

# Mitochondrial Serine Acetyltransferase Functions as a Pacemaker of Cysteine Synthesis in Plant Cells<sup>1</sup>[C][W][OA]

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Cysteine (Cys) synthesis in plants is carried out by two sequential reactions catalyzed by the rate-limiting enzyme serine acetyltransferase (SAT) and excess amounts of *O*-acetylserine(thiol)lyase. Why these reactions occur in plastids, mitochondria, and cytosol of plants remained unclear. Expression of artificial microRNA (amiRNA) against *Sat3* encoding mitochondrial SAT3 in transgenic *Arabidopsis* (*Arabidopsis thaliana*) plants demonstrates that mitochondria are the most important compartment for the synthesis of *O*-acetylserine (OAS), the precursor of Cys. Reduction of RNA levels, protein contents, SAT enzymatic activity, and phenotype strongly correlate in independent amiSAT3 lines and cause significantly retarded growth. The expression of the other four *Sat* genes in the *Arabidopsis* genome are not affected by amiRNA-SAT3 according to quantitative real-time polymerase chain reaction and microarray analyses. Application of radiolabeled serine to leaf pieces revealed severely reduced incorporation rates into Cys and even more so into glutathione. Accordingly, steady-state levels of OAS are 4-fold reduced. Decrease of sulfate reduction-related genes is accompanied by an accumulation of sulfate in amiSAT3 lines. These results unequivocally show that mitochondria provide the bulk of OAS in the plant cell and are the likely site of flux regulation. Together with recent data, the cytosol appears to be a major site of Cys synthesis, while plastids contribute reduced sulfur as sulfide. Thus, Cys synthesis in plants is significantly different from that in nonphotosynthetic eukaryotes at the cellular level.

Cys synthesis in plants is a fundamental process for protein biosynthesis and all anabolic pathways that require reduced sulfur. Bacteria and fungi are able to reduce sulfate by assimilatory sulfate reduction to sulfide and to integrate it into Cys in the cytosol. In contrast, mammals need to take up reduced sulfur as Met and can synthesize Cys via trans-sulfurylation in the cytosol. Protein biosynthesis in the mitochondria in animals and fungi receives Cys, therefore, from the cytosol. In plants, the assimilatory sulfate reduction pathway is localized to plastids, whereas Cys synthesis is found in the cytosol, plastids, and mitochondria. Why Cys synthesis in plants is localized in three compartments has long been speculated.

Serine acetyltransferase (SAT; also termed Serat; EC 2.3.1.30) catalyzes the activation of Ser by acetyl-CoA

to *O*-acetylserine (OAS). In *Arabidopsis* (*Arabidopsis thaliana*), five genes encoding SAT1 to SAT5 are localized on the five chromosomes (Hell et al., 2002). SAT1 is plastid localized, SAT3 is found in mitochondria, and SAT5 is cytosolic (Kawashima et al., 2005). SAT2 and SAT4 are also cytosolic proteins, but they differ substantially from the other SATs in their amino acid sequences and are much less expressed (Kawashima et al., 2005). The *Arabidopsis Sat* genes are almost constitutively expressed ([www.geneinvestigator.ethz.ch](http://www.geneinvestigator.ethz.ch)), and the encoded proteins are of low abundance in comparison with *O*-acetylserine(thiol)lyase (OAS-TL; EC 2.5.1.47), since the activity of the latter is 300-fold higher than SAT activity in leaves (Ruffet et al., 1994). SAT activity is generally regarded as the rate-limiting step of Cys synthesis (Wirtz and Droux, 2005; Wirtz and Hell, 2006). Fractionation of pea (*Pisum sativum*) leaf cells showed that about 80% of extractable SAT activity under substrate-saturating conditions resides in mitochondria, while about 10% each is found in plastids and cytosol (Ruffet et al., 1995; Droux, 2003). The second step of Cys synthesis consists of the  $\beta$ -substitution of the acetyl moiety in OAS by sulfide and is catalyzed by OAS-TL. OAS-TLs are members of the pyridoxal phosphate-dependent  $\beta$ -substituted Ala synthase family (Hatzfeld et al., 2000b). Of the nine OAS-TL-like genes in *Arabidopsis*, one is a pseudogene, three encode the cytosolic OAS-TL A, plastid OAS-TL B, and mitochondrial OAS-TL C enzymes, and one is a  $\beta$ -cyanoalanine synthase that localizes to mitochondria (Hatzfeld et al., 2000b; Jost et al., 2000;

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Hell et al., 2002; Watanabe et al., 2008). According to cell fractionation of spinach (*Spinacia oleracea*), cauliflower (*Brassica oleracea*), and *Datura innoxia*, 40% to 45% of maximal OAS-TL activity is localized in plastids and cytosol, while about 5% is attributed to mitochondria (Lunn et al., 1990; Rolland et al., 1992; Kuske et al., 1996). As a result of the low mitochondrial OAS-TL activity, this activity is believed to be a side activity of  $\beta$ -cyanoalanine synthase in spinach (Warrilow and Hawkesford, 2000). The other four OAS-TL-like genes in Arabidopsis are only weakly expressed and characterized and show little or no Cys synthase activities (Wirtz and Hell, 2006; Heeg et al., 2008).

The three major SATs, SAT5, SAT1, and SAT3, form the Cys synthase protein complex with the major OAS-TLs, A, B, and C, in the cytosol, plastids, and mitochondria, according to interaction studies of the Arabidopsis proteins (Bogdanova and Hell, 1997; Jost et al., 2000). The Cys synthase complex was hypothesized to function in the cellular control of Cys synthesis, because SAT requires the presence of OAS-TL to gain full activity (Droux et al., 1998). At the same time, complex formation inactivates OAS-TL, and thus the reaction intermediate OAS dissociates from the complex and is freely available. OAS is known from feeding experiments to activate the expression of numerous genes, including genes related to sulfate uptake and reduction (Hirai et al., 2003; Maruyama-Nakashita et al., 2005). At the same time, OAS is able to dissociate the Cys synthase complex, at least in vitro, which is antagonized through stabilization by sulfide (Droux et al., 1998; Berkowitz et al., 2002; Wirtz et al., 2004; Wirtz and Hell, 2006). This effector-driven, reversible protein-protein interaction supposedly functions in cytosol, plastids, and mitochondria, although cell fractionations suggest considerably different ratios of SAT and OAS-TL activities in these compartments (Droux, 2003; Wirtz and Droux, 2005).

The role of OAS-TL in the three compartments with independent protein biosynthesis was recently addressed using Arabidopsis T-DNA insertion lines. Knockout of cytosolic OAS-TL A apparently caused oxidative stress and lesions as well as sensitivity to heavy metals (Lopez-Martin et al., 2008). While this phenotype was not reported by Watanabe et al. (2008) and Heeg et al. (2008), the latter studies confirmed the major roles of OAS-TL A, B, and C. OAS-TL A and B together contribute 95% of OAS-TL activity in leaves, and OAS-TL C contributes only about 5%. Despite this small contribution, a growth rate retardation of approximately 20% was observed for the *oastlC* mutant line. However, an *oastlAB* double mutant with OAS-TL C as the only Cys-forming activity was fully viable, with only a small reduction in growth rate (Heeg et al., 2008). OAS contents in the *oastlC* mutant were decreased, but in the *oastlAB* mutant they were found to be elevated, while labeling studies with [<sup>35</sup>S]sulfate revealed lowered incorporation of <sup>35</sup>S into Cys only in *oastlA* mutants. From these findings and the high specific SAT activity in isolated mitochondria, it was

concluded that mitochondria are the major site of OAS synthesis and that the cytosol, but not chloroplasts, provides most of the cellular Cys (Heeg et al., 2008). Despite the partial redundancy of Cys synthesis between these compartments, the results point toward a specific role of SAT in mitochondria as a pacemaker for the rate of cellular Cys synthesis. The investigations presented here use specific down-regulation of the gene encoding mitochondrial SAT3 by artificial microRNA (amiRNA) expression in Arabidopsis. Direct evidence for the dominant role of mitochondrial OAS formation for cellular Cys synthesis and its significance for plant growth is provided.

