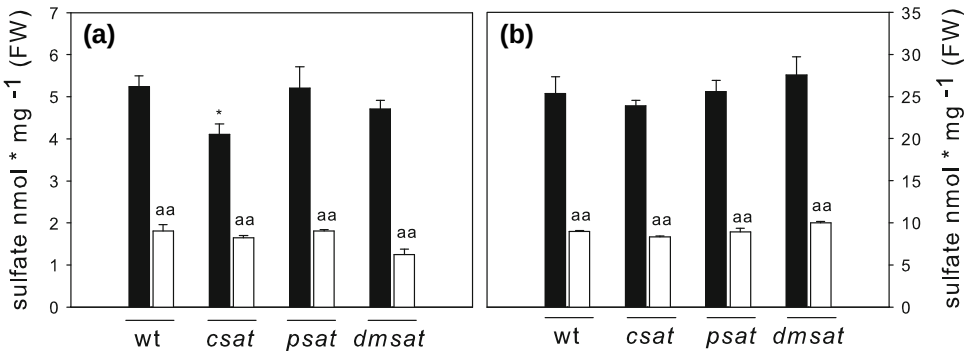

Fig. 4 Changes in sulfate content upon 5 days of sulfur starvation in leaf (**a**) and root (**b**) tissues. For each experiment, mean values and SD of three to four biological replicates collected from at least ten plants per replicate for each mutant and the wild type are presented. *Letters* indicate differences between starved plants and non-starved controls ($^{a}P < 0.05$, $^{aa}P < 0.001$), whereas *asterisks* mark significant differences between wild type and mutant ($P < 0.05$)

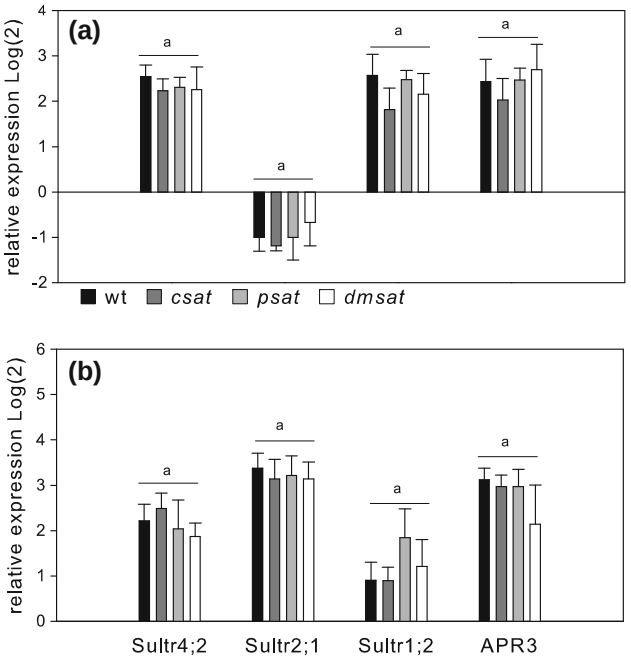

Fig. 5 Relative expression of different sulfur starvation marker genes in leaves (**a**) and roots (**b**) was analyzed by quantitative RT-PCR. *Black bars* represent wild type; *dark grey bars*, *csat*; *light grey bars*, *psat* and *white bars*, *dmsat* mutant, respectively. For each experiment, mean values and SD of three replicates collected from at least ten plants per replicate for each mutant and the wild type are presented. *Letters* indicate differences between starved plants and non-starved controls ($^{a}P < 0.05$)

level of all sulfur-responsive genes was similar for the mutants and wild type after 5 days of starvation.

