Amino Acids (2006) 30: 163–171
DOI 10.1007/s00726-005-0253-2

Amino Acids
Printed in Austria

In silico assessment of gene function involved in cysteine biosynthesis in Arabidopsis: expression analysis of multiple isoforms of serine acetyltransferase

M. Noji[1], **C. Goulart Kawashima**[1,*], **T. Obayashi**[1,2], and **K. Saito**[1,2,3]

[1] Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, Inage-ku, Chiba, Japan
[2] Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Japan
[3] RIKEN Plant Science Center, Tsurumi-ku, Yokohama, Japan

Received May 13, 2005
Accepted July 5, 2005
Published online March 10, 2006;

Summary. In plants, the inorganic sulfur is first fixed into cysteine by the cysteine biosynthetic pathway. This biosynthetic pathway of cysteine involves several enzymatic reactions. In *Arabidopsis thaliana*, multiple isoforms seem to participate in each enzymatic step for cysteine biosynthesis. To obtain more insights on the specific role of each isoform involved in the cysteine biosynthesis, *in silico* analysis of these isoforms using Arabidopsis expressed sequence tags (EST) database was carried out. This EST database analysis revealed distinct population distribution of ESTs among multiple isoforms, suggesting that each isoform has its particular expression pattern, presumably associated with its specific role in cysteine biosynthesis. As another *in silico* analysis, co-expression analysis of genes involved in sulfur metabolism in Arabidopsis was performed using a public transcriptome database of DNA microarrays. This co-expression analysis also suggested specific function and co-regulation of some isoform genes for cysteine biosynthesis by consideration on the clustering of co-expressed genes. From the results of sensitivity to feedback regulation, subcellular localization and expression of mRNA analyses, each serine acetyltransferase (SATase) isoform seems to have its specific role for cysteine biosynthesis. Similar expression patterns were observed between the experimental results of expression data for SATase isoforms and the *in silico* results of "digital northern" analysis using EST database.

Keywords: Cysteine biosynthesis – Serine acetyltransferase – *In silico* – Expressed sequence tags – Co-expression analysis

Abbreviations: APS = adenosine 5′-phosphosulfate, SATase = serine acetyltransferase, ESTs = Expressed sequence tags, OAS = *O*-acetyl-L-serine, PAPS = 3′-phosphoadenosine 5′-phosphosulfate

Introduction

L-Cysteine is one of the sulfur-containing amino acids in the twenty standard amino acids found in proteins. In plants, the inorganic sulfur, which is an essential nutrition for growth of plants, is first fixed into cysteine by the cysteine biosynthetic pathway (Leustek and Saito, 1999; Saito, 2000, 2004). Cysteine is then incorporated into proteins and glutathione, or serves as the sulfur donor of methionine and sulfur-containing secondary products in plants. This biosynthetic pathway of cysteine involves several enzymatic reactions (Fig. 1). The cysteine biosynthesis in plants consists of two biochemical pathways: the pathway of the activation and reduction of sulfate into sulfide, and the pathway supplying amino acid moiety, which is derived from serine through *O*-acetyl-L-serine (OAS), and then yielding cysteine by the reaction of incorporating sulfide moiety into β-position of alanine.

Three enzymes, ATP sulfurylase, adenosine 5′-phosphosulfate (APS) reductase and sulfite reductase, are committed in the reduction of sulfate into sulfide. This reduction pathway takes place almost exclusively in chloroplasts. On the other hand, two enzymes, serine acetyltransferase (SATase) and cysteine synthase are involved in the final step of cysteine biosynthesis. This final step of cysteine biosynthesis exists in three major compartments of plant cells, cytosol, chloroplasts, and mitochondria. The presence of SATase (Smith, 1972; Ascano and Nicholas, 1977; Brunold and Suter, 1982; Ruffet et al., 1995) and cysteine synthase (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.

* Present address: School of Biological Sciences, University of East Anglia, Norwich NR47TJ, U.K.

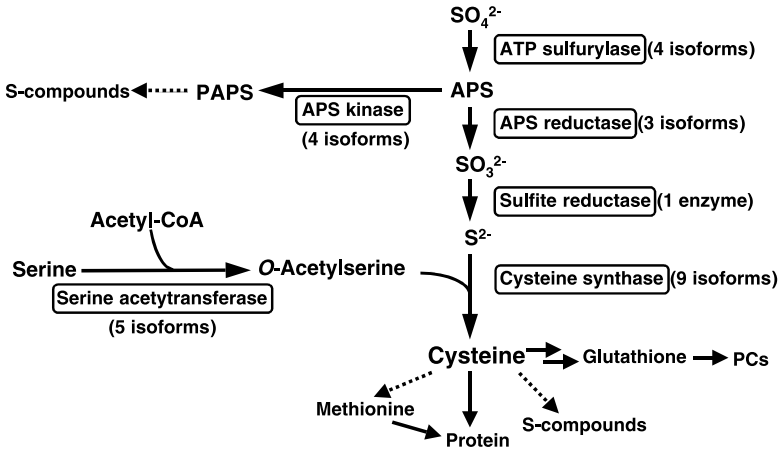

Fig. 1. Biosynthetic pathway for cysteine in plants. Indicated numbers of isoforms participate in cysteine biosynthesis in *Arabidposis thaliana*. *APS*, adenosine 5′-phosphosulfate; *PAPS*, 3′-phosphoadenosine 5′-phosphosulfate; *PCs*, phytochelatins