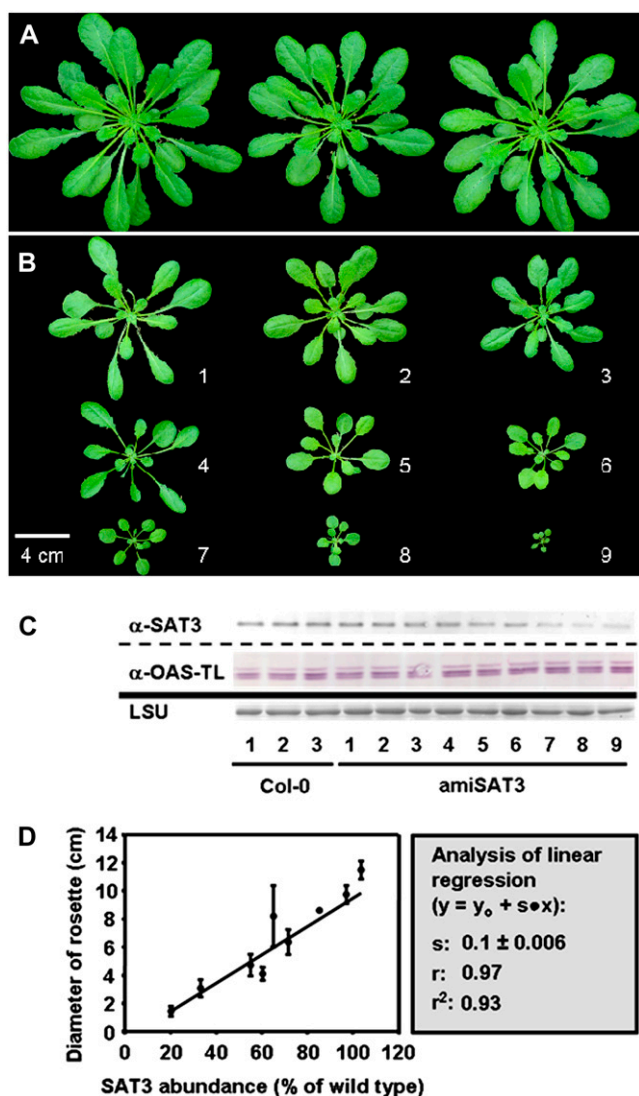
## RESULTS

### Expression of amiRNA against *Sat3* Causes a Severe Growth Phenotype

According to in vitro analyses, the majority of SAT activity resides in mitochondria in pea leaves and heterotrophic Arabidopsis cell cultures (Ruffet et al., 1995; Heeg et al., 2008). The expression of the gene that encodes the mitochondrial isoform SAT3 (*Sat3*, equivalent to *Serat2;2*; At3g13110) was down-regulated by RNA interference to determine (1) if mitochondria indeed provide most of the OAS in vivo and (2) if this step limits Cys biosynthesis in the plant cell. Highest specificity of the designed amiRNA construct to *Sat3* mRNA compared with the other four *Sat* messages was achieved using the WMD 2 - Web MicroRNA Designer ([www.weigelworld.org](http://www.weigelworld.org)). Furthermore, the sequence homology and hybridization energy of ami-SAT3 were carefully analyzed and revealed specific targeting of amiSAT3 to *Sat3* mRNA compared with RNAs of *Sat1*, *Sat2*, *Sat4*, and *Sat5* genes as well as the rest of the Arabidopsis genome (Supplemental Fig. S1). The amiSAT3 construct was expressed under the control of the cauliflower mosaic virus 35S promoter and used to transform Arabidopsis ecotype Columbia (Col-0; amiSAT3 lines).

Several independent transformants (T1) were identified using kanamycin as a selectable marker. Nine plants of quite variable size survived (Fig. 1, A and B), while numerous others failed to develop any further and died at the early rosette stage before production of flowers (data not shown). Determination of SAT3 protein levels in the surviving amiSAT3 lines by specific antibodies (Wirtz and Hell, 2003) revealed decreasing amounts with declining plant size. In comparison, protein contents of the major OAS-TL forms A, B, and C, which can be simultaneously detected by an OAS-TL-specific antibody (Fig. 1C; Heeg et al., 2008), were not affected at all. Indeed, SAT3 protein contents and rosette sizes of amiSAT3 lines showed a strong linear correlation ( $r = 0.97$ ; Fig. 1D).

Lines 4 and 5 showed intermediate growth retardation and were selected for further analyses. The T2 generation of amiSAT3 lines 4 and 5 confirmed the

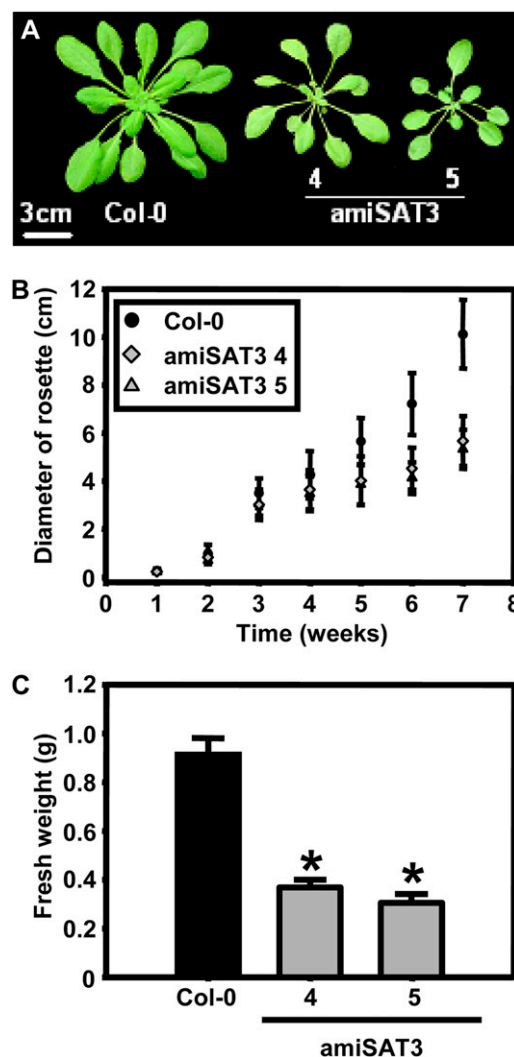


**Figure 1.** Impact of reduced mitochondrial SAT levels on the growth rate of Arabidopsis. A, Wild-type Arabidopsis plants were transformed with pBinAR-amiSAT3 and selected on kanamycin-containing medium. B, After 2 weeks, surviving transgenic plants were transferred to soil for an additional 6 weeks. C, The abundance of mitochondrial SAT3 was measured by immunological detection from soluble Arabidopsis protein extracts (15  $\mu$ g) using a SAT3-specific antibody (Wirtz and Hell, 2007). The large subunit of ribulose-1,5-bisphosphate carboxylase (LSU) served as a loading control after staining with Coomassie Brilliant Blue. D, At the same time of harvesting the sample for protein extraction, the rosette diameters of the transgenic plants were measured three times for each individual and plotted against the SAT abundance of the corresponding plant. The data were fitted using a linear equation. Error bars indicate sd. [See online article for color version of this figure.]

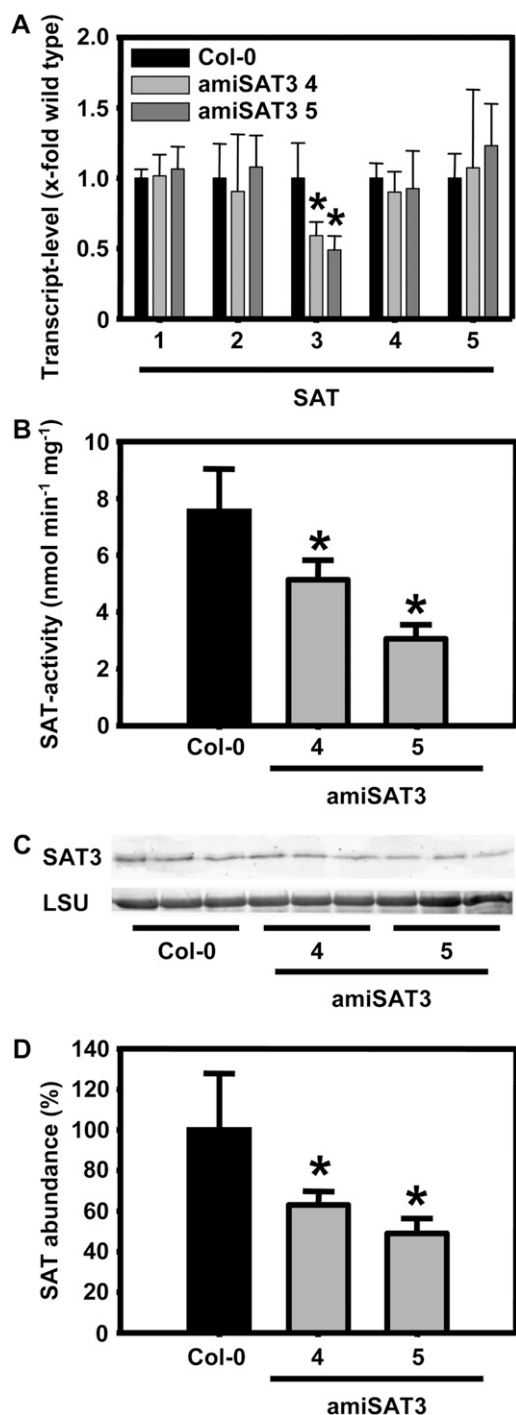
growth phenotypes of the T1 generation (Fig. 2). Growth rates of lines 4 and 5 differed significantly from those of the wild type from week 4 on after germination, and fresh weights of rosettes were  $40\% \pm 4\%$  and  $33\% \pm 4\%$ , respectively, of wild-type levels after 8 weeks.

