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Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolic engineering in plants

Review Article

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Summary. Serine acetyltransferase (SATase) and cysteine synthase (*O*-acetylserine (thiol)-lyase) (CSase) are committed in the final step of cysteine biosynthesis. Six cDNA clones encoding SATase have been isolated from several plants, *e.g.* watermelon, spinach, Chinese chive and *Arabidopsis thaliana*. Feedback-inhibition pattern and subcellular localization of plant SATases were evaluated. Two types of SATase that differ in their sensitivity to the feedback inhibition by L-cysteine were found in plants. In *Arabidopsis*, cytosolic SATase was inhibited by L-cysteine at a physiological concentration in an allosteric manner, but the plastidic and mitochondrial forms were not subjected to this feedback regulation. These results suggest that the regulation of cysteine biosynthesis through feedback inhibition may differ depending on the subcellular compartment. The allosteric domain responsible for L-cysteine inhibition was characterized, using several SATase mutants. The single change of amino acid residue, glycine-277 to cysteine, in the C-terminal region of watermelon SATase caused a significant decrease of the feedback-inhibition sensitivity of watermelon SATase. We made the transgenic *Arabidopsis* overexpressing point-mutated watermelon SATase gene whose product was not inhibited by L-cysteine. The contents of OAS, cysteine, and glutathione in transgenic *Arabidopsis* were significantly increased as compared to the wild-type *Arabidopsis*. Transgenic tobacco (*Nicotiana tabacum*) (F_1) plants with enhanced CSase activities both in the cytosol and in the chloroplasts were generated by cross-fertilization of two transgenic tobacco expressing either cytosolic CSase or chloroplastic CSase. Upon fumigation with $0.1\,\mu L\,L^{-1}$ sulfur dioxide, both the cysteine and glutathione contents in leaves of F_1 plants were increased significantly, but not in leaves of non-transformed control plants. These results indicated that both SATase and CSase play important roles in cysteine biosynthesis and its regulation in plants.

Keywords: Amino acids – Cysteine biosynthesis – Serine acetyltransferase – Cysteine synthase

Abbreviations: SATase = serine acetyltransferase, CSase = cysteine synthase, GSH = glutathione, Rubisco = ribulose-1,5-bisphosphate carboxylase

Introduction

Cysteine biosynthesis in plants plays a key role in the sulfur cycle in nature, because the inorganic sulfur in the environment (*e.g.*, sulfate ion in the soil and sulfur dioxide in the air) is fixed into cysteine mainly by this biosynthetic pathway in plants (Saito, 1999, 2000). Cysteine is incorporated into proteins and glutathione (GSH) or serves as the sulfur donor for the biosynthesis of methionine and sulfur-containing secondary products in plants. The cysteine biosynthetic pathway involves several enzymatic reactions (Brunold and Rennenberg, 1997; Leustek and Saito, 1999). The final step of cysteine biosynthesis is the reaction of incorporating sulfide moiety into β-position of alanine. The amino acid moiety is derived from serine through *O*-acetyl-L-serine (OAS). Two enzymes, serine acetyltransferase (SATase) (EC 2.3.1.30) and cysteine synthase (*O*-acetylserine (thiol)-lyase) (CSase) (EC 4.2.99.8) are committed in this step (Fig. 1). This final step of cysteine biosynthesis seems to exist necessarily in three major compartments of plant cells, cytosol, chloroplasts, and mitochondria, because the presence of SATase (Smith, 1972; Ascano and Nicholas, 1977; Brunold and Suter, 1982; Ruffet et al., 1995) and CSase (Brunold and Suter, 1989; Lunn et al., 1990; Droux et al., 1992; Rolland et al., 1992; Yamaguchi and Masada, 1995; Kuske et al., 1996) has been demonstrated in these three compartments from several plants.

Feedback inhibition pattern and subcellular localization of SATases

SATase catalyzes the formation of OAS from L-serine and acetyl-CoA.