Thus, many enzymes participate in the cysteine biosynthetic pathway in higher plants. Completion of genome sequencing (Arabidopsis Genome Initiative, 2000) allowed identification of all members of the genes concerned with the cysteine biosynthesis in the Arabidopsis genome. In contrast to bacteria, multiple isoforms, except for sulfite reductase, seem to be involved in each enzymatic step of cysteine biosynthetic pathway in Arabidopsis (Fig. 1). These multiple isoforms may be necessary for higher plants to respond to the environmental change for plant growth and/or the distinct demands for reduced sulfur in each organ or each intracellular compartment. Therefore, it is speculated that each isoform has a distinct tissue-specific and/or stress-inducible expression pattern, a distinct subcellular localization and/or enzymatic properties. Studies on differential roles among these isoforms are important for understanding the regulation of the cysteine biosynthesis in higher plants.

To clarify the specific role of each isoform involved in the cysteine biosynthesis, *in silico* analysis using expressed sequence tags (ESTs) database may provide some useful initial insights. At the present day (April 15, 2005), 418,564 ESTs from Arabidopsis, which are derived from libraries from different organs and plants treated with various stress, are registered in available databases. Because of this abundance of Arabidopsis ESTs, it is anticipated that the analysis of Arabidopsis ESTs databases will provide some clues as to possible physiological functions of multiple isoforms of cysteine biosynthetic enzymes. Such EST analyses have been applied in studies as diverse as toxicology (Fielden et al., 2002), inner-ear function (Klockars et al., 2003), and plant metabolic pathways (Allona et al., 1998; Strerky et al., 1998; White et al., 2000; Hertzberg et al., 2001; Costa et al., 2003).

As an alternative approach of *in silico* analysis, cross-experiment transcript co-expression analysis may describe the functional character of each isoform genes of cysteine biosynthetic enzymes. Co-expression analysis is based on the premise that common transcriptional control of genes should be reflected in synchronous changes in transcript level. This analysis demonstrates common change of transcript levels among gene pairs.

In the present report, we describe *in silico* analysis of multiple isoforms involved in the cysteine biosynthesis in Arabidopsis using EST database and transcript co-expression analysis. Furthermore, comprehensive analysis about SATase isoforms of Arabidopsis from the viewpoint of its expression pattern and enzymatic property was carried out. By comparing the results of expression analyses of SATase isoforms and the result of "digital northern" analysis using EST database, the usefulness of *in silico* analysis was examined.

In silico analysis of EST database for the genes involved in cysteine biosynthesis

We investigated Arabidopsis ESTs from The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/), and counted the number of ESTs of each isoform involved in the cysteine biosynthesis. ESTs were categorized by expressing organs and by growth condition of plants (Table 1). ESTs from the libraries derived from plants treated with various stress (dry, cold, NaCl, heat, and UV), and hormone (abscisic acid, auxin, ethylene, jasmonic acid, salicylic acid, gibberellic acid, and cytokinin) (Seki et al., 2002) were categorized as stressed plant in Table 1.

In the analysis of EST database like these, it is assumed that the population of ESTs implies the expression levels of each isoform gene. EST database analysis of all isoform genes involved in the cysteine biosynthesis in Arabidopsis revealed distinct population distribution of

Table 1. ESTs analysis of all isoforms involved in cysteine biosynthesis in Arabidopsis

Name	Gene name	Locus name	Total number of EST	Number of ESTs from						
				Leaf	Root	Mix	Silique and flower	Developing seed	Dark-grown plant	Stressed plant
ATP sulfurylase	*APS1*	At3g22890	61	7	5	3	11	1	2	32
	APS2	At1g19920	18	3	2	3	4	0	2	4
	APS3	At4g14680	8	4	2	1	1	0	0	0
	APS4	At5g43780	92	5	26	20	5	0	18	18
APS kinase	*akn*	At2g14750	10	1	4	1	1	0	0	3
	akn2	At4g39940	23	6	9	3	3	0	0	2
		At3g03900	0	0	0	0	0	0	0	0
		At5g67520	1	0	0	0	1	0	0	0
APS reductase	*APR1*	At4g04610	57	3	29	2	13	0	1	9
	APR2	At1g62180	43	9	6	5	11	1	0	11
	APR3	At4g21990	20	3	0	4	3	0	0	10
Sulfite reductase	*sir*	At5g04590	40	4	12	6	4	8	0	6
Serine acetyltransferase	*Serat1;1*	At5g56760	33	3	13	3	7	0	4	3
	Serat2;1	At1g55920	17	2	3	7	0	0	1	4
	Serat2;2	At3g13110	33	5	2	6	8	0	1	11
	Serat3;1	At2g17640	4	0	0	2	1	0	0	1
	Serat3;2	At4g35640	22	0	0	0	12	0	0	10
Cysteine synthase	*Bsas1;1*	At4g14880	151	8	26	24	33	2	18	40
	Bsas1;2	At3g22460	3	0	1	1	0	0	0	1
	Bsas2;1	At2g43750	45	12	4	5	6	0	0	18
	Bsas2;2	At3g59760	29	0	14	4	3	0	0	8
	Bsas3;1	At3g61440	78	14	12	12	15	0	8	17
	Bsas4;1	At5g28020	18	2	1	2	6	0	2	5
	Bsas4;2	At3g04940	9	0	0	4	0	0	1	4
	Bsas4;3	At5g28030	2	1	0	1	0	0	0	0
	Bsas5;1	At3g03630	4	0	0	2	0	0	0	2

ESTs among multiple isoforms (Table 1), suggesting that each isoform has its particular expression pattern presumably associated with its specific role in cysteine biosynthesis.