Changes in the subcellular distribution of OAS and thiols upon sulfur starvation

To determine alterations in the subcellular distribution of sulfur-containing metabolites upon sulfur starvation, non-aqueous fractionation (NAF) of *A. thaliana* leaf material was conducted as previously described by Krueger et al. (2009). Assignment of the metabolite distribution to the compartments cytosol, plastid and vacuole was made according to best fit calculation (Riens et al. 1991; Krueger et al. 2009). Previously, it was shown that NAF is not suitable for analyzing metabolite composition within mitochondria, as the mitochondrial marker was in no fraction superior and similarly distributed as the marker for the cytosolic compartment (Gerhardt and Heldt 1984; Farré et al. 2001; Tiessen et al. 2002; Fettke et al. 2005; Krueger et al. 2009). Therefore, mitochondrial localized metabolites are contained in the cytosolic fractions. The relative abundances of the different metabolites were normalized to the protein: chlorophyll ratio before fractionation and to the average ratio of organelle volumes to total cell volume published for spinach, wheat and potato (Winter et al. 1992, 1993; Leidreiter et al. 1995). Figure 6 depicts percent changes of subcellular metabolite concentration after 2, 3 and 4 days of sulfur starvation compared to the non-starved control. The subcellular distribution of OAS and thiols revealed that less than 5% of the cellular OAS is located in the chloroplasts (Fig. 6). Moreover, chloroplastically localized OAS did not accumulate upon sulfur starvation. OAS accumulated mainly in the fractions assigned to the cytosol upon sulfur starvation. After 4 days of sulfur starvation the OAS pool assigned to the cytosol was increased up to 70%, whereas the cytosolic cysteine pool was decreased only around 20%. In agreement with our previous observations, γ-glutamylcysteine did not accumulate in the chloroplasts to a major extent; however, in plants grown in hydroponic culture, 10% of the cellular γ-glutamylcysteine was located in the chloroplasts (Fig. 6). Interestingly, the plastidic γ-glutamylcysteine pool stayed relatively constant over 4 days of sulfur starvation, whereas the cytosolic γ-glutamylcysteine concentration was already decreased to 21% after 3 days and slightly enhanced to 29% after 4 days of sulfur starvation. At these