L-Serine + Acetyl-CoA → *O*-acetyl-L-serine (OAS) + CoA-SH

SATase is responsible for the entry step from serine metabolism to cysteine biosynthesis; therefore, it would be logical to postulate the existence of regu-

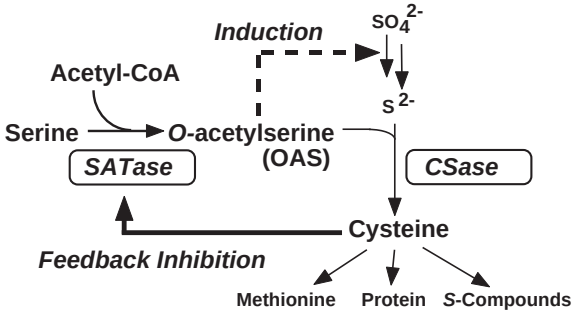

Fig. 1. Assimilation pathway of sulfur and cysteine biosynthesis

lation at this step. SATase has been characterized with the purified or partially purified preparations from several plants (Smith and Thompson, 1971; Smith, 1972; Brunold and Suter, 1982; Ruffet et al., 1994; Nakamura et al., 1988; Nakamura and Tamura, 1990). It is known that SATase can be multimeric, and is able to form a complex in association with CSase (Bogdanova and Hell, 1997; Ruffet et al., 1994; Droux et al., 1998; Nakamura et al., 1988; Nakamura and Tamura, 1990). In bacteria, SATase activity is inhibited by micromolar concentrations of L-cysteine (Kredich and Tomkins, 1966). OAS serves as an inducer of cysteine regulon besides as a substrate for cysteine synthesis in bacteria (Kredich, 1996), and a positive regulation by OAS has been also suggested for plants (Saito, 2000; Smith et al., 1997).

Until now, six cDNA clones encoding SATase have been isolated from watermelon (Saito et al., 1995), spinach (Noji et al., 2001a), *Arabidopsis thaliana* and *Allium tuberosum* (Chinese chive) (Urano et al., 2000). In particular, from *Arabidopsis*, cDNAs of three SATase isoforms, SAT-c (Howarth et al., 1997), SAT-p (Murillo et al., 1995; Ruffet et al., 1995), and SAT-m (Bogdanova et al., 1995; Hell and Bogdanova, 1995; Roberts and Wray, 1996), were cloned. Feedback inhibition pattern and subcellular localization of plant SATases were evaluated by analyzing the mode of inhibition using the recombinant SATases, and by the analysis of transient expression in *Arabidopsis* leaves using the fusion proteins of each SATase N-terminal region with green fluorescent protein (GFP) (Table 1). In plants, there are two types of SATase that differ in their sensitivity to the L-cysteine inhibition (Noji et al., 1998; Noji et al., 2001a; Saito et al., 1995; Urano et al., 2000). The activities of *Arabidopsis* SAT-c (a cytosolic isoform), watermelon SATase, spinach SATase and Chinese chive SATase are inhibited by L-cysteine in an allosteric manner. In contrast, *Arabidopsis* SAT-p (a plastidic isoform) and SAT-m (a mitochondrial isoform) were insensitive to feedback inhibition.

Difference of sensitivity to L-cysteine means that SATase have a regulatory role through the feedback inhibition in cysteine biosynthesis and it depends on the subcellular compartmentation (Saito, 2000; Noji, 1998). OAS is presumably a positive regulatory factor that derepresses the genes that

Table 1. Differences in feedback inhibition and subcellular localization of SATase isoforms from plants

Plant	Isoform	Feedback inhibition by L-cysteine	IC_{50} (μM)	Subcellular localization by GFP study
Arabidopsis	SAT-c	sensitive	1.8	cytosol
	SAT-p	insensitive	–	chloroplasts
	SAT-m	insensitive	–	mitochondria
Watermelon	SAT2	sensitive	2.9	cytosol
Spinach	SAT56	sensitive	7.6	chloroplasts
Chinese chive	ASAT5	sensitive	48.7	(Not experimentally examined)

encode enzymes involved in cysteine biosynthesis. Therefore, it has been proposed that the cytosolic SATase regulates the OAS concentration in cytosol strictly by the feedback inhibition by cysteine, whereas in chloroplasts, the high amount of cysteine is needed for full biosynthesis of methionine and GSH. Thus it is presumably necessary to produce a large amount of OAS even under the high concentration of cysteine without the feedback inhibition by cysteine in chloroplasts.