In the case of ATP sulfurylase, *APS4* (Hatzfeld et al., 2000a) showed characteristic expression patterns by digital northern analysis (Table 1). The number of ESTs of *APS4* from root and dark-grown plant was much larger than that of other ATP sulfurylase isoform genes, suggesting *APS4* is highly expressed in root and in dark-grown plant. Though the activity of ATP sulfurylase has been detected in chloroplasts and cytosol of plants (Lunn et al., 1990; Renosto et al., 1993; Klonus et al., 1994; Rotte and Leustek, 2000), all of four ATP sulfurylase genes likely encode plastidic isoforms (Hatzfeld et al., 2000a). Hence the identification and the significance of cytosolic isoform of ATP sulfurylase in Arabidopsis are still unclear. One of the SATase isoform genes, *Serat1;1*, and one of the cysteine synthase isoform genes, *Bsas1;1*, were also highly expressed both in root and in dark-grown plant (Table 1), which is an expression pattern similar to that of *APS4*. Both Serat1;1 and Bsas1;1 are major isoforms in each enzymatic reaction (Dominguez-Solis et al., 2001; Kawashima et al., 2005), and their subcellular localization is cytosol (Noji et al., 1998; Jost et al., 2000). APS4 might be a candidate for cytosolic ATP sulfurylase from the viewpoint of expression pattern by digital northern analysis, even though its nucleotide sequence of N-terminal region obviously encodes a signal peptide to chloroplasts (Hatzfeld et al., 2000a). In any case, the distinct expression pattern of *APS4* observed here suggests a role of APS4 in cysteine synthesis different from those of other ATP sulfurylase isoforms.

Among four isoforms of APS kinase, akn and akn2 seem to be major isoforms, because almost none of the ESTs of other two isoform genes were obtained. APS, which is produced by ATP sulfurylase, is further reduced by APS reductase or phosphorylated by APS kinase. APS kinase catalyzed the formation of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). PAPS serves as a source of activated sulfate for sulfotransferases that catalyzes sulfation of a variety of compounds such as flavonoids, glucosinolates, and jasmonates. At the branch point to cysteine synthesis or to sulfation reaction, APS kinase competes with APS reductase for APS as a substrate for phosphorylation or reduction. In fact, under sulfate-starved conditions, since the cells primarily need to produce cysteine, induction of APS reductase gene expression was observed in Arabidopsis, but not of APS kinase (Takahashi et al., 1997). The total number of ESTs of APS kinase was relatively small as compared to the total number of ESTs of APS reductase (Table 1), suggesting that the further reduction of APS into sulfite by APS reductase for cysteine synthesis is dominant over the PAPS synthesis by APS kinase for sulfation in Arabidopsis cells.

Though cysteine synthase makes a protein complex with SATase (Hell et al., 2002), the cellular activity of cysteine synthase is much higher than that of SATase (Ruffet et al., 1994). The population of ESTs of cysteine synthase also exhibited the abundance of cysteine synthase in cells in comparison with that of SATase (Table 1). Bsas1;1, Bsas2;1, and Bsas2;2 are known as major isoforms of cysteine synthase localized in different subcellular compartment, cytosol, plastid, and mitochondria, respectively (Hesse et al., 1999; Jost et al., 2000). *In silico* analysis also indicated that these three isoforms were major in 9 cysteine synthase isoforms (Table 1). From the result of the EST analysis, the expression level of *Bsas3;1*, one of the cysteine synthase-like isoforms, seems to be relatively higher compared with other cysteine synthase isoforms, however, Bsas3;1 is responsible for β-cyanoalanine formation in mitochondria as β-cyanoalanine synthase rather than cysteine synthase (Hatzfeld et al., 2000b).

Distinct population distribution of ESTs among five SATase isoforms was demonstrated in Table 1, also implying their different function for cysteine synthesis. Further consideration of SATase isoforms were mentioned in the following sections.