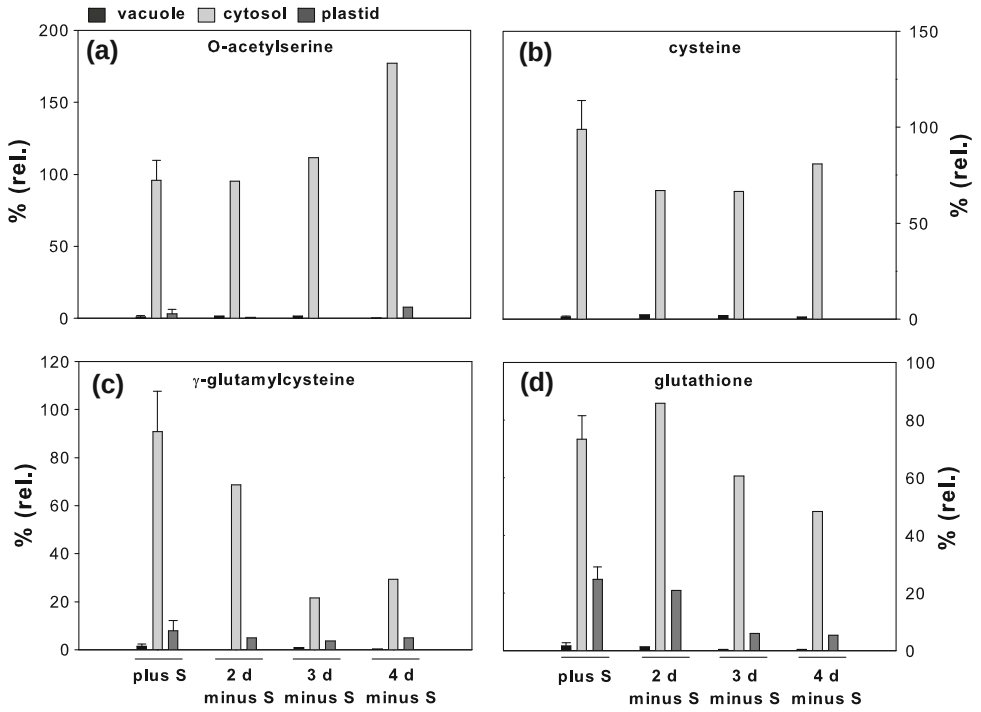

Fig. 6 Changes in subcellular distribution of metabolites involved in cysteine and glutathione biosynthesis under sulfur starvation conditions in *Arabidopsis thaliana* leaves. Percent distribution to the subcellular compartments vacuole, cytosol and plastid of OAS (**a**), cysteine (**b**), γ-glutamylcysteine (**c**) and glutathione (**d**) was calculated using the best fit algorithm (Riens et al. 1991). Subcellular concentrations were evaluated as described by Krueger et al. 2009. Changes in subcellular concentrations of the different metabolites upon sulfur starvation were expressed in percent changes relative to control conditions. Mean values and SD of four independent gradients for control conditions and mean values of three technical replicates of one gradient for the respective starvation condition are shown

time points, the cytosolic glutathione level was reduced to 60% at day 3 and 48% at day 4 of the non-starved control, whereas the plastidic glutathione pool was decreased from 25% under non-starved conditions to 5% after 4 days of starvation. Vacuolar concentrations of all metabolites analyzed were low compared to the cytosol.

Discussion

OAS rather than sulfide limits cysteine biosynthesis

To understand the regulation of cysteine, biosynthesis knowledge of the concentration and compartmentation of cysteine precursor molecules is necessary. Aside from *O*-acetylserine (OAS), sulfide is the direct precursor for cysteine. Therefore, a method to measure sulfide in lugworm body wall and colon fluid (Völkel and Grieshaber 1992; Wohlgemuth et al. 2000) was modified to determine sulfide levels in plant leaf extracts. Sulfide concentrations determined in *A. thaliana* leaves were 13.1 pmol mg^{-1} (FW) and therefore in a similar range as cysteine concentrations (Fig. 1). The observation that sulfide concentrations by far exceeded OAS concentrations indicates that under normal growth conditions, OAS is the limiting factor for the synthesis of cysteine. These results were supported by the finding that under sulfur starvation conditions OAS accumulated to the same extent as sulfide content decreased (Fig. 1). That sulfide alone is not the limiting factor for the cysteine biosynthesis was already speculated by Rennenberg (1983), who observed enhanced cysteine levels after OAS feeding at the cost of hydrogen sulfide emission in pumpkin leaves. In addition, in plants overexpressing an adenosine 5′-phosphosulfate-reductase (APR) from *Pseudomonas aeruginosa* thiol levels were significantly enhanced only after external application of OAS (Tsakraklides et al. 2002). In plants, sulfide is not only released from the sulfate assimilation pathway but also through the cysteine degrading activity of OASTL or cysteine-desulfhydrase (Riemenschneider et al. 2005). However, more than 50% of sulfide detected in leaf tissue could be assigned to the chloroplast, which indicates that the sulfate assimilation pathway is the main source for the cellular sulfide (Krueger et al. 2009). Determination of metabolite concentrations at the subcellular level is important to understand the tight regulation of CSC. OAS and sulfide-driven dissociation and association kinetics have recently been established for the CSC using BIAcore technology (Berkowitz et al. 2002). Cytosolic sulfide concentration (∼55 μM) under non-stressed conditions

would rather promote association of the CSC, whereas upon 5 days of sulfur starvation cytosolic sulfide concentration (~27 μM) would support the dissociation of the CSC (Krueger et al. 2009 and data published here; Fig. 1).

The performance of compartment-specific SAT knock-out mutants under sulfur starvation