The spinach SATase, the activity of which is significantly inhibited by L-cysteine, possesses a transit peptide for chloroplast translocation of pre-SATase protein (Noji et al., 2001a). In the transit peptide region of spinach SATase, there are two ATG codons at Met-1 and Met-59. The result from *in vitro* translation of spinach SATase suggests that the spinach SATase is presumably translated from Met-1 and is localized in chloroplasts, but a part of SATase may be translated from Met-59 and may be localized in cytosol. The result suggests that this SATase cDNA encodes both plastidic and cytosolic SATase, but the major product of this SATase cDNA is a plastidic SATase. The regulatory mechanism of cysteine biosynthesis through the feedback inhibition of SATase by L-cysteine may differ with plant species.

The sensitivity of Chinese chive SATase to L-cysteine (48.7μM of the concentration for 50% inhibition, IC_{50}) was quite low compared with other SATases (~10μM IC_{50}) from various plants (Urano et al., 2000). In Chinese chive, the level of cysteine was several-fold higher than that in *Arabidopsis* and tobacco. This higher concentration of cysteine in Chinese chive is likely due to the lower sensitivity of feedback inhibition of SATase to L-cysteine.

Determination of the site required for the allosteric inhibition of SATase by cysteine

In the protein structure of SATase, the domains responsible for catalytic activity and protein-protein interaction for complex formation with CSase have been suggested (Bogdanova and Hell, 1997). However, no study has yet reported the localization of the allosteric domain of SATase responsible for the cysteine inhibition. Intensive analysis of mutated watermelon SATase indicated that the sensitivity of SATase to L-cysteine was determined by the co-operation of the N-terminal and C-terminal regions outside of the catalytic domain. Futhermore, amino acid residues responsible for the cysteine inhibition in the C-terminal region were identified (Inoue et al., 1999).

The C-terminal region from Pro-276 to Phe-285 of watermelon SATase, where the amino acid sequences are completely identical among feedback-inhibition sensitive SATases, differs from SAT-p at 5 amino acid positions, Gly-277, Glu-278, Ser-279, His-282 and Phe-285. Thus, five site-directed SATase mutants were constructed for detailed analysis of this C-terminal region in watermelon SATase. Each five residue, Gly-277, Glu-278, Ser-279, His-282, and Phe-285 in watermelon SATase was substituted with the corresponding amino acid residue found in SAT-p at these positions (Fig. 2). The

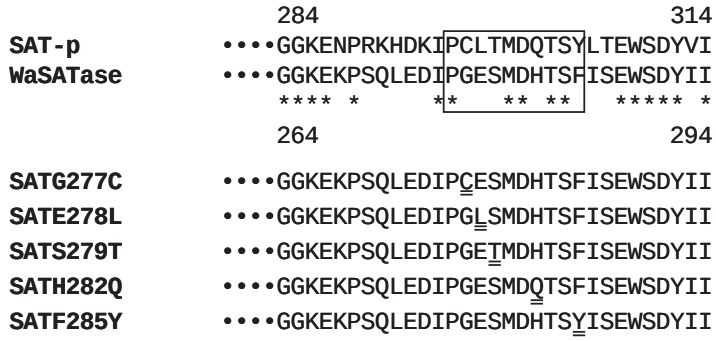

Fig. 2. Point mutations in the C-terminal region in watermelon SATase. The residues, Gly-277, Glu-278, Ser-279, His-282 and Phe-285 in watermelon SATase were replaced with corresponding amino acids of SAT-p to give mutants, SATG277C, SATE278L, SATS279T, SATH282Q and SATF285Y, respectively. *Asterisk* indicates an identical amino acid residue between SAT-p and WaSATase. *Boxed* region from Pro-276 to Phe-285 in WaSATase is shown as an essential region for the L-cysteine inhibition. The *double underlined* amino acids represent the point mutations in each SATase mutant. *Key.* WaSATase, watermelon SATase. (Adopted from Inoue et al., 1999)