### Specific Down-Regulation of Mitochondrial SAT Causes Growth Retardation

The amiSAT3 lines 4 and 5 were characterized in detail. First, the expression pattern of the *Sat* gene family was compared with the pattern in wild-type plants by quantitative real-time PCR. Lines 4 and 5 showed approximately 50% of wild-type *Sat3* mRNA levels but no changes in abundance of *Sat1*, *Sat2*, *Sat4*, and *Sat5* mRNA (Fig. 3A). This pattern was independently confirmed by microarray analyses of amiSAT3



**Figure 2.** Comparison of growth between wild-type and transgenic Arabidopsis plants with intermediate reduction of mitochondrial SAT. A, Phenotypes of a 7-week-old wild-type plant (Col-0) and two independent transgenic plant lines (amiSAT3 lines 4 and 5) with intermediate reduction of mitochondrial SAT. B, The growth of eight individuals from each genotype was monitored for 7 weeks by determining the diameter of the rosette three times for each individual ( $n = 24$ ). C, The fresh weights of 8-week-old wild-type plants and the two amiSAT3 lines ( $n = 4$ ) were determined. Error bars indicate sd, and asterisks mark significant differences using the unpaired *t* test. [See online article for color version of this figure.]



**Figure 3.** Molecular and biochemical characterization of transgenic Arabidopsis amiSAT3 plants. A, Transcript levels for all members of the SAT gene family in Arabidopsis were determined by quantitative real-time PCR after extraction of total mRNA from leaves of 8-week-old plants ( $n > 5$ ). Proteins were extracted from three individuals of wild-type plants (black bars) and amiSAT3 plants of lines 4 and 5 (gray bars) of the same age. B, The crude protein extracts (60–100  $\mu$ g) were tested three times for SAT activity ( $n = 9$ ). C and D, The abundance of mitochondrial SAT3 was analyzed ( $n = 9$ ) by immunological detection (C) and quantified by densitometry (D). The large subunit of ribulose-1,5-bisphosphate carboxylase (LSU) served as a loading control after

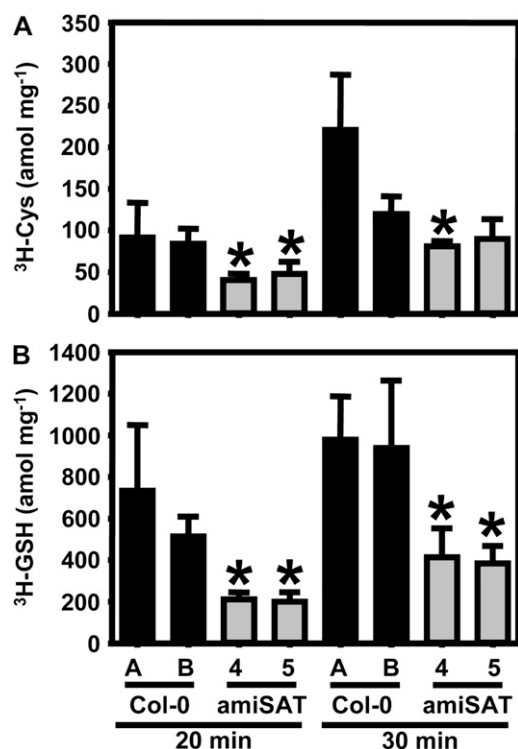
line 5 (see below). These findings confirmed the specificity of the amiRNA approach and demonstrated that no compensatory up-regulation of other *Sat* genes occurred in the transgenic lines. Second, SAT enzyme activities were determined from whole leaf protein extracts (Fig. 3B). The down-regulation of *Sat3* mRNA was mirrored by decreases of SAT enzyme activity in the two lines. Finally, the same extent of decrease in lines 4 and 5 was observed with respect to protein levels using a SAT3-specific antiserum for immunoblotting and subsequent densitometric quantification (Fig. 3, C and D). Thus, the phenotype of growth retardation is caused by gene-specific down-regulation of the gene encoding mitochondrial SAT.

#### Flux of Ser into Cys and Glutathione Is Reduced in amiSAT3 Lines

The causal relationship between reduced growth and specific down-regulation of the gene encoding mitochondrial SAT3 implies a reduced rate of OAS formation and ultimately of Cys synthesis, unless other SAT isoforms compensated for the decreased activity of the major SAT location in the cell in other ways than transcriptional increases. This assumption was tested by incubation of leaf discs in the light with radiolabeled [<sup>3</sup>H]Ser and quantification of label in Cys and glutathione (Fig. 4). Application of labeled Ser rather than sulfate was chosen to monitor flux via OAS but not of the assimilatory sulfate reduction pathway. [<sup>3</sup>H]Ser uptake of wild-type plants and amiSAT3 lines 4 and 5 was compared after 20 and 30 min, respectively. Radioactive labeling of Cys formed from [<sup>3</sup>H]OAS corresponded to half of the flux rate compared with the wild type at both time points due to down-regulation of mitochondrial SAT3 activity. An even stronger reduction of incorporation, down to 30% to 40%, was observed for glutathione. This difference suggests that, after uptake into the leaf cells, [<sup>3</sup>H]Ser was transported into the mitochondria to be used for the synthesis of [<sup>3</sup>H]OAS. This entered the cytosol to serve for the synthesis of Cys, which finally moved into the chloroplasts as substrate for the initial step of glutathione formation.

It is concluded that decreased mitochondrial SAT activity has a profound effect on flux into Cys and glutathione due to reduced overall OAS synthesis. As expected from the constant expression levels of the four unaffected SAT genes in Arabidopsis, no significant flux compensation could be observed, since the reductions in *Sat3* mRNA and SAT3 protein levels were in the same range as the reductions of incorporation rates into Cys. Furthermore, these matching quantitative molecular and metabolic changes due to amiSAT3 expression imply that the contributions of the potentially important plastid SAT1 and cytosolic

staining with Coomassie Brilliant Blue. Error bars indicate SD, and asterisks mark significant differences using the unpaired *t* test.



**Figure 4.** Incorporation of Ser into thiols in wild-type and transgenic plants with reduced mitochondrial SAT activity. Eight-week-old transgenic plants with reduced mitochondrial SAT activity (amiSAT3 lines 4 and 5; gray bars) were tested for incorporation of [ $^3\text{H}$ ]Ser into Cys and glutathione (GSH). Wild-type plants of the same age (Col-0 A) and of the same developmental stage (Col-0 B; 6 weeks old) as the analyzed amiSAT lines served as controls (black bars). All plants were grown on soil under the same conditions as plants used for analysis of metabolites, transcript levels, and protein abundance. Samples of three individuals of wild-type and transgenic plants were taken and analyzed for incorporation of the radioactive label from [ $^3\text{H}$ ]Ser into thiols after 20 and 30 min according to Heeg et al. (2008). Error bars indicate  $\text{SD}$ , and asterisks mark significant differences from both wild types using the unpaired  $t$  test.

