Isoform-dependent Differences in Feedback Regulation and Subcellular Localization of Serine Acetyltransferase Involved in Cysteine Biosynthesis from *Arabidopsis thaliana*

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Serine acetyltransferase (SATase; EC 2.3.1.30), which catalyzes the formation of *O*-acetyl-L-serine (OAS) from acetyl-CoA and L-serine, plays a regulatory role in the biosynthesis of cysteine by its property of feedback inhibition by cysteine in bacteria and certain plants. Three cDNA clones encoding SATase isoforms (SAT-c, SAT-p, and SAT-m) have been isolated from *Arabidopsis thaliana*. However, the significance of the feedback regulation has not yet been clear in these different isoforms of SATase from *A. thaliana*. We constructed the overexpression vectors for cDNAs encoding three SATase isoforms of *A. thaliana* and analyzed the inhibition of SATase activity by cysteine using the recombinant SATase proteins. In the case of SAT-c, the activity was feedback-inhibited by a low concentration of cysteine (the concentration that inhibits 50% activity; IC50 = 1.8 μM). By contrast, SAT-p and SAT-m were feedback inhibition-insensitive isoforms. We also determined the subcellular localization of three SATase isoforms by the transient expression of fusion proteins of each SATase N-terminal region with jellyfish green fluorescent protein (GFP) in 4-week-old Arabidopsis leaves. The SAT-c-GFP fusion protein was stayed in cytosol, whereas SAT-p-GFP and SAT-m-GFP fusion proteins were localized in chloroplasts and in mitochondria, respectively. These results suggest that these three SATase isoforms, which are localized in different organelles, are subjected to different feedback regulation, presumably so as to play the particular roles for the production of OAS and cysteine in Arabidopsis cells. Regulatory circuit of cysteine biosynthesis in the plant cells is discussed.

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 gas in the air) is fixed into cysteine mainly by this biosynthetic pathway in plants (1). Cysteine is incorporated into proteins and glutathione (GSH) or serves as the sulfur donor of methionine and sulfur-containing secondary products in plants. The cysteine biosynthetic pathway involves several enzymatic reactions (1–4). The final step of cysteine biosynthesis is the reaction of incorporating sulfide moiety into the 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*-acylserine (thio)-lyase) (CSase) (EC 4.2.99.8) are committed in this step. 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 (5–8) and CSase (9–14) has been demonstrated in these three compartments from several plants.

SATase, which catalyzes the formation of OAS from acetyl-CoA and L-serine, is responsible for the entry step from serine metabolism to cysteine biosynthesis; therefore, it would be logical to postulate the existence of regulation at this step. SATase has been characterized with the purified or partially purified preparations from *Phaseolus vulgaris* (5, 15), spinach (7, 16), rape (17), and *Allium tuberosum* (18). The activity of SATase was associated with that of CSase as a multienzyme complex during the purification procedures (16–18). The cellular activity of SATase is 100–300-fold less than that of CSase (16). The importance of OAS in regulation of the entire flux of cysteine formation has been suggested from several physiological studies (19). In fact, the availability of OAS has been found to be rate-limiting for cysteine biosynthesis in feeding experiments (20–22). Furthermore, addition of OAS to plants led to increase in mRNA accumulation of the sulfate transporter and the rate of sulfate uptake in barley roots (23). The activities of ATP sulfurylase and adenosine 5′-phosphosulfate sulfotransferase in *Lemna minor* were also enhanced by supplement of OAS (21). These studies suggest that SATase and OAS are the major regulatory factors in the biosynthesis of cysteine in plants.

We have previously isolated a cDNA clone encoding SATase of presumable cytosolic localization from *Citrus vulgaris* (watermelon) (24). The purified recombinant watermelon SATase produced in *Escherichia coli* was highly sensitive to feedback inhibition by *L*-cysteine, the end-product of the biosynthetic pathway, at the physiological concentration (~3 μM) in an allosteric manner (24). The cellular concentration of cysteine is normally ~10 nmol/g fresh weight (22, 25). Thus, we proposed that this feedback inhibition at the physiological concentration of *L*-cysteine certainly plays a central role in the regulation of cysteine biosynthesis. However, in *P. vulgaris* (15) and spinach (7), the inhibition for SATase was observed only at nonphysiologically high cysteine concentrations for partially

Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; CS-C, cysteine synthase C; NAS, *N*-acetyl-L-serine.
purified enzymatic preparations. Hence, from Arabidopsis thaliana, several cDNA clones of SATase have been isolated (8, 26–30), but no feedback inhibition by t-cysteine was reported about these Arabidopsis SATases. Thus, the significance of regulation by the feedback inhibition has not yet been clear in plant SATases, in particular, for different isoforms presumably localized in particular organelles.

