

# Comparative Genomics and Reverse Genetics Analysis Reveal Indispensable Functions of the Serine Acetyltransferase Gene Family in *Arabidopsis* <sup>WJ|OA</sup>

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**Ser acetyltransferase (SERAT), which catalyzes O-acetyl-Ser (OAS) formation, plays a key role in sulfur assimilation and Cys synthesis. Despite several studies on SERATs from various plant species, the in vivo function of multiple SERAT genes in plant cells remains unaddressed. Comparative genomics studies with the five genes of the SERAT gene family in *Arabidopsis thaliana* indicated that all three *Arabidopsis* SERAT subfamilies are conserved across five plant species with available genome sequences. Single and multiple knockout mutants of all *Arabidopsis* SERAT gene family members were analyzed. All five quadruple mutants with a single gene survived, with three mutants showing dwarfism. However, the quintuple mutant lacking all SERAT genes was embryo-lethal. Thus, all five isoforms show functional redundancy in vivo. The developmental and compartment-specific roles of each SERAT isoform were also demonstrated. Mitochondrial SERAT2;2 plays a predominant role in cellular OAS formation, while plastidic SERAT2;1 contributes less to OAS formation and subsequent Cys synthesis. Three cytosolic isoforms, SERAT1;1, SERAT3;1, and SERAT3;2, may play a major role during seed development. Thus, the evolutionally conserved SERAT gene family is essential in cellular processes, and the substrates and products of SERAT must be exchangeable between the cytosol and organelles.**

## INTRODUCTION

Sulfur plays a pivotal role in plant metabolism and development. Cys, as the first organic-reduced sulfur compound, contributes not only to life as building blocks in proteins, but it also serves as a precursor for the syntheses of Met, glutathione (GSH), cofactors, essential vitamins, sulfur esters, and other sulfur derivatives (Beinert, 2000; Leustek et al., 2000; Marquet, 2001; Gerber and Lill, 2002; Mendel and Hänsch, 2002; Noctor et al., 2002; Saito, 2004). Ser acetyltransferase (SERAT; EC 2.3.1.30), which catalyzes the formation of O-acetyl-Ser (OAS) from Ser and acetyl-CoA, links Ser metabolism to Cys biosynthesis. Subsequently, Cys is formed by the condensation of sulfide and OAS, catalyzed by OAS (thiol) lyase (OASTL; EC 4.2.99.8), which is classified within the  $\beta$ -substituted Ala synthase (BSAS) family (Leustek and Saito, 1999; Hatzfeld et al., 2000; Saito, 2004). In contrast with

sulfate reduction that occurs exclusively in the plastid, both SERAT and OASTL were found to be associated not only with plastids but also with the cytosol and mitochondria of plant cells (Lunn et al., 1990; Saito, 2000; Hell et al., 2002; Droux, 2003). The reason for the localization of SERAT and OASTL in three compartments where protein synthesis occurs has been conceived to be that the transport of Cys between cellular compartments may be limited (Lunn et al., 1990; Leustek and Saito, 1999; Saito, 2004). However, recent studies on *Arabidopsis thaliana* T-DNA knockout mutants of OASTL suggested that Cys or its derivatives must be exchangeable between the compartments and that the Cys biosynthesis in plant cells was under compartment-specific regulation (Heeg et al., 2008; Watanabe et al., 2008). In the regulation of Cys synthesis, SERAT also must play an important role since the Cys synthesis might be limited by the availability of OAS rather than sulfide (Saito et al., 1994; Takahashi and Saito, 1996; Blaszyk et al., 1999; Harms et al., 2000; Noji and Saito, 2002; Tsakraklides et al., 2002; Wirtz and Hell, 2003; Wirtz et al., 2004), and the cellular activity of OASTL exceeds that of SERAT, although the ratio of OASTL/SERAT activity differs with compartments (Ruffet et al., 1995; Saito, 2000; Hell et al., 2002; Droux, 2003; Heeg et al., 2008). In addition, two proposed mechanisms on the regulation of SERAT activity to maintain the OAS and Cys levels at a steady state in plant cells emphasize the significant role of SERAT: (1) feedback regulation on SERAT activity by Cys (Noji et al., 1998; Wirtz and Hell, 2003, 2006; Kawashima et al., 2005) and (2) modulation of SERAT activity

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through the formation of the Cys synthase complex with OASTL, the stability of which is regulated by sulfide and OAS (Kredich and Tomkins, 1966; Droux et al., 1998; Wirtz et al., 2004; Wirtz and Hell, 2006).