In silico co-expression analysis by transcriptome data

As another approach of *in silico* analysis to define the particular function of each isoform of cysteine biosynthetic enzymes, cross-experiment transcript co-expression analysis of genes involved in the metabolic pathway of sulfur-containing compounds was done using a publicly available transcriptome database, AtGenExpress (http://pfg.psc.riken.jp/AtGenExpress/ and http://www.weigelworld.org/resources/microarray/AtGenExpress/), and a co-expression database, ATTED (http://www.atted.bio.titech.ac.jp/ or http://prime.psc.riken.jp/). Positive correlations ($r > 0.6$) of transcript levels among 57 genes concerning metabolic pathway of cysteine, glutathione, phytochelatins, methionine and glucosinolates were analyzed using 298 data sets of 22 k Affymetrix ATH1 GeneChips under stress conditions in AtGenExpress. Twenty-four out of 57 genes exhibited a positive correlation of transcript levels among gene pairs (Fig. 2). As shown in Fig. 2, *SULTR4;1*, *SULTR4;2*, *APS1*, *APS3*,

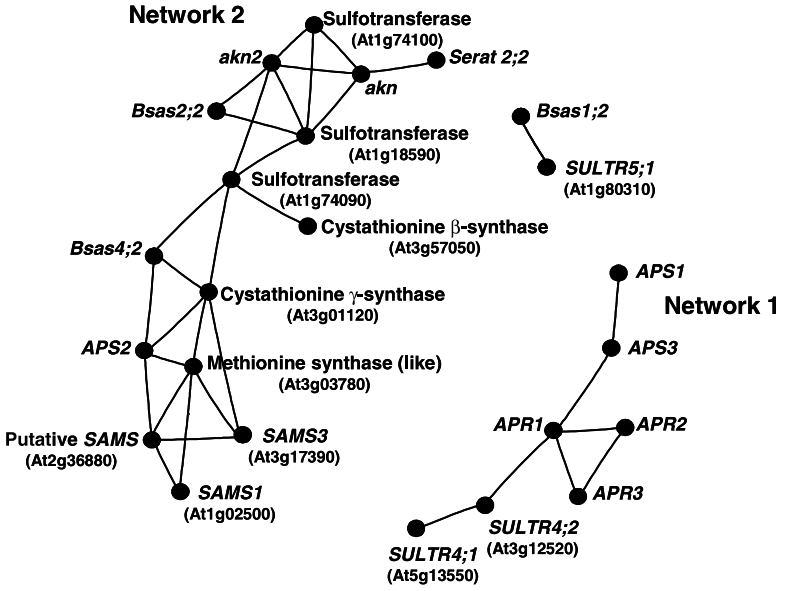

Fig. 2. Transcript co-expression analysis of genes involved in the metabolic pathway of sulfur-containing compounds. Combinations of positive correlation ($r > 0.6$) of transcript levels between gene pairs are shown as lines. Calculation of co-expression was carried out as described in ATTED (http://www.atted.bio.titech.ac.jp/ or http://prime.psc.riken.jp/) using the subset database of stress condition. The network 1 contains 7 genes, and the network 2 contains 15 genes. The network visualization was done using the Pajek program for large network analysis (http://vlado.fmf.uni-lj.si/pub/network/pajek/pajekman.htm). *SULTR*, sulfate transporter; *SAMS*, *S*-adenosylmethionine synthetase

and all three isoform genes of APS reductase showed a positive correlation of transcript levels under stress conditions (Network 1 in Fig. 2). SULTR4;1 and 4;2 are vacuolar sulfate transporters which facilitate the efflux of sulfate from the vacuoles and plays critical roles in optimizing the internal distribution of sulfate in Arabidopsis (Kataoka et al., 2004). This positive co-expression network suggests that the initial stages of cysteine biosynthesis in cells, i.e., supply of sulfate from vacuole by SULTR4-type transporter, activation of sulfate to APS by ATP sulfurylase, and reduction of APS by APS reductase, are subjected to the common transcriptional control of genes at least under stress conditions. APS1 and APS3, but not APS2 and APS4, are likely to participate in the sulfate activation for cysteine biosynthesis under stress conditions. Another positive co-expression network (Network 2 in Fig. 2) contains 15 genes for glucosinolates synthesis, e.g., 3 sulfotransferases (Hirai et al., 2005), and methionine metabolism, etc. Two APS kinase isoform genes, *akn* and *akn2*, which synthesize PAPS for sulfation reaction of compounds such as glucosinolates, also co-expressed with the genes clustered in network 2. Network 1 in Fig. 2 seems to be composed of the primarily important genes for the regulation of cysteine biosynthesis, whereas the genes clustered in network 2 are likely to engage indirectly in the cysteine biosynthesis. It is interesting that the genes for Serat2;2 and Bsas2;2, both localized in mitochondria, and the *APS2* gene clustered in the network 2, suggesting APS2 and mitochondrial SATase (Serat2;2) and cysteine synthase (Bsas2;2) may be apart from the co-regulation system of cysteine biosynthetic genes in Network 1.

Enzymatic properties and subcellular localization of SATase isoforms from Arabidopsis

SATase, which catalyzes the formation of OAS from serine and acetyl-CoA, is placed at the entry step from serine metabolism to cysteine biosynthesis. In the Arabidopsis genome, five genes (*Serat1;1*, *Serat2;1*, *Serat2;2*, *Serat3;1*, and *Serat3;2*) putatively encode SATase. The five *Serat* genes of Arabidopsis are each located on different chromosomes. All five SATase isoforms encoded by *Serat* genes were characterized regarding different sensitivity to cysteine feedback regulation, subcellular localization (Noji et al., 1998; Kawashima et al., 2005) (Table 2).