The present study was conducted to confirm that the feedback inhibition of SATase is actually effective in the regulation of cysteine biosynthesis in particular organelle by analyzing the mode of inhibition using the recombinant SATases from Arabidopsis. In view of amino acid sequences and the difference in subcellular localization, which is clarified in our present study, of the Arabidopsis SATase isoforms, these SATase isoforms can be divided into three groups: (i) SAT-c, that is a cytosolic isoform, identical to SAT52 (30); (ii) SAT-p, that is a plastidic isoform, identical to SAT1 (27) and almost identical to SAT5 (8) except for small amino acid changes (98% identity); (iii) SAT-m, that is a mitochondrial isoform, identical to Sat-1 (29), which is a different isoform from SAT1 (27) described above, and almost identical to SAT1–6 (26) and SAT-A (28) except for the length of N-terminal region. SAT1–6 and SAT-A lack the 97 and 55 N-terminal amino acid residues of SAT-m, respectively. Therefore, we refer these three SATase isoforms as SAT-c, SAT-p, and SAT-m throughout this paper. Here we report that there are two types of SATase isoforms in Arabidopsis regarding cysteine feedback inhibition, one feedback-sensitive and the other feedback-insensitive. We also clarified the subcellular localization of these SATase isozymes in Arabidopsis leaves, and then we discuss the implication of the differences in feedback inhibition of SATase for the regulation of cysteine biosynthesis in different organelles.

MATERIALS AND METHODS

Plant Growth—A. thaliana (Columbia ecotype) plants were grown on germination medium agar plates (31) under 16-h light (approximately 2500 lux) and 8-h dark cycles at 22 °C. After 3–6 weeks, the plants were used for isolating total RNA and experiments of particle gun bombardment.

Preparation of cDNA Templates for the Polymerase Chain Reaction (PCR)—Total RNA was isolated by the guanidine hydrochloride/phenol method (32) from whole plants of Arabidopsis that have been grown for 3 weeks. Poly(A) + RNA was purified by oligo(dT)-cellulose column chromatography as described elsewhere (32). First-stranded cDNA was synthesized from poly(A) + RNA by use of First-strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Differently, total RNA was isolated from the above-ground part of 3-week-old Arabidopsis plants by the phenol/SDS method and precipitated by LiCl (32). Double-stranded cDNA was synthesized from the total RNA by using CapFinder cDNA Synthesis Kit (Clontech, Palo Alto, CA). SATase cDNAs from A. thaliana—The plasmids used in this study are described in Table I. SAT-p cDNA (pSAT-p) was kindly provided by Dr. T. Leustek, Rutgers University, New Brunswick, NJ (27). Truncated SAT-m cDNA (pSAT-m, identical to SAT-A) was kindly provided by Dr. R. Hell, Ruhr Universitat, Bochum, Germany (28).

Table I

| Plasmid       | Host               | Description                                                                 |
|---------------|--------------------|------------------------------------------------------------------------------|
| pSAT-c        | E. coli XL1-Blue   | cDNA encoding SAT-c (identical to SAT52 in Ref. 30)                         |
| pSAT-p        | E. coli XL1-Blue   | cDNA encoding SAT-p (identical to SAT1 in Ref. 27)                          |
| pSAT-m        | E. coli XL1-Blue   | cDNA encoding truncated SAT-m (identical to SAT-A in Ref. 28)               |
| pAS-c         | E. coli BL21 (DE3) | cDNA encoding SAT-c (identical to SAT1-1 in Ref. 29)                        |
| pAS-p         | E. coli BL21 (DE3) | Overexpression vector for SAT-c recombinant protein                          |
| pAS-m         | E. coli BL21 (DE3) | Overexpression vector for truncated SAT-m protein                            |
| sGFP (S65T)   | E. coli XL1-Blue, A. thaliana cells | GFP expression vector                                                      |
| 3SSO-TP-GFP   | E. coli XL1-Blue, A. thaliana cells | Fusion of transit peptide of Rubisco small subunit with GFP                  |
| pCS-C-GFP     | E. coli XL1-Blue, A. thaliana cells | Fusion of transit peptide of CS-C with GFP                                  |
| pSAT-c-GFP    | E. coli XL1-Blue, A. thaliana cells | Fusion of N-terminal region of SAT-c with GFP                               |
| pSAT-p-GFP    | E. coli XL1-Blue, A. thaliana cells | Fusion of N-terminal region of SAT-p with GFP                               |
| pSAT-m-GFP    | E. coli XL1-Blue, A. thaliana cells | Fusion of N-terminal region of SAT-m with GFP                               |
| pWMSAT-GFP    | E. coli XL1-Blue, A. thaliana cells | Fusion of N-terminal region of watermelon SATase with GFP                   |