*SERAT* enzymes in *Arabidopsis* are encoded by a multigene family consisting of five genes (*SERAT1;1*, *SERAT2;1*, *SERAT2;2*, *SERAT3;1*, and *SERAT3;2*) (Arabidopsis Genome Initiative, 2000). Judging from the kinetic properties of recombinant proteins and gene expression levels, *SERAT1;1*, *SERAT2;1*, and *SERAT2;2*, which are localized in the cytosol, plastids, and mitochondria, respectively, seem to play major roles in OAS formation (Noji et al., 1998). Two minor forms, *SERAT3;1* and *SERAT3;2*, which are localized in the cytosol, have low substrate affinities and low gene expression levels (Kawashima et al., 2005). These findings have led to the controversy of whether both minor isoforms actually contribute to the OAS formation in vivo. The activities of *SERAT1;1* and *SERAT3;2* among the five isoforms have been reported to be inhibited by Cys at a physiological concentration in an allosteric manner (Noji et al., 1998; Kawashima et al., 2005).

To date, biochemical characterization in vitro and subcellular localization of *Arabidopsis* *SERAT* are well studied; however, the difference among all *SERAT* isoforms in terms of contribution and capacity for OAS formation and significance of tissue-specific and subcellular compartmentation in vivo has remained largely unaddressed. Furthermore, the question of how the *SERAT* gene family has evolved with functional speciation of each gene in *Arabidopsis* and in other plant species remains to be answered. To address these questions, we performed an evolutionary study of the *SERAT* gene family in several plant species and an analysis of *Arabidopsis* mutants deficient in the *SERAT* gene. The evolutionary study revealed the importance of the presence of three *SERAT* subfamilies. The comprehensive analysis of multiple knockout mutants along with single knockout mutants of all members in the *SERAT* gene family demonstrated the indispensable role of this gene family for plant viability and defined the extent of contribution of each member to OAS formation in vivo. Based on these results, we discuss the evolutionary and functional divergence of plant multigene families with regard to an important biochemical pathway in general.

## RESULTS

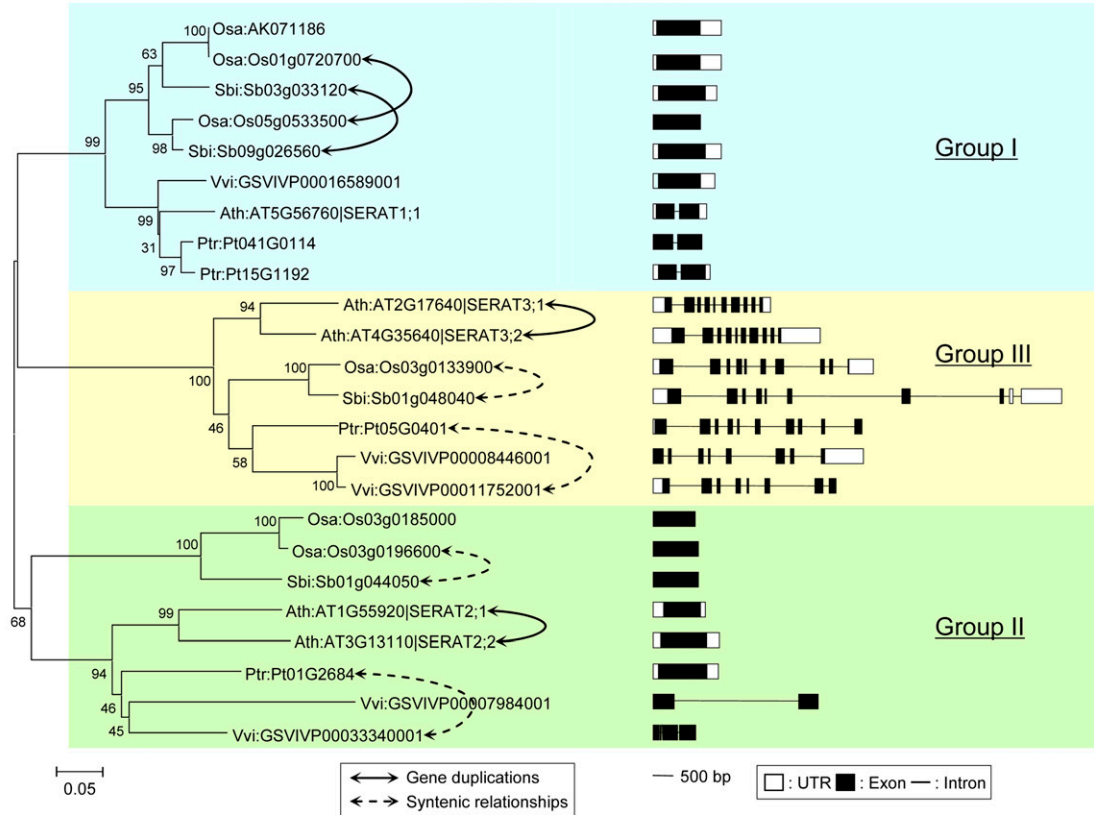
### Three *SERAT* Gene Subfamilies Are Evolutionarily Conserved across Plant Species