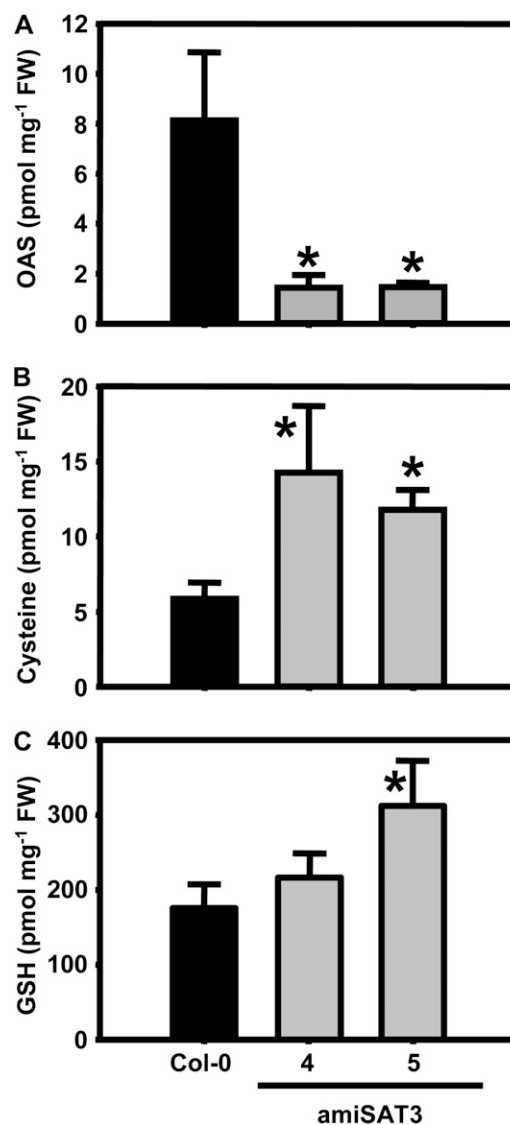
SAT5 to cellular OAS synthesis are rather small *in vivo* and by far exceeded by mitochondrial SAT3.

#### Steady-State Levels of Sulfur-Related Metabolites

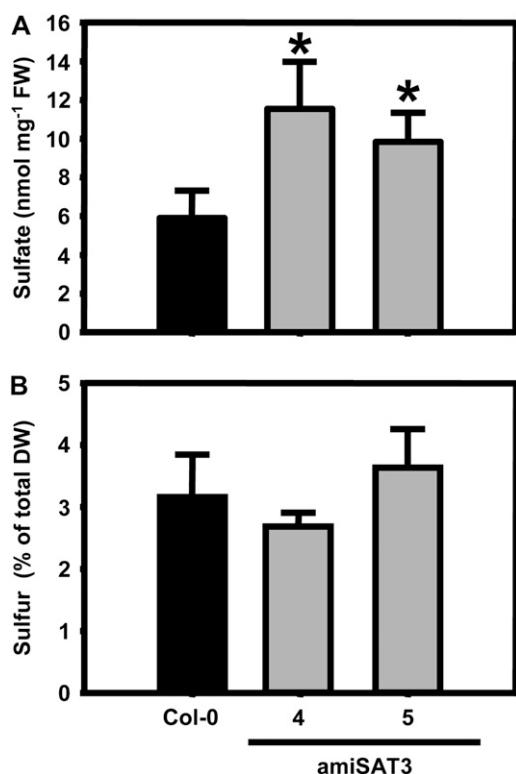
The overall cellular contents of major metabolites of primary sulfur metabolism were determined in leaves. OAS concentrations in lines 4 and 5 were decreased by  $82\% \pm 2\%$  and  $83\% \pm 6\%$  compared with the wild type and confirmed the down-regulation of the flux rate (Fig. 5A). Remarkably, steady-state levels of Cys were doubled and those of glutathione increased up to 1.5-fold compared with wild-type levels (Fig. 5, B and C). Therefore, despite strongly reduced rates of synthesis, the free concentration of both thiols was maintained, at least at the whole cell level.

Also, the content of sulfate was significantly increased in the amiSAT3 lines (Fig. 6A). It should be

noted that phosphate contents increased 1.5- and 2-fold, respectively, in amiSAT lines 4 and 5, but not the levels of nitrate (Supplemental Fig. S2). Despite elevated sulfate levels, the total sulfur contents of these lines were not affected (Fig. 6B), as were the total carbon and nitrogen contents (Supplemental Fig. S3). The significant increase of sulfate in combination with unchanged levels of total sulfur levels point toward a deregulation of the ratio between oxidized and reduced sulfur in amiSAT3 lines.



**Figure 5.** OAS and thiol contents in leaves of wild-type and transgenic Arabidopsis plants. Rosette leaves of three Arabidopsis wild-type plants (black bars) and three individuals of each independent transgenic plant line with reduced mitochondrial SAT activity (gray bars) were analyzed for OAS (A), Cys (B), and glutathione (GSH; C) after extraction of metabolites with 0.1 M hydrochloric acid. Error bars indicate  $\text{SD}$ , and asterisks mark significant differences using the unpaired  $t$  test. FW, Fresh weight.



**Figure 6.** Sulfate and total sulfur contents in leaves of wild-type and transgenic *Arabidopsis* plants. A, Rosette leaves of three *Arabidopsis* wild-type plants (black bars) and three individuals of each independent transgenic plant line with reduced mitochondrial SAT activity (gray bars) were analyzed for sulfate contents. FW, Fresh weight. B, The total sulfur content in dried rosette leaves of 8-week-old wild-type plants (black bars) and the amiSAT3 lines 4 and 5 (gray bars) was analyzed using an elemental analyzer ( $n = 4$ ). Error bars indicate SD, and asterisks mark significant differences using the unpaired  $t$  test. DW, Dry weight.

#### Impact of Reduced OAS Synthesis on Expression of Sulfur Metabolism-Related Genes

The transcriptome response of amiSAT3 lines was investigated with an array containing 912 genes designed for the detection of even small changes of mRNA abundance, as is often observed with genes of primary metabolism. Sixty genes with different expression intensity by robust constitutive patterns according to Czechowski et al. (2005) were included to optimize the evaluation of all genes of sulfur metabolism and key regulatory genes of other metabolic pathways, as well as stress response, redox homeostasis, hormone metabolism, and membrane transport. In addition, 152 candidate genes were included, which are reported to be regulated in response to sulfur availability (Hirai et al., 2003; Higashi et al., 2006; Maruyama-Nakashita et al., 2006). The whole list of investigated genes is shown in Supplemental Table S1.

Based on three biological repetitions of the wild type and amiSAT3 line 5 with four technical replicates, each including dye swaps for each set, 26 genes were found

to be significantly up- or down-regulated according to  $P$  values lower than 0.05 (Table I). Most of the significantly regulated genes (eight) belong to the category of sulfur metabolism, while a lower number of redox-related genes (six) and genes involved in hormone function (five), primary metabolism (four), and stress response (three) were also found to be significantly altered in abundance.

Microarray analyses of amiSAT3 line 5 confirmed the specificity and extent of down-regulation of *Sat3* expression and the unchanged mRNA levels of the other four *Sat* genes that had been observed by real-time PCR (Fig. 3). Most interestingly, two genes upstream of SAT in assimilatory sulfate reduction were down-regulated: *Aps4*, encoding a ubiquitously expressed plastidic isoform of ATP-sulfurylase, which is responsible for the activation of sulfate prior to reduction (Hatzfeld et al., 2000a); and *Apr2*, encoding adenosine 5'-phosphosulfate reductase (APR), the major plastid isoform for the first reduction step. None of the other genes of primary sulfur metabolism was significantly affected (Fig. 7A). The mRNA data of selected genes were complemented by immunoblotting of leaf proteins. Protein contents of sulfite reductase (SIR) and OAS-TL A, B, and C were unchanged, while APR protein was decreased by 40% in amiSAT3 line 5 (Fig. 7B). Thus, all three protein species precisely reflected the mRNA contents determined by microarray analysis, although it should be noted that the APR2 antiserum can probably cross-react with all three APR isoforms (Koprivova et al., 2000).