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S. aureus SATase cDNA fragment was obtained from the first-stranded cDNAs of A. thaliana by PCR amplification using a set of primers derived from the nucleotide sequence of SAT-c coding region, which has been reported previously as SAT52 (30). Purification of SATase cDNA was performed with the AmpliPrep kit (Amersham Pharmacia Biotech). The PCR mixture contained a 1 m M concentration of dATP, dGTP, dCTP, and dTTP, 0.1 m M of the primers described above, and 250 m M of MgCl2. The amplification was performed in a 20-μL reaction volume using a Biometra Thermoblock (Biometra, Goettingen, Germany). The PCR conditions were as follows: a preincubation step at 97 °C for 2 min, followed by 35 cycles of amplification each consisting of 30 s at 97 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final extension at 72 °C (30). The amplification products were purified with a gel extraction kit (Qiagen, Hilden, Germany) and then cloned into the plasmid vector pT7Blue-T-Vector (Novagen) to afford pSAT-m, and the nucleotide sequence of the cloned SAT-m cDNA fragment was confirmed to be identical to that reported as SAT52 (30) by the dyeodeoxy chain termination method using Thermo Sequenase (Amersham Pharmacia Biotech) and a Shimadzu DNA sequencer model DSQ1000.

The full-length version of SAT-m cDNA, which is identical to Sat-1 (29), was obtained from Arabidopsis double-stranded cDNAs by PCR using two synthetic primers (forward, 5′-GGAGATCCACGGGCGGAGAACT-3′; reverse, 5′-CGCTACCATGACAAAGATTATGATGTAATC-3′). We designed these primers to create the NdeI sites to both ends of amplified SAT-c DNA fragment. The oligonucleotide primers were phosphorylated with T4 polynucleotide kinase as described elsewhere (32). The amplified cDNA fragment of SAT-c was subcloned into the EcoRV site of pBluescript II KS + (Stratagene) and the resulting plasmid was designated as pSAT-c. Then, the nucleotide sequence of SAT-c cDNA was confirmed to be identical to that reported as SAT52 (30) by the dyeodeoxy chain termination method using Thermo Sequenase (Amersham Pharmacia Biotech) and a Shimadzu DNA sequencer model DSQ1000.

The amplified cDNA fragment of SAT-c was subcloned into the pT7Blue T-Vector (Novagen) to afford pSAT-m, and the nucleotide sequence of the cloned cDNA fragment was confirmed by sequencing as described above.

Construction, Expression, and Partial Purification of Recombinant SATase Proteins—The 0.95-kilobase pair NdeI fragment containing the full-length cDNA of SAT-c was recovered from the plasmid, pSAT-c, described above and was inserted into NdeI site of pET3a (33) (Novagen) to afford pAS-c. For the overexpression of SAT-p, NcoI sites were created on both ends of SAT-p coding region by PCR engineering using two synthetic primers: forward, 5′-CTCCCACATGCTGTAATCAATCA-3′; reverse, 5′-ACTCCCTATGCTGTAATCAATCA-3′; reverse, 5′-ACTCCCTATGCTGTAATCAATCA-3′. The engineered cDNA fragment was inserted into the NcoI site of pET3b (32) (Novagen), and the resulting plasmid was designated as pAS-p. Likewise, we constructed the overexpression vector of truncated SAT-m, which was designated as pAS-m, using primers: forward, 5′-ATTCCCCATGCTGTAATCAATCA-3′; reverse, 5′-ATTCCCCATGCTGTAATCAATCA-3′; reverse, 5′-ATTCCCCATGCTGTAATCAATCA-3′. In these plasmids, pAS-c, pAS-p, and pAS-m, each SATase cDNA was placed under the strong dAT promoter in the sense orientation.

E. coli BL21 (DE3) pLysE cell (Novagen), in which the gene for t-cysteine was expressed and induced by isopropyl-1-thio-D-galactopyranoside, was used as host for overexpression of the recombinant SATase proteins. Overnight cultures of E. coli cells carrying the recombinant plasmid were inoculated at 1:100 in 1 liter of LB medium (10 g of Bacto-tryptone, 5 g of Bacto yeast extract, 10 g of NaCl) containing 100 mg/ml carbenicillin and were grown for 3 h at 37 °C. Isopropyl-1-thio-D-galactopyranoside was added to a final concentration of 1 mM, and incubation was continued for an additional 3 h at 37 °C.