Sulfate starvation was extensively used as a tool to investigate the regulation of sulfate uptake and assimilation in plants (Takahashi et al. 1997; Lappartient et al. 1999; Nikiforova et al. 2003; Hirai et al. 2003; Hopkins et al. 2005). It has been proposed that sulfur limitation might induce SAT activity to enhance OAS production and, in consequence, as OAS influences sulfate assimilation, accelerate sulfate uptake and ensure cysteine biosynthesis (Saito 2000; Ravina et al. 2002; Kawashima et al. 2005). In the present study modulation of SAT activity in leaves and roots of wild type and mutant plants under sulfur starvation could not be observed (Fig. 2). Furthermore, also under sulfur starvation conditions, SAT activity was slightly but significantly reduced in leaves and roots of *csat* mutant and in leaves of *dmsat* mutant compared to wild type. In addition, the observation that reduced SAT activity in the *csat* and *dmsat* mutant resulted in moderately lower concentrations of OAS and thiol compounds indicates that SERAT1.1 is at least partially active, despite high cytosolic cysteine concentrations (Fig. 6; Krueger et al. 2009). These results are supported by the finding that CSC formation enhances SAT activity and prevents SAT from feedback inhibition by cysteine (Kumaran et al. 2009). As OASTL present in the cytosol is in 200-fold excess over SAT almost all SAT is likely bound in the CSC and therefore presumably not longer subject to feedback regulation by cysteine. That SERAT1.1 is functional and to some extent involved in cysteine biosynthesis was also described by Watanabe et al. (2008b). The high residual SAT activity in *csat*, *psat* and *dmsat* mutants analyzed in this study can be explained by the action of mitochondrial SAT, which was shown to be the predominant SAT in *A. thaliana* (Haas et al. 2008; Watanabe et al. 2008b; Krueger et al. 2009).

With respect to OASTL activity, no alteration was observed upon sulfur starvation in SAT mutants and wild type (Fig. 2). These results are in agreement with earlier studies showing that OASTL activity is only slightly affected by sulfur starvation in plants (Takahashi and Saito 1996). One of the first responses to sulfur starvation in plants is accumulation of OAS (Nikiforova et al. 2003; Hirai et al. 2003; Hopkins et al. 2005). Although OAS levels increased upon 5 days of sulfur starvation in all plants analyzed, the mutants exhibited significantly lower OAS concentrations in comparison to wild type (Fig. 3). Thus, it appears that due to sulfide limitation, the flux of OAS into thiols is reduced and OAS produced by all SAT isoezymes accumulates. This shift between mutant and wild type may indicate the real contribution of SERAT1.1 and SERAT2.1 to OAS formation. That SERAT1.1 and SERAT2.1 contribute to cysteine biosynthesis is supported by the fact that both enzymes are able to compensate for the whole SAT activity in plants (Watanabe et al. 2008b). Concentration of cysteine, γ-glutamylcysteine and glutathione decreases upon 5 days of sulfur starvation to nearly equal levels and no significant difference could be observed between wild type and mutant plants. This might have two reasons: first, upon starvation, sulfide rather than OAS becomes limiting for the cysteine biosynthesis, which is supported by the accumulation of OAS. Secondly, the cell might try to keep thiol concentration on a certain level, even if the flux into thiols is reduced. Analysis of plants with reduced mitochondrial SAT activity supports this hypothesis as here thiols were even higher concentrated in comparison to wild type (Haas et al. 2008).

Does knock out of compartment-specific SAT isoenzymes influence the regulation of gene expression upon sulfate assimilation?

Bacteria possess a cysteine regulon which is strongly controlled by the metabolic state of the cell. A decrease in cysteine which results from sulfate limitation leads to activation of the cysteine-sensitive SAT which, in consequence, increases OAS levels. OAS rapidly converts to *N*-acetylserine (NAS), which further positively regulates the cysteine regulon by binding to the cysB activator protein (Ostrowski et al. 1987; Lynch et al. 1994). Similarly, the plant genome contains a set of genes involved in sulfate uptake and assimilation. Expression of these genes is affected upon sulfate starvation (Neuenschwander et al. 1991; Koprivova et al. 2000; Vauclare et al. 2002; Hirai et al. 2003; Nikiforova et al. 2003; Buchner et al. 2004; Hawkesford and De Kok 2006). In contrast to the plant system, the bacterial mechanisms of regulation of gene expression through metabolites are well understood (Ostrowski et al. 1987; Ostrowski and Kredich 1990; Lynch et al. 1994). An elegant model has been proposed for the regulation of sulfate uptake and assimilation upon sulfur limitation in plants. Upon sulfate starvation the decrease in cytosolic cysteine concentration would activate the cysteine-sensitive SERAT1.1 and enhanced cytosolic OAS levels would promote the expression of genes involved in sulfate uptake and accumulation (Saito 2000; Ravina et al. 2002; Kawashima et al. 2005). However, expression of sulfate transporters and APR3, which are known to respond to sulfur starvation and OAS feeding (Hirai et al. 2003; Nikiforova et al. 2003), was induced to the same extent in *csat* or the other mutants compared to wild type upon 5 days