Only two genes out of the 152 candidate genes known for sulfur-dependent regulation (Hirai et al., 2003) were found to be significantly regulated in amiSAT3 line 5. Both genes, At1g33960 (avirulence-responsive protein) and At1g75300 (putative isoflavone reductase), are known to be transcriptionally regulated also by other environmental factors or hormone stimuli. Especially At1g75300 is regulated more strongly by methyl jasmonate (MJ) than by sulfur availability ([www.geneinvestigator.ethz.ch](http://www.geneinvestigator.ethz.ch)). In this respect, it is noteworthy that the gene with the highest up-regulation (2.6-fold) is *ST2a*, a hydroxyjasmonate sulfotransferase (At5g0710), which is supposed to inactivate MJ by sulfonation (Gidda et al., 2003). Responses to disease and pathogen attack have been known for a long time to be coordinated by MJ. All significantly regulated genes of the category stress response in this experiment (PR1, AIG1, and PRP1) are involved in responses to diseases or pathogens. In agreement with the idea of inactivation of MJ by induction of *ST2a*, all of them are down-regulated. Besides genes responding to MJ, two genes (GASA1 and GASA4) that were known as markers for GA stimulus are induced. Taken together, the expression data point toward a down-regulation of the sulfate assimilation pathway in amiSAT3 lines in order to coordinate plastid sulfate reduction with the reduced synthesis of OAS in mitochondria. Additionally, an inactivation of stress-responsive genes was observed,

**Table 1.** Genes regulated in response to reduced mitochondrial SAT activity

The list comprises all genes that were regulated significantly ( $P < 0.05$ ) in response to the down-regulation of mitochondrial SAT. The list of all tested genes is deposited as Supplemental Table S1. The annotation of genes and grouping by functional category is according to The Arabidopsis Information Resource ([www.arabidopsis.org](http://www.arabidopsis.org)).

Gene ID	Annotation	Change	<i>P</i>	Category
At5g07000	ST2b, sulfotransferase	+1.45	0.022	Sulfur metabolism
At5g27380	GSH2, glutathione synthetase	+1.28	0.017	Sulfur metabolism
At5g43780	APS4, ATP-sulfurylase 4	-1.20	0.041	Sulfur metabolism
At5g43850	ARD4, acireductone dioxygenase	-1.23	0.048	Sulfur metabolism
At5g61420	Myb28, transcription factor	-1.25	0.028	Sulfur metabolism
At2g47730	GSTF8, $\phi$ -class glutathione transferase	-1.45	0.028	Sulfur metabolism
At1G62180	APR2, APS reductase 2	-1.78	0.028	Sulfur metabolism
At3G13110	SAT3, Ser acetyltransferase 3	-2.01	0.008	Sulfur metabolism
At2g32880	TRAF homology domain-containing protein	+1.33	0.006	Redox related
At3g24170	GR1, cytosolic GSH reductase	-1.27	0.047	Redox related
At1g75300	Isoflavone reductase	-1.34	0.027	Redox related
At5g21100	L-Ascorbate oxidase	-1.55	0.004	Redox related
At1g10960	FD1, ferredoxin	-2.03	0.037	Redox related
At1g64500	Glutaredoxin family protein	-2.08	0.005	Redox related
At5g07010	ST2a, hydroxyjasmonate sulfotransferase	+2.66	0.001	Hormone function
At1g75750	GASA1, GA regulated	+1.87	0.003	Hormone function
At5g05690	CPD, cytochrome P450 monooxygenase	+1.34	0.022	Hormone function
At5g15230	GASA4, GA regulated	+1.27	0.035	Hormone function
At4g39800	MIPS, myoinositol-1-P synthase	-2.32	0.006	Hormone function
At2g18700	TPS11, trehalose-6-P synthase	+1.67	0.006	Primary metabolism
At4g39330	CAD9, cinnamyl alcohol dehydrogenase	-1.30	0.027	Primary metabolism
At3g19450	CAD4, cinnamyl alcohol dehydrogenase	-1.39	0.022	Primary metabolism
At1g17745	PGDH, 3-phosphoglycerate dehydrogenase	-1.57	0.009	Primary metabolism
At3g44480	RPP1, TIR-NB-LRR R protein	-1.43	0.033	Stress response
At1g33960	ALG1, avirulence-responsive protein	-1.51	0.041	Stress response
At2g14610	PR1, pathogen-related protein 1	-1.74	0.048	Stress response

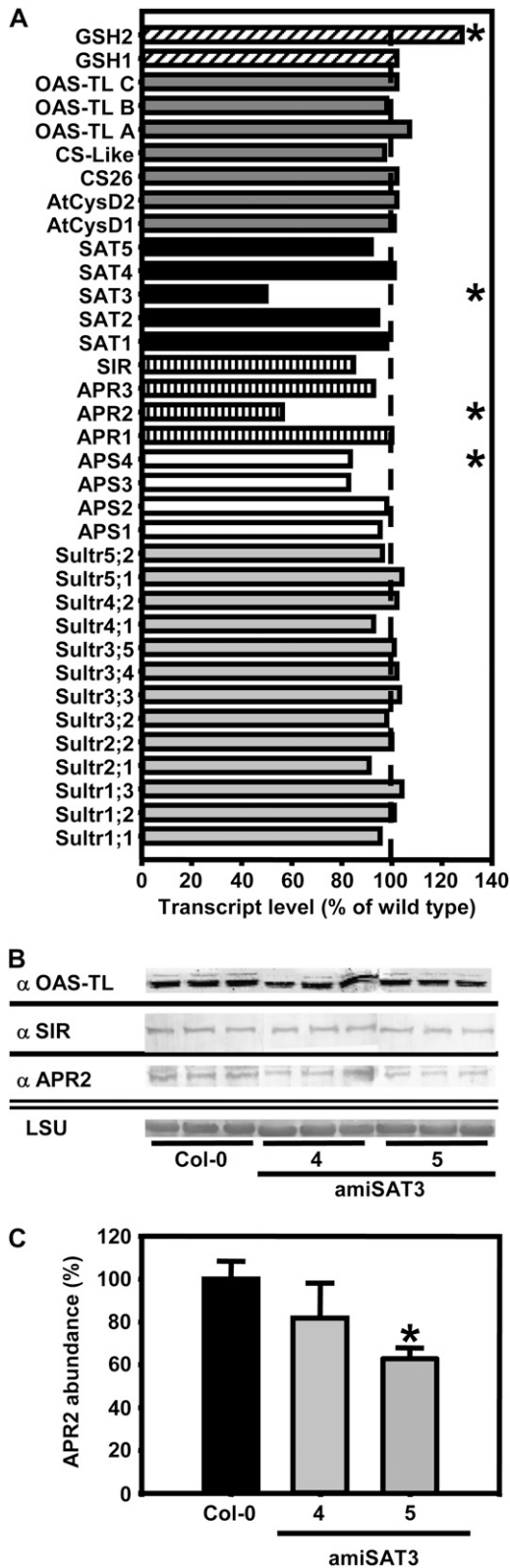
which could be a result of perturbed hormone metabolism.

## DISCUSSION

The subcellular organization of Cys synthesis in plants differs strongly from that in other eukaryotes. Why plant SAT and OAS-TL form Cys in the cytosol, plastids, and mitochondria, the reasons for their strongly different activity ratios, and whether this distribution reflects redundancy or specific functions remained enigmatic (Fig. 8, A and B). With respect to the second step of Cys synthesis, recent analyses of the OAS-TL protein family members in Arabidopsis using T-DNA insertion lines indicated redundancy as well as compartment-specific functions (Heeg et al., 2008; Lopez-Martin et al., 2008; Watanabe et al., 2008). In nonstressed plants, cytosolic OAS-TL turned out to be the most important isoform in terms of enzymatic activity (Heeg et al., 2008; Watanabe et al., 2008).

However, a mitochondrial OAS-TL C mutant showed a mild growth phenotype despite a measurable OAS-TL C contribution of less than 5% of total activity. On the other hand, this little OAS-TL C activity alone was sufficient for comfortable growth under nonstress conditions in a double mutant without cytosolic OAS-TL A and plastid OAS-TL B (Heeg et al., 2008). At the same time, these mutants allowed us to conclude that Cys must be exchangeable between cytosol and organelles, at least in Arabidopsis, making an earlier hypothesis of a lack of such exchange as reason for the three sites of Cys very unlikely (Lunn et al., 1990). Feeding experiments with [<sup>35</sup>S]sulfate to *oastl* mutants furthermore confirmed the importance of the second step of Cys synthesis in the cytosol. The explanation for these findings was that the rate of formation of the reaction intermediate OAS in mitochondria dominates the overall rate of Cys synthesis in the cell (Fig. 8C; Heeg et al., 2008).