The cells (5 g) were harvested, washed with saline once, and resuspended in buffer A (50 ml) containing 200 mM potassium phosphate (pH 8.0), 250 mM sucrose, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA. The
Fig. 1. Comparison of amino acid sequences of SATase.

internal methionine residue that was the start point for translation of truncated recombinant SAT-m is indicated by double underlining. An arrow indicates the N-terminal regions of SATases that were fused to GFP for construction of SAT-c-GFP, SAT-p-GFP, and SAT-m-GFP. Dashes indicate gaps in sequence for best alignment. Black shading indicates identical amino acid residue. Gray shading indicates similar amino acid residue. SATase sequences compared are: SAT-m, Arabidopsis SAT-m (identical to Sat-1 (29)); SAT-p, Arabidopsis SAT-p (identical to SAT1 (27)); SAT-c, Arabidopsis SAT-c (identical to SAT52 (30)); Watermelon, watermelon SAT2 (24).

cell suspensions were disrupted by sonication, for 10 times at 100 watts for 20 s each on ice. The soluble protein extracts of *E. coli* BL21 transformants were obtained by centrifugation at 10,000 × g and then were subjected to fractional precipitation with ammonium sulfate (20–80% saturation). The precipitates redissolved in buffer B, containing 10 mM potassium phosphate (pH 8.0), 10 mM 2-mercaptoethanol, and 0.5 mM EDTA, were desalted by passage through a Sephadex G-25 column and then applied to a DEAE-Sepharose column. The absorbed proteins were eluted with a linear gradient of NaCl (0.1–0.5 M) in buffer B. The combined fractions containing each SATase isozyme were concentrated by ultrafiltration using Desal Ultrafilters (Amicon).

Western blotting and immunostaining were carried out on an Immobilon-P membrane (Millipore) using phosphatase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and 5-bromo-4-chloro-3-indolyolphosphate *p*-toluidine/nitro blue tetrazolium chloride (Life Technologies, Inc.) as substrates. The rabbit primary antibody against the recombinant SATase of watermelon (24) was used at 1:400 dilution. Protein concentrations were determined with a Bio-Rad Protein Assay kit using bovine serum albumin as the standard.

**Enzymatic Activity Assay**—The SATase activities were determined by monitoring the decrease in *A* 420 due to the cleavage of thioester bond of acetyl-CoA (35). The standard mixture contained 50 mM Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, 1 mM *L*-serine, and enzyme solution, in a final volume of 1 ml. The reaction was initiated by the addition of *L*-serine and carried out at 37 °C. The initial velocity of the decrease in absorbance at 232 nm was monitored. The molar extinction coefficient of acetyl-CoA of ε = 4500 was used for calculation.

**Construction of Chimeric Genes Encoding Fusion Protein of SATase**—

Construction of *SAT*ases with Green Fluorescent Protein (GFP)—The cDNA fragments of SATase N-terminal regions were obtained from each full-length SATase cDNA by PCR and fused to *SuI*-NcoI site at the 5′-end of GFP. A 176-base pair fragment of SAT-c (PCR-amplified with primers: forward, 5′-TACGCGTCGACATGGCAACTGT-3′; reverse, 5′-TCTACCCATGGCGCGCCTGCTGTTTAC-3′), a 173-base pair fragment of SAT-p (amplified using primers: forward, 5′-TACGCGTCGACATGGCAACTGT-3′; reverse, 5′-TCTACCCATGGCGCGCCTGCTGTTTAC-3′), and a 416-base pair fragment of SAT-m (amplified using primers: forward, 5′-TACGCGTCGACATGGCAACTGT-3′; reverse, 5′-TCTACCCATGGCGCGCCTGCTGTTTAC-3′) were subcloned into the GFP expression vector, sGFP (S65T) (36), in which GFP gene is driven by cauliflower mosaic virus 35S promoter, and the resulting plasmids were designated pSAT-c-GFP, pSAT-p-GFP, and pSAT-m-GFP, respectively. The plasmid, pCS-C-GFP, containing the transit peptide sequence of spinach mitochondrial cysteine synthase C (CS-C) (37, 38) fused to GFP, was used as a positive control for localization in mitochondria. The primers (forward, 5′-TACGCGTCGACATGGCAACTGT-3′; reverse, 5′-TCTACCCATGGCGCGCCTGCTGTTTAC-3′) were used for PCR amplification of CS-C transit peptide sequence. The plasmid, sGFP (S65T), without any fusion protein was used as a positive control for localization in cytosol and partly in nuclei as intrinsic nature of GFP.

**Tissue Bombardment and Fluorescence Microscopy**—

Particle gun bombardment on Arabidopsis tissues was carried out by Helios Gene Gun System (Bio-Rad) by following standard protocol provided by the supplier. Gold particle (diameter: 1.0 μm), microcarrier loading quantity of 0.5 mg gold/target and DNA loading ratio of 2 μg DNA/mg gold
were chosen for the particle bombardment, and 4- or 6-week-old Arabidopsis plants were bombarded at pressure 100 p.s.i./shot. After bombardment, Arabidopsis plants were cultivated for 20 h under illumination at 22 °C. Individual leaf was visualized with a fluorescent microscope (BX50-PLA, Olympus) using the Chroma’s Dual Band filter, FITC & TRITC (Olympus), which provides excitations at 475–490 and 545–565 nm and emissions at 510–530 and 585–620 nm.