Table 2. Comparison of the L-cysteine inhibition (IC_{50}) and Kinetic constants of watermelon SATase and several point mutants

Mutant	L-cysteine inhibition IC_{50} (μM)	*Km* (mM) for	
		L-Ser	acetyl-CoA
watermelon SATase	2.9 ± 0.3	0.59	0.13
SATG277C	77.8 ± 5.2	0.97	0.06
SATE278L	1.7 ± 0.1	0.26	0.38
SATS279T	2.6 ± 0.1	0.42	0.16
SATH282Q	10.1 ± 0.6	1.1	0.10
SATF285Y	4.9 ± 0.1	0.63	0.09

mutant proteins (SATG277C, SATE278L, SATS279T, SATH282Q and SATF285Y) were expressed in *E. coli*, and the catalytic and regulatory properties were investigated using partially purified recombinant proteins.

The sensitivity of SATG277C to L-cysteine was markedly decreased from IC_{50} = 2.9μM to IC_{50} = 77.8μM (Table 2). The sensitivity of the mutant, SATH282Q, was slightly decreased to IC_{50} = 10.1μM. However, replacement of 3 other amino acid residues, Glu-278, Ser-279, and Phe-285, did not substantially change the sensitivity to L-cysteine. Several point mutations affected the *Km* values of watermelon SATase for L-serine and acetyl-CoA (Table 2). These results confirm that Gly-277 in watermelon SATase is primarily responsible for inhibition by L-cysteine, and that His-282 is secondarily involved.

Transgenic *Arabidopsis* overexpressing SATase gene

To reveal the role of SATase in the regulation of cysteine synthesis and to enhance the ability of cysteine synthesis in plants, we made four kinds of transgenic *Arabidopsis*, SATW, SATM, RuSATW, and RuSATM, in which watermelon SATase gene or mutant SATase gene, SATG277C (Inoue et al., 1999), is overexpressed. In SATW and SATM, produced SATase was localized in cytosol; whereas in RuSATW and RuSATM produced SATase was localized in chloroplasts, since SATase gene was fused with ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit transit peptide gene in these chimeric genes. In SATW and RuSATW, wild-type SATase produced was highly sensitive to the feedback inhibition by L-cysteine. However, mutated SATase produced in SATM and RuSATM was insensitive to this feedback regulation because of the point mutation of Gly-277 to Cys. These gene expressions were controlled by the strong enhancer element El2 and 35S promoter. The contents of OAS and cysteine in 3-week-old transgenic *Arabidopsis* overexpressing SATG277C were increased significantly as compared to the wild-type *Arabidopsis* (Fig. 3). The overexpression of SATG277C caused the overaccumulation of OAS and cysteine, but overexpression of wild-type SATase gene did not. In cell-free extracts of SATW and RuSATW, we detected highly enhanced SATase activity by *in vitro* assay, however, the enzymatic activity of SATase is presumably inhibited by L-cysteine that exists in plant cells. Therefore, overaccumulation of OAS and cysteine did not remarkably occur in the transgenic plants, SATW and RuSATW, overexpressing wild-type SATase gene compared to SATM and RuSATM overexpressing mutated SATase gene. Interestingly, GSH contents were increased not only in SATM and RuSATM plants but also in SATW and RuSATW plants. These results suggest that the metabolic flux through cysteine to GSH is likely enhanced in all transgenic plants, although the steady-state concentration of cysteine is not increased in SATW and RuSATW plants. These *in vivo* results through transgenic plant experiments confirmed that feedback inhibition to SATase activity by L-cysteine is important for the regulation of the cysteine synthesis at lease for cytosolic SATase-catalyzed cysteine formation.