If this assumption was correct, one has to conclude that plastids and mitochondria provide most of the



**Figure 7.** Protein abundances and transcript levels of sulfur metabolism-related genes in leaves of transgenic Arabidopsis plants. A, The transcript levels of sulfur metabolism-related genes in leaves of amiSAT3 line 5 and wild-type Arabidopsis plants were compared using a targeted microarray

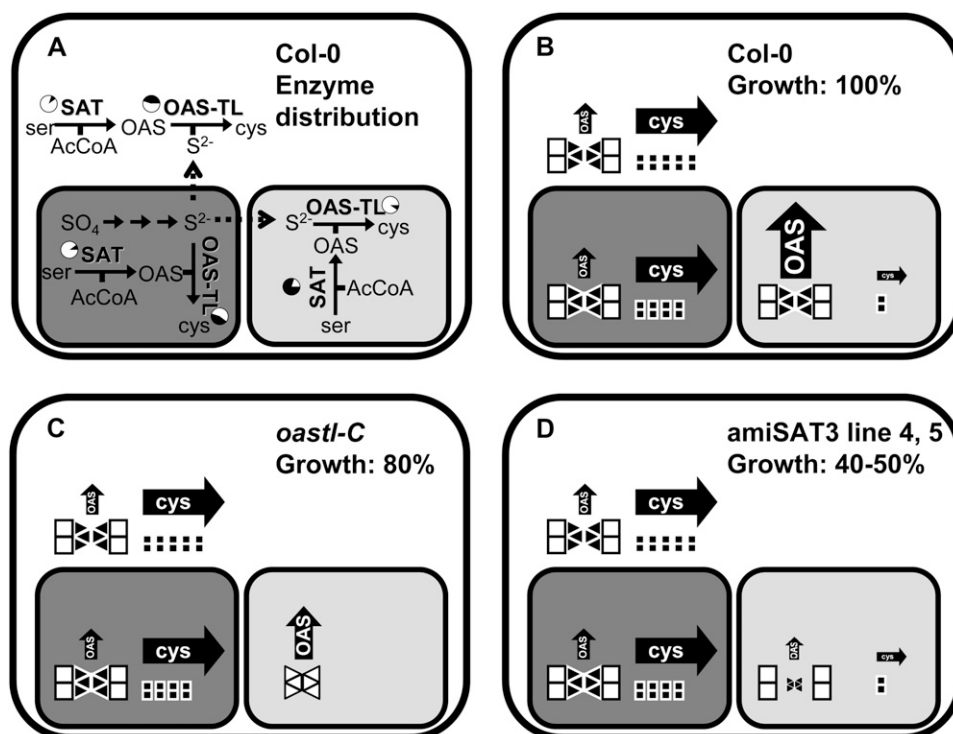
substrates sulfide and OAS, respectively, while the majority of Cys synthesis actually takes place in the cytosol. The experimental down-regulation of mitochondrial SAT activity in vivo, therefore, should result in reduced formation of OAS and Cys. Indeed, targeting of mitochondrial SAT3 by amiRNA resulted in reduced OAS contents, reduced rates of Cys synthesis, and reduced growth rates. Importantly, the degree of down-regulation of *Sat3* mRNA, SAT3 protein levels, and overall SAT enzyme activity quantitatively matched the lowered OAS levels, Cys synthesis rates, and growth reduction. The amiRNA approach was shown by two independent methods to be specific with respect to the expression levels of the other four *Sat* genes. This demonstrates that mitochondrial SAT3 activity is indeed responsible for the bulk of OAS production under these conditions, and since SATs in the other compartments showed no compensation for the reduction, this isoform limited Cys production, as shown by retarded growth (Fig. 8D).

This strong dependence of total cellular Cys production on the pacemaking role of mitochondrial synthesis of OAS is unexpected, because until now cytosol and plastids were believed to be the major production sites of both OAS and Cys (Urano et al., 2000; Droux, 2003). In this respect, numerous transgenic approaches aimed to enhance Cys synthesis in these two compartments by overexpression of SAT or OAS-TL indeed achieved moderate 1.5- to 3-fold increases of total Cys and glutathione contents (Blaszczyk et al., 1999; Harms et al., 2000; Noji and Saito, 2002; Wirtz and Hell, 2003, 2007; Sirko et al., 2004). The mitochondria have not been a target for overexpression of SAT or OAS-TL until now.

The cytosol was assumed to be the major site for regulation of OAS synthesis as a consequence of the strong feedback inhibition of cytosolic SAT isoforms by Cys (50% inhibition of initial activity at 2–10  $\mu$ M Cys; Noji et al., 1998; Saito, 2000; Urano et al., 2000). Organelle-localized SAT isoforms showed much less feedback inhibition by Cys in several higher plants species (Saito et al., 1995; Noji et al., 1998; Saito, 1998). Indeed, overexpression of less feedback-inhibited SAT

approach. Total mRNA was extracted from three individuals of each plant line, labeled independently two times with Cy3 and Cy5, and cohybridized with the microarray two times ( $n = 11$ ). The transcript levels of sulfate transporters (gray bars), ATP-sulfurylases (white bars), sulfate-reducing genes (dashed bars), SATs (black bars), OAS-TLs (dark gray bars), and genes participating in glutathione (GSH) synthesis (inclined dashed bars) in amiSAT3 line 5 are shown as percentages of wild-type levels. B, Proteins were extracted from three individuals of wild-type plants and amiSAT3 lines 4 and 5 of the same age. The abundances of OAS-TL A, B, and C, SIR, and APR2 were determined by immunoblotting using specific antibodies from crude extracts (15  $\mu$ g). The large subunit of ribulose-1,5-bisphosphate carboxylase (LSU) served as a loading control after staining with Coomassie Brilliant Blue. C, The reduction of APR2 protein in amiSAT3 lines 4 and 5 (gray bars) in comparison with wild-type plants (black bars) was quantified densitometrically. Error bars indicate SD, and asterisks mark significant differences using the unpaired *t* test.





**Figure 8.** Organization of Cys biosynthesis pathways in plant cells and transgenic *Arabidopsis* plants with reduced mitochondrial SAT activity. A, Schematic overview of enzymatic activities and metabolites involved in synthesis of Cys in the cytosol (white panel), the mitochondrion (light gray panel), and the chloroplast (dark gray panel). Enzymes are indicated in boldface letters. Black fields of pie charts show the ratio of enzymatic activities present inside the respective compartment as percentages of total extractable activity calculated from subcellular fractionation experiments with pea and spinach leaves (Droux, 2004). Full arrows represent enzymatic reactions, while dashed arrows indicate transport of metabolites across membranes. B, Relative fluxes of OAS and Cys (indicated by the sizes of filled arrows) in different subcellular compartments as deduced from the enzymatic activities of SAT and OAS-TL. Most likely, hexameric SAT (triangles) is associated with two OAS-TL dimers (cubes) to form the CSC. Black filling of triangles and cubes indicates the active form of enzyme, while white filling indicates inactivated or less active enzyme. The impacts on growth rate and subcellular fluxes of metabolites as a consequence of disturbed CSC formation in the mitochondria of OAS-TL C knockout plants (*oastl-C*) and specific down-regulation of mitochondrial SAT activity in *amiSAT3* plants are shown in C and D, respectively. The sizes of triangles in C and D represent the amount of SAT with respect to the wild-type level (B).

isoforms compared with stronger feedback-inhibited SATs in the cytosol of transgenic plants resulted in a higher increase of Cys steady-state levels in *Arabidopsis* and tobacco (*Nicotiana tabacum*; Blaszczyk et al., 1999; Wirtz and Hell, 2007). On the other hand, the estimated cytosolic Cys steady-state levels in wild-type plants led to the question: is cytosolic feedback-inhibited SAT active at in vivo concentrations of Cys (Saito, 1998; Wirtz and Hell, 2006)? The SAT of the enterobacterium *Escherichia coli* has similar inhibition constant values for Cys ( $1 \mu\text{M}$ ) compared with strongly feedback-inhibited plant SATs, but it is enzymatically almost completely inactive during normal growth (Kredich and Becker, 1971). In the case of *E. coli*, such a strict down-regulation makes sense due to the lifestyle of *E. coli*, which supports the organism with exogenous Cys. It remains unclear why cytosolic SAT, which contributes less than 10% of the total SAT activity (Ruffet et al., 1995), would be potently inhibited by Cys. Possible explanations for the presence of this

SAT could be the production of OAS in this compartment during a stress situation when cytosolic Cys pools are depleted, or perhaps its role is not the synthesis of OAS but instead the formation of the CSC complex. A regulatory model proposes the reversible dissociation of the CSC in response to OAS and sulfide as a sensor of the actual sulfur supply in a subcellular compartment and allows the regulation of SAT activity in response to the actual demand for OAS for the production of Cys (Wirtz and Hell, 2006). In this model, the function of the feedback-inhibited cytosolic SAT would be signal recognition of OAS and sulfide supply of the cytosol rather than production of OAS. The latter scenario would provide an elegant explanation for the presence of SAT in all three compartments, although total Cys synthesis depends almost entirely on the synthesis of OAS in mitochondria, according to the results presented here.