RESULTS

Expression of Recombinant SATase Proteins in E. coli—We constructed three recombinant genes, pAS-c, pAS-p, and pAS-m, for overexpression of SATase cDNA in E. coli BL21 (DE3) pLysE cells to obtain the recombinant SATase proteins. The recombinant genes, pAS-c and pAS-p, were designed to produce the full-length recombinant proteins of SAT-c (32.7 kDa) and SAT-p (34.2 kDa), respectively, whereas pAS-m was constructed to produce the truncated recombinant SAT-m (36.5 kDa), which was translated from internal ATG codon (methionine 56) of the full-length SAT-m cDNA (Fig. 1). This lack of 55 N-terminal amino acid residues of SAT-m protein does not presumably influence the enzymatic property of mature SAT-m, because the lacking region is in the part of transit peptide (see later).

The overexpressed recombinant SATase isoforms were obtained from E. coli transformants and then partially purified as described under “Materials and Methods.” The partially purified SATase isoforms were analyzed by SDS-PAGE with subsequent dye staining (Fig. 2A) and immunostaining (Fig. 2B). The protein bands corresponding to recombinant SATase proteins evidently detected by both dye staining and immunostaining in each transformant with pAS-c, pAS-p, and pAS-m. The apparent molecular mass of SAT-c (34.5 kDa) was larger than the predicted molecular mass of SAT-c on SDS-PAGE gel. The partially purified SAT-m exhibited two bands of 36.5 and 33 kDa that reacted with antibody. The 33-kDa protein was presumably resulted from translation starting at the internal ATG codon (methionine 88) of SAT-m (Fig. 1). The calculated value for translatable product beginning at Met-88 is 32,904 Da, being well matched with the observed value.

The concentration of the recombinant SATase proteins was increased up to approximately 5% of the total soluble proteins of E. coli transformants, and the SATase activities in the crude extracts of transformants were ~1000-fold higher than that of negative control E. coli BL21 (DE3) pLysE (data not shown).

Catalytic and Regulatory Properties of Recombinant SATase Isoforms: Feedback Inhibition Analysis—The catalytic and regulatory properties of SAT-c, SAT-p, and SAT-m were investigated using the partially purified recombinant proteins. The \( K_m \) values of these SATases for L-serine and acetyl-CoA were comparable with those reported previously for plant SATases (16–18, 24) (Table II). Then, we analyzed the feedback inhibition by L-cysteine using these recombinant proteins of SATase (Fig. 3).

The activity of SAT-c was inhibited by L-cysteine in a noncompetitive manner with L-serine, but competitive with acetyl-CoA. The \( K_i \) values of L-cysteine were determined to be 10.8 \( \mu \)M for L-serine and 7.4 \( \mu \)M for acetyl-CoA (Table II). The concentration for 50% inhibition (IC\text{\textsubscript{50}}) under the standard assay conditions was 1.8 \( \mu \)M (Fig. 3), being of the same order for that of watermelon SATase (2.9 \( \mu \)M) (24). In contrast to SAT-c, the activities of SAT-p and SAT-m were feedback-insensitive up to 100 \( \mu \)M L-cysteine (Fig. 3).

We examined the inhibition of SATase activity by various sulfur containing organic compounds, d-cysteine, N-acetyl-L-cysteine, L-cystine, dl-homocysteine, L-methionine, and glutathione. These sulfur-containing compounds did not inhibit the activities of SAT-p and SAT-m. The inhibitory effect to SAT-c was specific to L-cysteine, only weak activities being detected with N-acetyl-L-cysteine (20% inhibition at 1 mM) and L-cystine (49% inhibition at 1 mM), d-Cysteine (0.1 mM) and 1 mM dl-homocysteine, L-methionine, and glutathione exhibited no inhibitory action.

Subcellular Localization of SATase-GFP Fusion Proteins—To clarify the subcellular localization of SATase isoforms in Arabidopsis cells, we constructed SATase-GFP fusion genes from each SATase isoform by PCR engineering. In the fusion proteins, SAT-c-GFP, SAT-p-GFP, and SAT-m-GFP, N-terminal regions of SAT-c (amino acid positions 1–51), of SAT-p (amino acid positions 1–54), and of SAT-m (amino acid positions 1–131) (Fig. 1) were fused to N terminus of GFP, respectively. An alanine residue was additionally placed at the junction of SATase and GFP in all fusion proteins as a consequence of PCR engineering. All plasmid constructs were introduced into the leaves of 4-week-old (vegetative stage) and 6-week-old (reproductive stage) A. thaliana by DNA bombardment.