The activity of cytosolic SATase (Serat1;1) was inhibited by L-cysteine at a physiological concentration in an allosteric manner, but the plastidic (Serat2;1) and mitochondrial (Serat2;2) forms were not subject to this feedback regulation. The cytosolic Serat1;1 regulates the OAS concentration strictly by the feedback inhibition by cysteine, since OAS is not only the key intermediate of cysteine formation but also regarded as a regulatory molecule of the whole sulfur assimilation pathway (Leustek et al., 2000; Saito, 2000, 2004). In chloroplasts, however, high OAS levels have to be maintained even at high cysteine concentrations for the biosynthesis of methionine and glutathione by feedback-insensitive SATase isoforms in this compartment (Noji et al., 1998). Cytosolic localization of Serat3;1 and Serat3;2 was indicated by the analyses of the GFP fusion proteins directed by the transit peptides of these SATases. Surprisingly, Serat3;2 and Serat3;1 showed contrasting properties with respect to sensitivity of cysteine feedback inhibition (Table 2). Both

Table 2. Kinetic constants of recombinant Arabidopsis Serat proteins

Isoforms	Subcellular localization	K_m [mM]		Feedback inhibition by L-Cys	Inhibition (K_i) by L-Cys [μM]	
		L-Ser	Acetyl-CoA		L-Ser	Acetyl-CoA
Serat1;1	cytosol	2.71	0.28	sensitive	10.8 (noncompetitive)	7.4 (competitive)
Serat2;1	chloroplasts	1.64	0.16	insensitive	–	–
Serat2;2	mitochondria	1.68	0.02	insensitive	–	–
Serat3;1	cytosol	121.4	24.5	insensitive	–	–
Serat3;2	cytosol	39.5	45.1	sensitive	17.3 (noncompetitive)	2.5 (competitive)

–, No inhibition by L-cysteine

Serat3;1 and Serat3;2 have low substrate affinities (Table 2), suggesting low enzyme activities in vivo. From these findings, a minor role of these two isoforms in flux regulation towards cysteine synthesis may be inferred.

These results leave open the possibility that the OAS-producing activities of Serat3;1 and Serat3;2 might be side activities of those proteins that may have another unknown function(s) similar to cysteine synthase and β-cyanoalanine synthase (Hatzfeld et al., 2000b; Warrilow and Hawkesford, 2000).

Developmental and stress-inducible expression of SATase genes

Expression of mRNA during development and under stress was characterized about five SATase isoforms. The mRNA abundance of the SATase genes was examined by Northern blot analysis of total RNA from 3-week-old leaves (Fig. 3). A high level of expression was observed for *Serat 1;1*, *Serat2;1*, and *Serat2;2*. In contrast, the expression of *Serat3;1* and *Serat3;2* was low, indicating distinct expression patterns among the *Serat* genes.

Because of the low expression of *Serat3;1* and *Serat3;2*, further analysis was conducted with the more sensitive method of real-time quantitative PCR. *Serat1;1*, *Serat2;1*, and *Serat2;2* expressed higher amounts of mRNA (approximately 10-fold) compared with *Serat3;1* and *Serat3;2*, consistent with the results of Northern blotting. Among them, *Serat2;1* was the dominant form in most tissues examined, followed by *Serat1;1* and *Serat2;2*. Expression analysis of the five *Serat* genes revealed the gene-specific unique regulation during the course of plant development (Fig. 4). *Serat* genes exhibited two types of expression patterns during plant development. The three major genes, *Serat1;1*, *Serat2;1* and *Serat2;2*, had a similar trend of expression increasing until 3 weeks, whereas *Serat3;1* and *Serat3;2* showed an opposite pattern of expression increasing even in the reproductive stage (5–6 weeks). Expression of *Serat* genes is therefore switched from group 1 (*Serat1;1*, *Serat2;1* and *Serat2;2*) to group 2 (*Serat3;1* and *Serat3;2*) by the developmental transition from the vegetative stage to the reproductive stage, for provision of OAS and subsequently cysteine in parallel to the export of cysteine and glutathione from source tissues to the growing shoots and seeds.

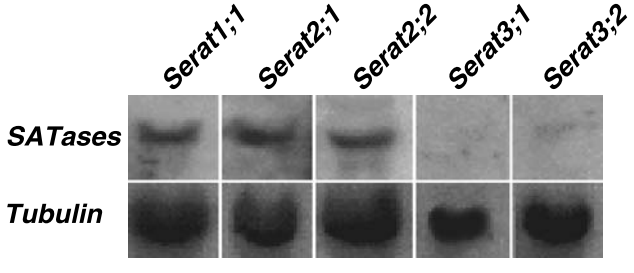

Fig. 3. Northern blot analysis of SATase mRNA from 3-week-old rosette leaves in Arabidopsis. 20 μg of total RNA was separated under denatured conditions on 1.2% (w/v) agarose gel containing formaldehyde, and transferred to a Hybond N^+ membrane, and then probed with a ^{32}P-labelled cDNA clone. The final wash was performed in 0.1 × SSPE, 0.1%SDS at 65 °C for 10 min