It is remarkable that radiolabel from [ $^3\text{H}$ ]Ser feeding appears in glutathione. This implies an uptake of Ser

into mitochondria, formation of OAS, transport to the cytosol for Cys synthesis, and import into plastids to serve as substrate of  $\gamma$ -glutamylcysteine, the precursor of glutathione that is exclusively formed in plastids in Arabidopsis (Wachter et al., 2005). The possibility of growth retardation in amiSAT3 lines due to deficiency of Cys in the mitochondria themselves can be excluded, since Cys can be imported into mitochondria from the cytosol (Heeg et al., 2008). Consequently, the retarded growth phenotype must be a consequence of reduced total Cys synthesis rates in the cell, although cytosolic SAT5 and plastid SAT1 are still present.

OAS contents were decreased in Arabidopsis amiSAT3 lines 4 and 5, but surprisingly, steady-state levels of Cys and glutathione were increased despite lowered flux into these thiols. Maintenance of Cys and glutathione contents was also observed in the Arabidopsis *oastlC* T-DNA mutant line, which lacked mitochondrial OAS-TL C and showed reduced growth (Heeg et al., 2008). In this respect, the different functions of OAS and thiols for cell metabolism have to be considered: OAS functions as reaction intermediate and potential signal for gene expression during sulfate starvation (Kim et al., 1999; Hirai et al., 2003), Cys is essential for protein biosynthesis and as a donor of reduced sulfur in numerous reactions (Hell and Bergmann, 1990; Pilon-Smiths et al., 2002; Picciocchi et al., 2003; Wirtz and Droux, 2005), and glutathione is an integral component of redox homeostasis (Meyer and Hell, 2005; Meyer et al., 2007). It is conceivable that the maintenance of thiol levels for these reasons is a prerequisite for the proper function of cell metabolism. Nonetheless, this would not fully explain the elevation of Cys steady-state levels. More likely, in amiSAT3 lines, Cys steady-state levels are up-regulated as a signal to serve as a signal to adjust cell metabolism to reduced fluxes of OAS.

The microarray analysis of leaves of the Arabidopsis amiSAT3 line 5 in comparison with wild-type plants revealed that none of the OAS-TL genes and none of the genes of sulfate uptake and primary sulfur metabolism were changed in expression, with the exception of one member of the *Aps* and *Apr* gene family each. Down-regulation of APR2 protein was confirmed by immunoblotting. Reduced activation and reduction of sulfate would explain the increased contents of sulfate in amiSAT3 lines. Especially the strong down-regulation of APR2 could be of particular importance, since lower APR2 activity in leaves was shown to be responsible for the higher sulfate content of the Arabidopsis ecotype Shahdara in comparison with ecotype Bay-0 (Loudet et al., 2007). High levels of Cys as well as glutathione were shown to down-regulate genes for sulfate activation and reduction in roots of several higher plants (Koprivova et al., 2000; Vauclare et al., 2002). It seems conceivable, therefore, that the high steady-state levels of Cys and glutathione in amiSAT3 lines 4 and 5 are needed to efficiently down-regulate sulfate reduction in order to avoid the accumulation of toxic sulfide, which may not be entirely scavenged by

Cys synthesis in the transgenic lines. In agreement with this hypothesis, sulfate accumulates 2-fold in amiSAT3 lines 4 and 5 in comparison with the wild type.

Further putative signals for the down-regulation of APR2 are OAS and MJ. It has been repeatedly observed that OAS, possibly due to the dissociation of the CSC, acts as a signal for the entire sulfur assimilation pathway. Feeding of OAS regulates more than 650 and 850 genes in leaves and roots of Arabidopsis, respectively (Hirai et al., 2003). At least in roots of Arabidopsis, all APR isoforms are up-regulated in response to exogenous application of OAS (Koprivova et al., 2000). Induction of APR by application of OAS was also shown in *Lemna minor*, indicating that regulation of APR by OAS is a general mechanism in higher plants (Kopriva et al., 2002). Thus, the 4-fold lowered OAS and doubled thiol steady-state levels in amiSAT3 lines may, independently or in combination, act as signals for the repression of *Apr2* in amiSAT3 lines. MJ has been shown to modulate expression levels of sulfate assimilation-related genes (Jost et al., 2005; Sasaki-Sekimoto et al., 2005). According to microarray analysis of the amiSAT3 line 5 *ST2a*, a hydroxyjasmonate sulfotransferase, which is supposed to inactivate jasmonic acid signals (Gidda et al., 2003), is one of the strongest responding genes. In agreement with such a possible mechanism, several genes of the stress response to pathogens and diseases were down-regulated and none was up-regulated on the microarray in this study. Therefore, a diminished octadecanoic acid signal may have contributed to the reduced *Aps4* and *Apr2* expression.

## CONCLUSION

Specific down-regulation of mitochondrial SAT3 activity in Arabidopsis by amiRNA expression demonstrated that mitochondria, but not chloroplasts or the cytosol, are the dominant source of OAS in vivo. Despite strongly reduced flux into Cys in amiSAT3 plants, the steady-state levels of Cys and glutathione were elevated and presumably contributed to the repression of assimilatory sulfate reduction and accumulation of free sulfate. This, together with previous evidence, strongly suggests that OAS from mitochondria and sulfide from chloroplasts serve as substrates for the bulk of Cys synthesis in the cytosol.

## MATERIALS AND METHODS

### General Cloning

Standard molecular biology technologies like growth of bacteria, plasmid isolation, and PCR were performed as described by Sambrook et al. (1989) according to GLP standards. The amiRNA-SAT3-specific sequences were selected with the WMD 2 - Web MicroRNA Designer ([www.weigelworld.org](http://www.weigelworld.org)). The amiRNA of SAT3 was constructed using an overlapping PCR approach with primers P1 (5'-gaTAAAATACAAGTCCAGCCCTctctctttgtattcc-3'), P2 (5'-gaGGGCTGGGACTTGTATTTTAtcaagagaatcaatga-3'), P3 (5'-gaGAGCTGGGACTTCTATTTTtcacaggtcgtgatg-3'), P4 (5'-gaAAAAATA-GAAGTCCCAGCTCctacatatattct-3'), pRS300-A (5'-CTGCAAGCGCAT-

TAAGTTCGGTAAC-3'), and pRS300-B (5'-GCGGATAACAATTCACACAGGAAACAG-3') according to Schwab et al. (2006), which resulted in the amplification of the amiRNA loop structure, the SAT-specific sequence, and terminal restriction sites for *KpnI* and *BamHI* at the 5' and 3' ends, respectively. The resulting PCR product was digested with *KpnI* and *BamHI* and cloned in the plant transformation vector pBINAR under the control of the cauliflower mosaic virus 35S promoter, resulting in pBINAR-amiSAT3 (Supplemental Fig. S1). The following cDNA was employed in this study: *AtSAT3* (X80938; Bogdanova et al., 1995).

## Plant Growth and Transformation

Transformation of *Agrobacterium tumefaciens* C58 with binary vectors and subsequent transformation and selection of *Arabidopsis* (*Arabidopsis thaliana* Col-0) were carried out as described by Clough and Bent (1998). Wild-type plants and plants expressing amiRNA-SAT3 (F1 or F2 generation) were grown in climate chambers in growth medium containing one-half soil and one-half substrate 2 (Klasmann-Deilmann) under controlled conditions: 8.5 h of light, 100  $\mu$ E light photon flux density, 24°C and 18°C at day and night, respectively, and 50% humidity. Rosette leaves of each 8-week-old plant were pooled and used to analyze metabolites as well as the expression of sulfur-related genes at the transcript and protein levels.