GFP without any targeting sequence accumulated diffusely in cytosol and partially in nucleus in both 4-week-old (Fig. 4a) and 6-week-old (data not shown) leaves. SAT-c-GFP fusion protein stayed in cytosol both in 4-week-old (Fig. 4b) and 6-week-old (data not shown) leaves. The targeting sequence of positive control carrying the transit peptide of CS-C could direct the transportation of GFP into mitochondria in both 4-week-old (Fig. 4c) and 6-week-old (data not shown) leaves. However, subcellular localization of SAT-p-GFP differed with the stage of Arabidopsis plants. In 4-week-old leaves, the green fluorescence of SAT-p-GFP was observed only in chloroplasts (Fig. 4f); however, in 6-week-old leaves, SAT-p-GFP fusion

| SAT-c | SAT-p | SAT-m |
|-------|-------|-------|
| L-Serine | Acetyl-CoA | L-Serine | Acetyl-CoA |
| \( K_m \) | 2.71 | 0.28 | 10.8 | 7.4 |
| Inhibition \( K_i \) by L-cysteine | (noncompetitive) | (competitive) | — | — |

\( a \) — no inhibition by L-cysteine.

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proteins were localized both in chloroplasts (Fig. 4g) and cytosol (Fig. 4h). We observed more than 100 cells bombarded with pSAT-p-GFP in 6-week-old leaves in several separated experiments, and the ratio of chloroplast to cytosol localization was in the ratio of 1 to 9.

PSORT system (GenomeNet Service, Osaka University), which is the program predicting the subcellular localization of given protein by sequence analysis with the transit peptide data base of defined subcellular translocation, also predicted that SAT-c, SAT-p, SAT-m would be most probably localized in cytosol, chloroplast stroma, and mitochondrial matrix space, respectively (Table III). This prediction by PSORT program agreed with our experimental results of subcellular localization of SATase-GFP fusion protein by particle bombardment experiments.

We also determined the subcellular localization of watermelon SATase (24), of which activity is inhibited by L-cysteine, by the expression of N-terminal region-GFP fusion protein, WMSAT-GFP (Fig. 1, Table I). This fusion protein was detected in cytosol in both 4- and 6-week-old Arabidopsis leaves (data not shown).

**DISCUSSION**

Until now, unequivocal evidence for feedback inhibition by cysteine of SATase from plants has been reported only for the cytosolic SATase from watermelon (24). However, for other plants, no data of feedback inhibition were presented with recombinant, purified, or native SATases. Thus, it has been unclear whether the feedback inhibition of SATase is of importance for the regulation of cysteine biosynthesis in plants or not. In the present study, we have clearly demonstrated that the activity of SAT-c was feedback-inhibited by micromolar concentration of L-cysteine (IC$_{50} = 1.8 \mu M$); however, SAT-p and SAT-m were feedback-insensitive isoforms as summarized in Table IV. These results suggest that plants, at least A. thaliana, have two types of SATase isoforms regarding inhibition by L-cysteine, one feedback-sensitive and the other feedback-insensi-
It was indicated from physiological evidence that SATase is localized in mitochondria of *P. vulgaris* (5), in the particulate fraction of extracts of wheat leaves (6), and in chloroplasts of spinach leaves (7). The recent data indicated that the major activity was localized in mitochondria (approximately 76% of total cellular activity), and the minor activity is found in cytosol (14% of total activity) and in chloroplasts (10% of total activity) of pea leaves (8). In this study, we provide the first direct evidence for different subcellular localization of Arabidopsis SATase isoforms by the transient expression analysis of fusion proteins of SATase N-terminal region and GFP in Arabidopsis leaves using particle gun bombardment. In the 4-week-old leaves, SAT-c-GFP fusion protein was located in cytosol, whereas SAT-p-GFP and SAT-m-GFP fusion proteins were localized in chloroplasts and mitochondria, respectively (Fig. 4 and Table IV).

Interestingly, in 4-week-old Arabidopsis leaves, SAT-p-GFP was localized only in chloroplasts; however, in 6-week-old leaves, SAT-p-GFP was localized both in chloroplasts and cytosol, although the chloroplastic positive control, 3S5S-TP-sGFP (S65T), was localized only in chloroplasts both in 4- and 6-week-old leaves. These results suggest that import of SAT-p-GFP into chloroplasts may be susceptible to the vital condition or developmental stage of chloroplasts. The stage of 4-week-old Arabidopsis plants was distinct from that of 6-week-old plants. The 4-week-old Arabidopsis plants were at the vegetative stage, whereas the 6-week-old plants were at the reproductive stage. The physiological conditions of Arabidopsis cells and subsequent ability of transport machinery into chloroplasts may differ with the developmental stage, and these may affect the transportation of SAT-p-GFP. It has been reported that SAT-p protein translated in vitro failed to enter isolated intact chloroplasts under conditions that promoted the import of another chloroplast enzyme (27), supporting the idea that SAT-p import ability into chloroplasts may be susceptible to the physiological conditions. Another feedback-sensitive SATase from plants, watermelon SATase, was localized in cytosol, which was confirmed by the expression of watermelon SATase-GFP fusion protein. Combining these data available so far, it is concluded that SATase isoform localized in organelles (chloroplasts and mitochondria) is feedback-insensitive, and cytosolic SATase is feedback-sensitive (Table IV).