Expression of each *Serat* gene was further analyzed under the stress conditions of sulfur nutritional deficiency and cadmium exposure. Three-week-old Arabidopsis plants were transferred onto sulfur-deficient medium or medium containing $CdCl_2$ (50 μM). After 12 and 96 h treatment of these stresses, real-time quantitative PCR analysis was performed using 5 ng of total RNA isolated from stress-treated plants. Under sulfur-starved conditions, the expression of *Serat3;2* was induced in both roots and shoots, indicating a special role of *Serat3;2* under sulfur starvation (Table 3). In addition, *Serat3;1* expression was induced after long-term sulfur starvation. No induction was observed with the other genes. It is known that OAS levels increase by sulfur starvation (Kim et al., 1999). The induced expression of *Serat3;1* and *Serat3;2* can partially contribute to the increasing level of OAS by

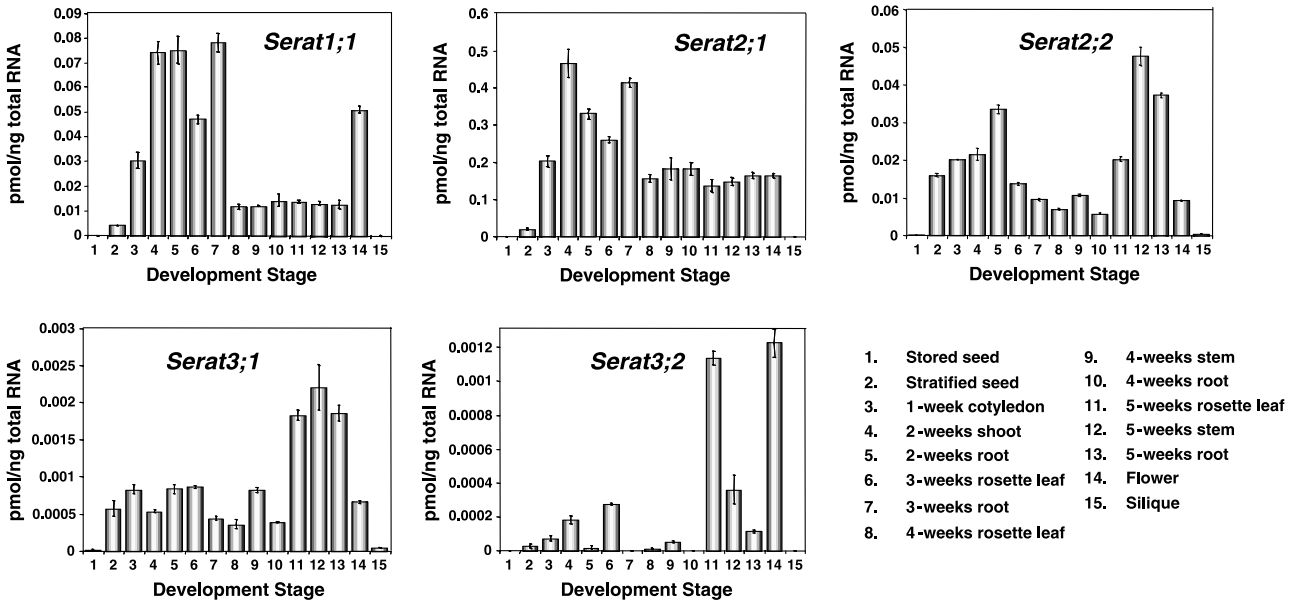

Fig. 4. Real-time quantitative PCR analysis of *Serat* mRNA accumulation during development. Total RNA (5 ng) isolated from stored seeds, stratified seeds, cotyledons, leaves, stems, roots, siliques, and flower tissues was used for amplification. Data are the means of triplicate determinations ± SD

Table 3. Relative fold-change of *Serat* mRNA accumulation under stress conditions

Name	Sulfur deficiency				Cadmium treatment			
	Leaf		Root		Leaf		Root	
	12 h	96 h	12 h	96 h	12 h	96 h	12 h	96 h
Serat1;1	0.69 ± 0.06	0.90 ± 0.07	0.86 ± 0.08	1.20 ± 0.06	0.91 ± 0.05	1.19 ± 0.03	2.51 ± 0.38	3.00 ± 0.11
Serat2;1	0.60 ± 0.03	0.81 ± 0.05	0.87 ± 0.08	0.86 ± 0.11	1.01 ± 0.04	1.01 ± 0.04	2.37 ± 0.33	2.09 ± 0.26
Serat2;2	0.99 ± 0.03	0.83 ± 0.03	0.92 ± 0.04	0.94 ± 0.03	0.93 ± 0.08	1.22 ± 0.15	2.54 ± 0.17	2.55 ± 0.29
Serat3;1	0.89 ± 0.02	1.02 ± 0.05	1.03 ± 0.01	2.03 ± 0.09	1.00 ± 0.09	1.24 ± 0.03	1.42 ± 0.04	0.98 ± 0.08
Serat3;2	6.51 ± 0.36	39.8 ± 0.22	7.24 ± 0.43	44.8 ± 0.27	12.0 ± 0.31	3.02 ± 0.28	4.01 ± 1.75	2.55 ± 0.32