of sulfur starvation (Fig. 5). These data indicate that SERAT1.1 and SERAT2.1 do not function as a regulator for gene expression upon sulfur starvation. However, these data do not exclude a metabolite-mediated signal. Differences in thiol concentration between mutants and wild type plants grown under full nutrient conditions disappeared after 5 days of starvation, whereas at this time point, OAS accumulation was less pronounced in mutants compared to the wild type. Besides OAS, cysteine and glutathione were shown to regulate expression of genes involved in sulfate uptake and assimilation (Koprivova et al. 2000; Vauclare et al. 2002). Therefore, it may be speculated that the decrease in thiol concentration leads to an enhanced expression of APR3 and sulfate transporter and might overwrite the OAS-mediated response. Although no differences in the expression of genes involved in sulfate uptake and assimilation could be observed in the mutants, the content of sulfate in leaves of the *csat* mutant was significantly reduced under non-stress conditions (Fig. 4a). A similar behavior was observed in mutants lacking the cytosolic OASTL (Heeg et al. 2008). Therefore, influencing the cytosolic cysteine biosynthesis seems to interfere with sulfate uptake. These results might become important for understanding the regulation of sulfate uptake as it was shown that cytosolic OASTL interact with sulfate transporters via the STAS domain (Shibagaki and Grossman 2007). Thus, in plants, the cytosolic CSC might represent a link between cysteine biosynthesis and sulfate uptake.

Sulfur starvation leads to distinct changes in the subcellular distribution of OAS and thiols

Recently, several reverse genetic approaches have partly clarified the function of cysteine biosynthesis in the different subcellular compartments (Heeg et al. 2008; Haas et al. 2008; Watanabe et al. 2008a, b; Krueger et al. 2009). That cysteine synthesis takes place mainly in plastids was one of the thoughts which could be falsified. Here, we show that OAS accumulates outside the plastids when plants are grown for 4 days under sulfur starvation conditions, which further supports these findings.

NAF does generally not allow sufficient separation of mitochondria (Krueger et al. 2009), therefore it cannot be resolved to which extent OAS accumulates within this organelle. Upon sulfur starvation the cytosolic cysteine concentration decreases compared to the non-starved control, whereas no changes are observed for the vacuolar cysteine pool. Surprisingly, the plastidic cysteine pool is very low and even below the detection limit in plants grown in hydroponic culture. These results indicate a rapid incorporation of cysteine into other metabolites as, for example, into sulfur-containing proteins or γ-glutamylcysteine (Abdel-Ghany et al. 2005; Wachter et al. 2005).

Interestingly, the main pool of γ-glutamylcysteine is located in the cytosol, whereas the amount located in the plastids is rather low. As GSH1 is strictly feedback regulated by its own product and glutathione is mainly synthesized in the cytosol, the export of γ-glutamylcysteine from the plastid into the cytosol would guarantee an efficient biosynthesis of glutathione even under stress conditions or during different developmental stages (Hell and Bergmann 1990; Vernoux et al. 2000; Noctor et al. 2002; Jez and Cahoon 2004; Wachter et al. 2005; Pasternak et al. 2008). Furthermore, the low K_m value of GSH2 for γ-glutamylcysteine (38 µM; Jez and Cahoon 2004) and its relatively high cytosolic concentration under non-stressed conditions ($\sim$225 µM; Krueger et al. 2009) indicates that the cytosol represents a reservoir for γ-glutamylcysteine to allow a demand-driven synthesis of glutathione. This hypothesis is further supported by the finding that after 3 and 4 days of sulfur starvation, the cytosolic γ-glutamylcysteine pool was significantly diminished, whereas no decrease in the plastidic pool could be observed. In comparison, glutathione concentration was decreased in plastids and the cytosol. The decrease in both compartments in combination with the unchanged concentration of γ-glutamylcysteine within plastids might support the speculation that at least in *A. thaliana* glutathione is imported into plastids (Wachter et al. 2005; Pasternak et al. 2008).

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