In Arabidopsis, three SATase isoforms of different sensitivity to feedback regulation and subcellular localization may play the different roles for cysteine biosynthesis (Fig. 5). Since the concentration of cysteine is kept at the steady state level about 10–20 μM in cells (22, 25), cysteine biosynthesis seems to be strictly regulated in plant cells. The concentration of L-cysteine in cytosol can be properly regulated by the feedback regulation of cytosolic isoform, SAT-c.

In bacteria, SATase plays a regulatory role of cysteine biosynthesis through feedback control (40). The expression of cysE gene for SATase is constitutive, but SATase activity is feedback-inhibited by micromolar concentration of cysteine (IC_{50} = 1.1 μM) (41). OAS and its derivative, N-acetyl-L-serine (NAS), serves not only as substrate for cysteine synthesis, but as an inducer of Cys regulon (40). In a situation of cysteine limitation, SATase activity is derepressed from feedback inhibition by cysteine, and the derepressed SATase provides OAS and NAS for the activation of the Cys regulon. SAT-c and watermelon SATase, which are localized in cytosol, are similar to the bacterial SATase in view of the expression pattern and the feedback inhibition by L-cysteine. Thus, cytosolic SATase, by analogy with bacterial SATase, seems to be responsible for strict regulation of the OAS concentration in cytosol by feedback inhibition by L-cysteine (Fig. 5). Under the condition of cysteine (sulfur) limitation, cytosolic SATase can be derepressed by the canceling of feedback inhibition and provide OAS for the sufficient production of cysteine and possibly for the induction of the gene expression of cysteine biosynthetic enzymes.

**TABLE IV** Differences in feedback inhibition and subcellular localization of SATase isoforms from plants

| Plant | Isoform | Feedback inhibition by L-cysteine | 4-Week-old leaves (vegetative stage) | 6-Week-old leaves (reproductive stage) |
|-------|---------|----------------------------------|--------------------------------------|---------------------------------------|
| Arabidopsis | SAT-c | Sensitive | Cytosol | Cytosol |
|        | SAT-p | Insensitive | Chloroplasts | Chloroplasts (10%) |
|        | SAT-m | Insensitive | Mitochondria | Mitochondria |
|        | SAT2 | Sensitive | Cytoplase | Cytoplase |
| Watermelon | SAT-c | Sensitive | Cytosol | Cytosol |
|         | SAT-m | Insensitive | Mitochondria | Mitochondria |

**Fig. 5. Regulation and compartmentation of SATase isoforms in A. thaliana.** Arabidopsis SATase isoforms are localized in the different organelles. SAT-c seems to be responsible for strict regulation of the OAS concentration in cytosol by feedback inhibition by cysteine. Under the cysteine limiting condition, SAT-c can be derepressed and provide OAS for the cysteine biosynthesis and possibly for the induction of the gene expression of cysteine biosynthetic enzymes.
SAT-m, were almost constitutive (47), suggesting again that SAT-p is responsible for production of cysteine needed for biosynthesis of methionine and GSH in chloroplasts.