sulfur depletion. Similar trends of induction of gene expression were seen by Cd stress (Table 3). *Serat3;2* responded to Cd treatment both in roots and shoots, indicating again the particular function of *Serat3;2* during the stress response. It may be speculated that the strong induction of the *Serat3;2* gene compensates for the low catalytic efficiency of the encoded Serat3;2 protein, thus contributing significantly to the flux of cysteine synthesis towards glutathione and ultimately to phytochelatins for Cd complexation. The other *Serat* genes were slightly induced in the roots. Increased heavy metal tolerance was confirmed in the transgenic plants overexpressing the cysteine synthase gene (Dominguez-Solis et al., 2001; Kawashima et al., 2004). These studies suggest that the increased cysteine and glutathione contents in these transgenic plants are likely to be responsible for tolerance to heavy metals. Since *Serat3;2* is inducible by Cd, a more detailed investigation on the induction and mechanism of tolerance would be applicable for further study and engineering of Cd resistance in plants.

Comparison between expression analysis and "digital northern" analysis about SATase isoforms

To verify the usefulness of *in silico* digital northern analysis using EST databases for the speculation of specific roles of multiple isoforms involved in cysteine biosynthesis, we compared the result of expression analysis and with the result of digital northern analysis. A high level of expression of *Serat 1;1*, *Serat2;1*, and *Serat2;2*, and a low expression of *Serat3;1* and *Serat3;2* were observed

by Northern blot analysis using total RNA isolated from 3-week-old leaves (Fig. 3). From the result of digital northern analysis (Table 1), the expression level of *Serat3;2* seems to be higher, since the total number of EST of *Serat3;2* is 22. Nevertheless, these 22 ESTs of *Serat3;2* were obtained from siliques and flowers and from stressed plants only. EST clones of *Serat3;1* and *Serat3;2* scarcely exist in vegetative tissues (leaf, root, and mix in Table 1), confirming the result of northern blot analysis (Fig. 3). *Serat3;2* showed the expression at low levels in young stages and increased expression at the late stage after 5 weeks (Fig. 4). This increased expression of *Serat3;2* at the late stage, especially in flower, was also indicated as the EST expression in siliques and flowers (Table 1).

By the expression analysis of SATase isoforms under sulfur deficiency and cadmium exposure condition, *Serat3;2* seems to be a stress-inducible gene (Table 3), which is suggested by the *in silico* EST analysis. Although the stress treatment was different from sulfur deficiency and cadmium exposure, *Serat3;2* expression was clearly induced in stress-treated plants (Table 1). The digital northern analysis using EST database seems to provide some useful initial information concerning possible physiological functions of multiple isoforms of cysteine biosynthetic enzymes, especially about the isoform like Serat3;2, whose expression pattern is distinctive compared to other isoforms. On the basis of initial information from the EST database analysis, further investigation, for instance, knock-out mutant analysis, should be conducted in order to identify the specific function of each isoform involved in cysteine biosynthesis.

Acknowledgments

This research was supported in part by Core Research for Evolutional Science and Technology of Japan Science and Technology Agency, by Grants-in-Aids from the Ministry of Education, Science, Culture, Sports and Technology, Japan.

References

Allona I, Quinn M, Shoop E, Swope K, St Cyr S, Carlis J, Riedl J, Retzel E, Campbell MM, Sederoff R, Whetten RW (1998) Analysis of xylem formation in pine by cDNA sequencing. Proc Natl Acad Sci USA 95: 9693–9698

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815

Ascano A, Nicholas DJD (1977) Purification and properties of *O*-acetyl-L-serine sulphydrylase from wheat leaves. Phytochemistry 16: 889–893

Brunold C, Suter M (1982) Intracellular localization of serine acetyltransferase in spinach leaves. Planta 155: 321–327

Brunold C, Suter M (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. Planta 179: 228–234

Costa MA, Collins RE, Anterola AM, Cochrane FC, Davin LB, Lewis NG (2003) An *in silico* assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in *Arabidopsis thaliana* and limitations thereof. Phytochemistry 64: 1097–1112

Dominguez-Solis JR, Gutierrez-Alcala G, Romero LC, Gotor C (2001) The cytosolic *O*-acetylserine (thiol) lyase gene is regulated by heavy-metals and can function in cadmium tolerance. J Biol Chem 276: 9297–9302

Droux M, Martin J, Sajus P, Douce R (1992) Purification and characterization of *O*-acetylserine (thiol) lyase from spinach chloroplasts. Arch Biochem Biophys 295: 379–390

Fielden MR, Matthews JB, Fertuck KC, Halgren RG, Zacharewski TR (2002) *In silico* approaches to mechanistic and predictive toxicology: an introduction to bioinformatics for toxicologists. Crit Rev Toxicol 32: 67–112

Hatzfeld Y, Lee S, Lee M, Leustek T, Saito K (2000a) Functional characterization of a gene encoding a fourth ATP sulfurylase isoforms from *Arabidopsis thaliana*. Gene 248: 51–58

Hatzfeld Y, Maruyama A, Schmidt A, Noji M, Saito K (2000b) β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and *Arabidopsis thaliana*. Plant Physiol 123: 1163–1171

Hell R, Jost R, Berkowitz O, Wirtz M (2002) Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. Amino Acids 22: 245–257

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlen M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G (2001) A transcriptional roadmap to wood formation. Proc Natl Acad Sci USA 98: 14732–14737