## Determination of Metabolites

Hydrophilic metabolites were extracted from leaves of *Arabidopsis* plants according to Wirtz and Hell (2003). Thiols, OAS, anions, and total contents of carbon, nitrogen, and sulfur were quantified as described by Heeg et al. (2008) in order to ensure the comparability of data sets.

## Determination of Enzyme Activities

Total soluble proteins were isolated with 0.5 mL of 50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 10% glycerol, 30 mM dithiothreitol, and 0.5% phenylmethylsulfonyl fluoride from 0.2 g of leaf material that was ground to a fine powder in liquid nitrogen. Cell debris was removed by centrifugation at 16,000g and 4°C for 10 min. Proteins were quantified as described by Bradford (1976) using bovine serum albumin as a standard. The enzymatic activities of SAT and OAS-TL were determined according to Nakamura et al. (1987): SAT activity was assayed by coupling to the OAS-TL reaction. All SAT activity determinations were supplemented with 2 units of purified recombinant OAS-TL (Wirtz et al., 2004) to ensure high excess of OAS-TL activity during coupling of both reactions.

## Immunological Detection of Proteins

Total proteins from leaves were separated according to Laemmli (1970) by discontinuous SDS-PAGE in Mini-Protean II cells (Bio-Rad). Transfer of proteins, washing, and development of signals were performed as described by Heeg et al. (2008) using the alkaline phosphatase-anti-rabbit IgG conjugate (Promega; 1:10,000) detection of SAT and OAS-TL proteins. APR and SIR were detected with primary antibodies (1:2,000; incubation at 4°C overnight) after blocking of the nitrocellulose membrane with 3% milk powder. The primary antibodies were detected with a 1:5,000 dilution of the above-mentioned secondary antibody. The resulting signals were quantified by densitometry using the INTAS gel documentation system in combination with Gel-Pro Express 4.0 software (INTAS).

## Determination of Incorporation Rates Using <sup>3</sup>H-Labeled Ser

Twelve hours prior to the labeling experiment, soil-grown plants were enclosed in transparent plastic bags to ensure opening of the stomata. Leaf pieces (30 mg) were cut out from the interveinal fields of leaves and floated on the labeling solution (half-strength Hoagland medium containing 2.5  $\mu$ M [<sup>3</sup>H] Ser [185–925 GBq mmol<sup>-1</sup>; Hartmann Analytic]) on a horizontal shaker with 60 rpm in the light (17  $\mu$ E). Samples were taken after 20 and 30 min, washed twice with half-strength Hoagland medium [2.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.5 mM KNO<sub>3</sub>, 0.5 mM MgSO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 40 mM Fe-EDTA, 25 mM H<sub>3</sub>BO<sub>3</sub>, 2.25 mM

MnCl<sub>2</sub>, 1.9 mM ZnSO<sub>4</sub>, 0.15 mM, CuSO<sub>4</sub>, and 0.05 mM (NH<sub>4</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub>, pH 5.9], and frozen in liquid nitrogen. A negative control for contamination of the leaf surface with [<sup>3</sup>H]Ser was taken by dipping leaf pieces into the labeling solution for 1 s, prior to washing and harvesting like the samples. The samples were powdered using the Bio101 ThermoSavant Fast Prep system (Qiogene) according to the manufacturer's instructions. The metabolites were extracted and separated prior to quantification of the incorporated <sup>3</sup>H label as described by Heeg et al. (2008).

## Determination of Transcript Levels Using a Custom-Made Microarray

### Design and Production of the Microarray

50mer oligonucleotides of 912 selected genes were synthesized by Ocimum Biosolutions with an N-terminal amino linker and checked for cross-hybridization with the Smith-Waterman alignment. Oligonucleotides were diluted to a concentration of 0.01 to 1 mM in 5× SSC (0.75 M sodium chloride and 0.075 M sodium citrate) containing 2.5 M betaine and spotted on epoxy-coated NexterionE slides (Peglab) with a MicroGrid robot (Biorobotics) using SMP3 pins (TeleChem International). A microarray contains all probes in four replicates, while the assembly of replicates on the microarray was randomized to avoid positional effects.

### Sample Preparation, Hybridization, and Evaluation

Fluorescently labeled cDNA samples were prepared from 10  $\mu$ g of total RNA that had been isolated from *Arabidopsis* leaf tissue with the RNeasy Plant Kit (Qiagen) according to the manufacturer's protocol. Routinely, Cy3/Cy5-dCTP was directly incorporated during first-strand synthesis according to The Institute for Genomic Research direct labeling protocol (www.tigr.org). The labeled cDNA probe was purified using the QIAquick Purification Kit (Qiagen) according to the manufacturer's instructions, and the incorporation rate of label was determined by photometric measurement. A Cy3-labeled wild-type cDNA sample and a Cy5-labeled amiSAT3 cDNA sample were mixed after concentration of each sample in a vacuum concentrator to a final volume of 20  $\mu$ L. The resulting sample was immediately denatured by the addition of 20  $\mu$ L of prewarmed hybridization buffer h (5× SSC, 0.1% [v/v] SDS, 0.06% [w/v] DNA from fish sperm, MB grade, and 40% [v/v] formamide) followed by incubation at 100°C for 5 min. The microarray was prehybridized with buffer p (5× SSC, 0.2% SDS, and 0.5% bovine serum albumin) for 45 min and washed three times with 250 mL of double-distilled water, before the denatured sample was applied and incubated for 20 h at 50°C in a Slide Booster (Advalytix). The hybridized microarray was washed at 30°C by subsequently applying buffer w1 (2× SSC and 0.5% SDS), w2 (0.2% SSC and 0.5% SDS), and w3 (0.1% SSC) for 10 min each. Signals were detected using the ScanArray5000 confocal laser scanner (Packard BioChip Technologies) and quantified with the GenePix Pro 4.1 analysis software (Axon Instruments). Data quality assessment, normalization, and correspondence cluster analysis were performed with the analysis and data warehouse software M-CHIP (DKFZ) according to Fellenberg et al. (2001; www.mchips.org). Signal intensities of repeated hybridizations were normalized by the majority of spots, and the minimal signal intensity was at least above twice the SD of the background signal.

## Determination of Transcript Levels by Real-Time PCR

Total RNA from leaf tissue was extracted with the RNeasy Plant Kit (Qiagen) according to the manufacturer's protocol. Total RNA was transcribed into cDNA and analyzed by quantitative real-time PCR as described by Talke et al. (2006). The following primers were used for specific amplification of actin7 and all members of the SAT gene family from *Arabidopsis*: SAT1-f (5'-CACATGCCGAACCGTAATAC-3'), SAT1-r (5'-GGTGAATCTCCGGTTACAGAGA-3'), SAT2-f (5'-ACGCTAAGGGAAGCTATAAGTCAGA-3'), SAT2-r (5'-TCTTCTCTATAGCATCCAAATAGCA-3'), SAT3-f (5'-AATGGAACCCAGACAAAACC-3'), SAT3-r (5'-GCCCAAACATCATCGACTCA-3'), SAT4-f (5'-CTCTTCCAATGATTGTCTCCCG-3'), SAT4-r (5'-CCTCTCGAAAGGAACTCGTCA-3'), SAT5-f (5'-TGGACACAGATCAAGGCGG-3'), SAT5-r (5'-ATGAGAAAGAAATCGTCAATATAGATAGC-3'), actin7-f (5'-CAACCCGTATTGTGCTCGATTC-3'), and actin7-r (5'-GAGTGAGTCTGTGAGATCCCG-3').

## Statistical Analysis

Regression analyses of data sets were performed with SigmaPlot 8.0 that uses the Marquardt-Levenberg algorithm for determination of independent variables. Comparison of means from different sets of data was analyzed for statistical significance with the unpaired *t* test. Constant variance and normal distribution of data were carefully checked with SigmaStat 3.0 prior to statistical analysis.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Specificity of the amiRNA-SAT3 approach.

**Supplemental Figure S2.** Nitrate and phosphate contents in leaves of wild-type and transgenic *Arabidopsis* plants.

**Supplemental Figure S3.** Carbon and nitrogen contents in leaves of wild-type and transgenic *Arabidopsis* plants.

**Supplemental Table S1.** List of genes that were analyzed by the targeted microarray approach.

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