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REFERENCES
1. Saito, K. (1998) in Plant Amino Acids: Biochemistry and Biotechnology (Singh, B., ed) pp. 267–291, Marcel Dekker, New York
2. Leustek, T. (1996) Physiol. Plant. 97, 411–419
3. Hell, R. (1997) Planta 202, 138–148
4. Brunold, C. & Rennenberg, H. (1997) Prog. Bot. 58, 164–186
5. Smith, I. (1972) Biocatalysis Biophysics 16, 889–893
6. Brunold, C. & Suter, M. (1982) Planta 155, 321–327
7. Ruffet, M. L., Lebron, M., Droux, M. & Douce, R. (1995) Eur. J. Biochem. 227, 560–569
8. Brunold, C. & Suter, M. (1989) Planta 179, 228–234
9. Lunn, J. E., Droux, M., Martin, J. & Douce, R. (1990) Plant Physiol. 94, 1345–1352
10. Droux, M., Martin, J., Sajus, P. & Douce, R. (1992) Arch. Biochem. Biophys. 295, 379–390
11. Rolland, N., Droux, M. & Douce, R. (1992) Plant Physiol. 98, 927–935
12. Yamaguchi, T. & Masuda, M. (1995) Biochem. Biophys. Acta 1251, 91–98
13. Kuske, C. R., Hill, K. K., Guzman, E. & Jackson, P. J. (1996) Plant Physiol. 112, 659–667
14. Smith, I. K. & Thompson, J. F. (1971) Biochim. Biophys. Acta 227, 288–295
15. Ruffet, M. L., Droux, M. & Douce, R. (1994) Plant Physiol. 104, 697–704
16. Nakamura, K., Hayama, A., Masada, M., Fukushima, K. & Tamura, G. (1988) Plant Cell Physiol. 29, 689–693
17. Nakamura, K., Hayama, A., Masada, M., Fukushima, K. & Tamura, G. (1988) Agric. Biol. Chem. 54, 649–656
18. Brunold, C. (1993) in Sulfur Nutrition and Assimilation in Higher Plants-Regulatory Agricultural and Environmental Aspects (De Kok, L. J., Stulen, I., Rennenberg, H., Brunold, C., and Rauser, W. E., eds) pp. 61–75, SPB Academic Publishing, The Hague, Netherlands
19. Rennenberg, H. (1983) Physiol. Plant. 73, 560–565
20. Neuenhawener, U., Suter, M. & Brunold, C. (1991) Plant Physiol. 97, 295–301
21. Saito, K., Kurusawa, M., Tatsuguchi, K., Takagi, Y. & Murakoshi, I. (1994) Plant Physiol. 106, 887–895
22. Smith, F. W., Hawkesford, M. J., Ealing, P. M., Clarkson, D. T., Vanden Berg, P. J., Belcher, A. R. & Warrillow, A. G. S. (1997) Plant J. 12, 875–884
23. Saito, K., Yokoyama, H., Noji, M. & Murakoshi, I. (1995) J. Biol. Chem. 270, 16321–16326
24. Rennenberg, H. (1982) Phytochemistry 21, 2771–2781
25. Bogdanova, N., Bork, C. & Hell, R. (1995) FEBS Lett. 358, 43–47
26. Murillo, M., Foglia, R., Diller, A., Lee, S. & Leustek, T. (1995) Cell. Mol. Biol. Res. 41, 425–433
27. Hell, R. & Bogdanova, N. (1995) Plant Physiol. 109, 1498
28. Roberts, M. A. & Wray, J. L. (1996) Plant Mol. Biol. 30, 1041–1049
29. Howarth, J., Roberts, M. A. & ray, J. L. (1997) Biochym. Biophys. Acta 1350, 123–127
30. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory, New York
31. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorf, J. W. (1990) Methods Enzymol. 185, 60–89
32. Laemmli, U. K. (1970) Nature 227, 680–683
33. Baecker, P. & Wedding, R. T. (1980) Anal. Biochem. 102, 16–21
34. Chiu, W. L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. & Sheen, J. (1996) Curr. Biol. 6, 325–330
35. Krebs, E., Seutinck, J., Herdies, L., Cashmore, A. R. & Timko, M. P. (1988) Plant Mol. Biol. 11, 745–759
36. Saito, K., Tatsuguchi, K., Takagi, Y. & Murakoshi, I. (1994) J. Biol. Chem. 269, 28187–28192
37. Takahashi, H. & Saito, K. (1996) Plant Physiol. 112, 273–280
38. Kredich, N. M. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhart, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E., eds) Second Ed., Vol. 1, pp. 514–527, American Society for Microbiology Press, Washington, D. C.
39. Kredich, N. M. & Tomkins, G. M. (1966) J. Biol. Chem. 241, 4955–4965
40. Kim, H., Awazuhara, M., Hayashi, H., Chino, M. & Fujitawa, T. (1997) in Sulphur Metabolism in Higher Plants-Molecular, Ecophysiological and Nutritional Aspects (Cram, W. J., De Kok, L. J., Stulen, I., Brunold, C. and Rennenberg, H. eds) pp. 307–399, Backhuys Publishers, Leiden, The Netherlands
41. Rolland, N., Droux, M. & Douce, R. (1995) Arch. Biochem. Biophys. 316, 585–595
42. Droux, M., Ravelan, S. & Douce, R. (1995) Arch. Biochem. Biophys. 316, 379–397
43. Engler, J. A., Engler, G., Van Montagu, M. & Saito, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11162–11167

Arabidopsis Serine Acetyltransferase

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Isoform-dependent Differences in Feedback Regulation and Subcellular Localization of Serine Acetyltransferase Involved in Cysteine Biosynthesis from *Arabidopsis thaliana*

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