Transgenic tobacco overexpressing CSase gene

CSase catalyzes the formation of cysteine from OAS and hydrogen sulfide, with the release of acetic acid at the final step of the cysteine biosynthetic pathway.

$$\text{OAS} + \text{H}_2\text{S} \rightarrow \text{cysteine} + \text{acetic acid}$$

This enzyme requires pyridoxal phosphate as a cofactor. The enzymatic activity was detected in several plant species and in three subcellular compartments (*i.e.*, plastids, mitochondria, and cytosol). In fact, several isoforms of CSase have been characterized from a variety of plant species (Brunold and Suter, 1989; Nakamura and Tamura, 1989; Lunn et al., 1990;

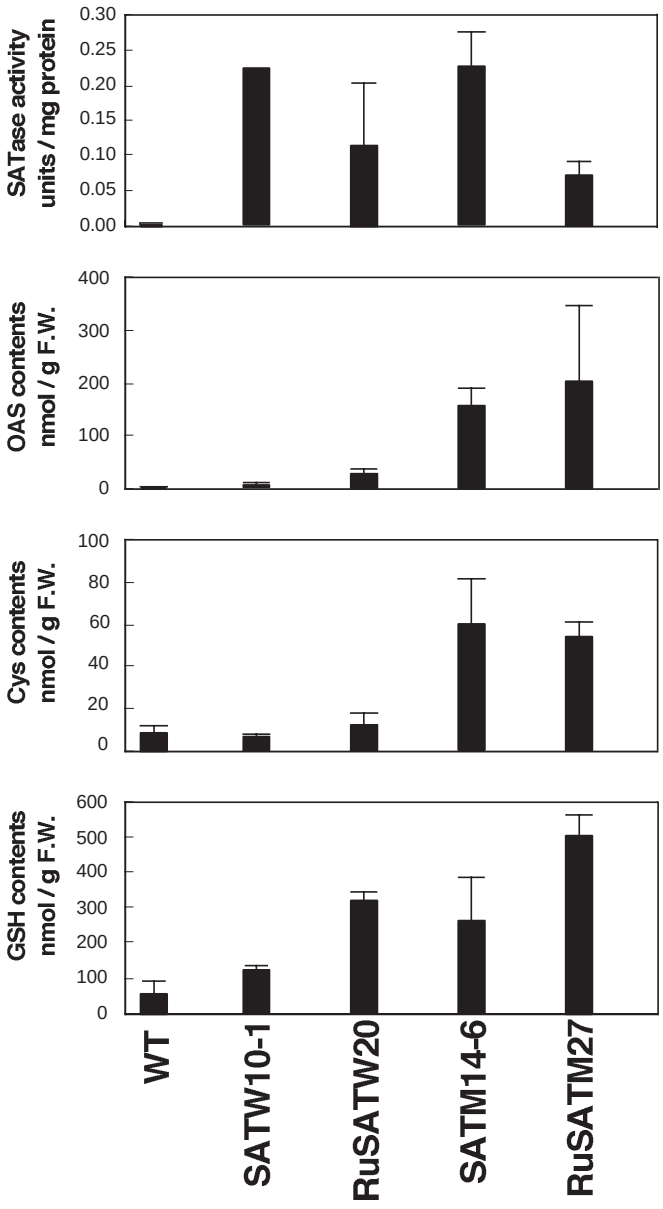

Fig. 3. SATase activity and OAS, cysteine and GSH contents of transgenic Arabidopsis overexpressing SATase. SATase activity and OAS, cysteine and GSH contents were determined from thirty transgenic plants from each transgenic line. Data are the means of triplicate analyses ± S.D. (Noji et al., unpublished)

Droux et al., 1992; Rolland et al., 1992; Ikegami et al., 1993; Kuske et al., 1994; Yamaguchi and Masada, 1995; Kuske et al., 1996).