Hesse H, Lipke J, Altmann T, Hoefgen R (1999) Molecular cloning and expression analyses of mitochondrial and plastidic isoforms of cysteine synthase (*O*-acetylserine(thiol)lyase) from *Arabidopsis thaliana*. Amino Acids 16: 113–131

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem 280: 25590–25595

Jost R, Berkowitz O, Wirtz M, Hopkins L, Hawkesford MJ, Hell R (2000) Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene 253: 237–247

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. Plant Cell 16: 2693–2704

Kawashima CG, Noji M, Nakamura M, Ogra Y, Suzuki KT, Saito K (2004) Heavy metal tolerance of transgenic tobacco plants over-expressing cysteine synthase. Biotech Lett 26: 153–157

Kim H, Hirai MY, Hayashi H, Chino M, Naito S, Fujiwara T (1999) Role of *O*-acetyl-L-serine in the coordinated regulation of the expression of a soybean seed storage-protein gene by sulfur and nitrogen nutrition. Planta 209: 282–289

Klockars T, Perheentupa T, Dahl H-HM (2003) In silico analyses of mouse inner-ear transcripts. J Assoc Res Otolaryngol 4: 24–40

Klonus D, Hoefgen R, Willmitzer L, Riesmeier J (1994) Isolation and characterization of two cDNA clones encoding ATP-sulfurylases from potato by complementation of a yeast mutant. Plant J 6: 105–112

Kuske CR, Hill KK, Guzman E, Jackson PJ (1996) Subcellular location of *O*-acetylserine sulfhydrylase isoenzymes in cell cultures and plant tissues of *Datura innoxia* mill. Plant Physiol 112: 659–667

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. Plant Physiol 120: 637–643

Leustek T, Martin MN, Bick J-A, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51: 141–165

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP-sulfurylase and *O*-acetylserine (thiol) lyase in spinach leaves. Plant Physiol 94: 1345–1352

Noji M, Inoue K, Kimura N, Gouda A, Saito K (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine-acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J Biol Chem 273: 32739–32745

Rolland N, Droux M, Douce R (1992) Subcellular distribution of *O*-acetylserine (thiol) lyase in cauliflower (*Brassica oleracea* L.) inflorescence. Plant Physiol 98: 927–935

Renosto F, Patel H, Martin H, Thomassian C, Zimmerman G, Segel I (1993) ATP sulfurylase from higher plants: kinetic and structural characterization of the chloroplast and cytosol enzymes from spinach leaf. Arch Biochem Biophys 307: 272–285

Rotte C, Leustek T (2000) Differential subcellular localization and expression of ATP sulfurylase and 5′-adenylylsulfate reductase during ontogenesis of Arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. Plant Physiol 124: 715–724

Ruffet ML, Droux M, Douce R (1994) Purification and kinetic properties of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Plant Physiol 104: 597–604

Ruffet ML, Lebrun M, Droux M, Douce R (1995) Subcellular distribution of serine acetyltransferase from *Pisum sativum* and characterization of an *Arabidopsis thaliana* putative cytosolic isoform. Eur J Biochem 227: 500–509

Saito K (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr Opin Plant Biol 3: 188–195

Saito K (2004) Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol 136: 2443–2450

Seki M, Narusaka M, Kamiya A, Ishida J, Satou M, Sakurai T, Nakajima M, Enju A, Akiyama K, Oono Y, Muramatsu M, Hayashizaki Y, Kawai J, Carninci P, Itoh M, Ishii Y, Arakawa T, Shibata K, Shinagawa A, Shinozaki K (2002) Functional annotation of a full-length Arabidopsis cDNA collection. Science 296: 141–145

Smith IK (1972) Studies of L-cysteine biosynthetic enzymes in *Phaseolus vulgaris* L. Plant Physiol 50: 477–479

Sterky F, Regan S, Karlsson J, Hertzberg M, Rhode A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R, Van Montagu M, Sandberg G, Olsson O, Teeri TT, Boerjan W, Gustafsson P, Uhlen M, Sundberg B, Lundeberg J (1998) Gene discovery in the wood-forming tissues of poplar: analysis of 5692 expressed sequence tags. Proc Natl Acad Sci USA 95: 13330–13335

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, de Almeida Engler J, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 94: 11102–11107

Warrilow A, Hawkesford M (2000) Cysteine synthase (O-acetyl-serine (thiol) lyase substrate specificities classify the mitochondrial isoform as a cyanoalanine synthase. J Exp Bot 51: 985–993

White JA, Todd J, Newman T, Focks N, Girke T, Martinez de Ilarduya O, Jaworski JG, Ohlrogge JB, Benning C (2000) A new set of Arabidopsis expressed sequence tags from developing seeds: the metabolic pathway from carbohydrates to seed oil. Plant Physiol 124: 1582–1594

Yamaguchi T, Masada M (1995) Comparative studies on cysteine synthase isozymes from spinach leaves. Biochem Biophys Acta 1251: 91–98

Authors' address: Kazuki Saito, Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1–33, Inage-ku, Chiba, 263-8522, Japan,
Fax: +81 43 290 2905, E-mail: ksaito@faculty.chiba-u.jp