CSase cDNA clone have been isolated from *Arabidopsis* (Hell et al., 1994; Barroso et al., 1995; Hesse and Altmann, 1995), spinach (Saito et al., 1992, 1993, 1994a; Hell et al., 1993; Rolland et al., 1993), watermelon (Noji et al., 1994), wheat (Youssefian et al., 1993), maize (Brander et al., 1995), bell

pepper (Römer et al., 1992), and Chinese chive (Urano et al., 2000). It is known that the mRNAs of cytosolic and chloroplastic isoforms of CSase were slightly induced by sulfate starvation (Takahashi and Saito, 1996; Hell et al., 1994; Barroso et al., 1995).

Environmental pollution by sulfur-containing compounds, *e.g.* sulfur dioxide (SO_2), hydrogen sulfide, sulfite and sulfate ions, is a serious problem for the global environment. In particular, gaseous SO_2 influences human health and the global ecological system of animals and plants (Wellburn, 1994; Murray, 1997). Moreover, when the gaseous SO_2 encounters moisture, considerable amounts of SO_2 are converted into sulfite and sulfate. These are critical components of acid rain and haze (Wellburn, 1994).

Although sulfur-containing compounds are toxic for plants at higher concentrations, sulfur is also an essential nutrition for plants. Plant assimilates the inorganic sulfur in the environment into cysteine through the cysteine biosynthetic pathway (Saito, 1999; Saito, 2000). Thus, the engineering of this cysteine biosynthetic pathway may be promising for development of the transgenic plant tolerant to sulfur-containing pollutants.

We constructed transgenic tobacco (*Nicotiana tabacum*) carrying either spinach cytosolic CSase A cDNA (Saito et al., 1992), designated 3F plants, or chimeric CSase A cDNA fused with the sequence for chloroplast-targeting transit peptide of pea Rubisco small subunit, designated 4F plants (Saito et al., 1994b). The 3F and 4F transgenic plants showed enhanced CSase activity in the cytosol and in the chloroplasts, respectively. The leaf discs of these transgenic tobacco showed partial tolerance to SO_3^{2-} (Saito et al., 1994b). To obtain transgenic plants highly tolerant to sulfur-containing pollutants, we crossed 3F plants with 4F plants to generate F_1 transgenic tobacco, in which CSase activities were enhanced both in the cytosol and in the chloroplasts (Noji et al., 2001b).

CSase activity in the cell-free extract of F_1 was about 5-fold higher than that of non-transformant plant. In the chloroplast fraction, CSase activity of F_1 was at the same level as that of 4F, and about 6-fold higher than those of 3F and non-transformant. In the cytosol fraction, CSase activity of F_1 was at the same level as that of 3F, and about 4-fold higher than those of 4F and non-transformant. These results indicated that F_1, in which CSase activities were enhanced both in the chloroplasts and in the cytosol, had the highest activity of CSase in these transgenic tobacco plants.

The F_1 transgenic plants were highly tolerant to toxic $1\mu L L^{-1}$ sulfur dioxide and 20 mM sulfite. Upon fumigation with $0.1\mu L L^{-1}$ sulfur dioxide, there was no visible damage on the leaves of control or transgenic plants. However, cysteine contents in F_1 plants after 4-day fumigation was increased significantly (Fig. 3). GSH contents in F_1 plants were also increased significantly after 2-day fumigation. Both cysteine and GSH contents were not changed in control plants. An unexpected finding was that both cysteine and GSH contents in 4F plants were not increased during the fumigation. These results suggest that transgenic plants overexpressing CSase, especially overexpressing in cytosol, can fix the atmospheric SO_2 into cysteine and GSH more efficiently than the control plant, since the contents of cysteine

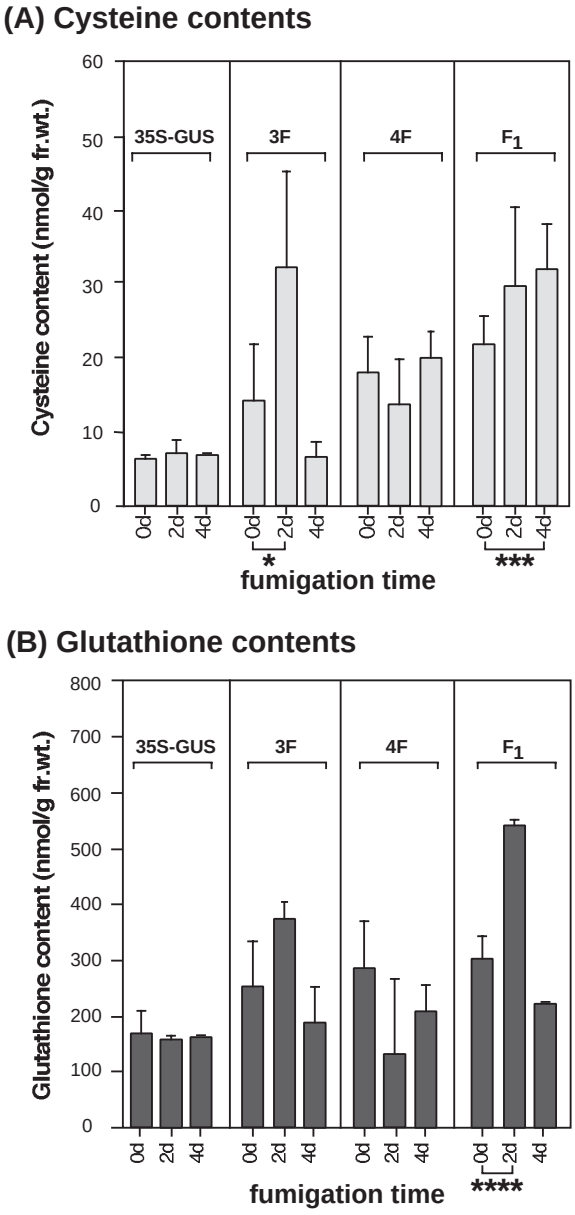

Fig. 4. Contents of cysteine and glutathione in transgenic plants exposed to $0.1\,\mu L\,L^{-1}$ SO_2. Six transgenic plants from each transgenic line were exposed to $0.1\,\mu L\,L^{-1}$ SO_2 for 4 days. The third leaves from the top of the transgenic plants were prepared and immediately subjected to quantification of the sulfhydryl compounds. The differences of the thiol content between 0-day and 2-day or 4-day treatment were analyzed statistically using Student's *t*-test. (*, $P < 0.1$; ***, $P < 0.025$; ****, $P < 0.005$). Data are the means of triplicate analyses ± S.D. 35S-GUS, tobacco plants transformed with a bacterial *UidA* gene encoding β-glucuronidase (GUS) using as a negative control. (Adopted from Noji et al., 2001b)

and GSH in the control plant were unchanged during the fumigation with $0.1\,\mu L\,L^{-1}$ SO_2. Furthermore, the leaves of F_1 plants exhibited the increased resistance to paraquat, a herbicide generating active oxygen species (Noji et al., 2001b).

Genomic organization of SATase and CSase genes in *Arabidopsis* and future prospects

After the completion of whole genome sequencing of *A. thaliana* in the end of 2000 (The Arabidopsis Genome Initiative, 2000), one can estimate the numbers of genes exhibiting homologous sequences to SATase and CSase. Five SATase-like genes and around ten CSase-like genes can be identified in the genome sequence of *A. thaliana*. Now it would be necessary to conduct functional genomics study to reveal the function of these putative genes experimentally. Recently, the CSase-like proteins localized in mitochondria of spinach, *Arabidopsis* and potato have been identified to act as β-cyanoalanine synthase rather than CSase (Hatzfeld et al., 2000; Warrilow and Hawkesford, 2000; Maruyama et al., 2001). From these observations, we proposed a new systematic nomenclature for CSase-like genes as *Bsas* (β-substituted alanine synthase) (Hatzfeld et al., 2000). In the near future, through the detailed conjunctive study of biochemical analysis of recombinant proteins and genetic analysis of knock-out or transgenic mutants, one can clarify the biological function of these SATase-like and CSase-like genes in *A. thaliana* for the further application of metabolic engineering of sulfur-related metabolism in plants.

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