In *Arabidopsis*, *SERAT* enzymes are encoded by a multigene family with five members that are subdivided into three classes termed groups I to III (Kawashima et al., 2005). The multigene family of *SERAT* enzymes is also found in other plant species. To investigate the evolutionary relationship among the distinct *SERAT* isoforms, phylogenetic analysis was performed on 24 translated *SERAT* sequences from five plant species (*Arabidopsis*, five genes; *Populus trichocarpa*, four genes; *Vitis vinifera*, five genes; *Oryza sativa*, six genes; and *Sorghum bicolor*, four genes), the genomes of which have been either completely or almost completely sequenced. A neighbor-joining tree of the amino acid sequences of *SERAT* from these five species clearly separates

the three groups, each containing genes from all five species (Figure 1A). All species possess *SERAT* isoforms of all three groups, which shows that their evolution predates monocot/dicot divergence. This result also suggests that the presence of each isoform in groups I, II, and III, which have been conserved throughout plant evolution, could be profitable for Cys metabolism of plants. Furthermore, we found that each group exhibited a characteristic exon-intron structure (Figure 1A), with a high degree of conservation of amino acid sequences within the three groups (see Supplemental Figure 1 online). The *SERAT* genes in groups I and II contain no or one intron, except for one gene from *V. vinifera* that contains five introns. In group III, the *SERAT* genes from *Arabidopsis*, *P. trichocarpa*, *O. sativa*, and *S. bicolor* contain nine introns and that from *V. vinifera* contains seven introns; however, the number and size distribution of exons within the coding sequences of *SERAT* members in group III are highly conserved in the five species (Figure 1B). This high degree of conservation of gene structure across different plant species suggests that the *SERAT* isoforms in group III, which includes *SERAT3;1* and *SERAT3;2* isoforms of *Arabidopsis*, play roles not only in plant development, but also, or perhaps primarily, in metabolism. This idea was supported by the result from the phylogenetic analysis including two lower species, *Physcomitrella patens* (four genes) and *Selaginella moellendorffii* (three genes), in addition to the five higher plant species (24 genes) (see Supplemental Figure 2 online). The two lower species also have the *SERAT* members in group III with the characteristic exon-intron structure.

The plant genome contains many putative paralogs that probably develop via segmental chromosome duplication events (Blanc et al., 2000; Vision et al., 2000). The search for such paralogs within the *SERAT* family of these five species using the Plant Genome Duplication Database (PGDD; <http://chibba.agtec.uga.edu/duplication/>) revealed that *SERAT2;1/SERAT2;2* and *SERAT3;1/SERAT3;2* in *Arabidopsis*, Os01g0720700/Os05g0533500 in *O. sativa*, and Sb03g033120/Sb09g026560 in *S. bicolor* form paralogous gene pairs (Figure 1A; see Supplemental Figure 3A online). Figure 1C illustrates the chromosome locations and duplication events of the region surrounding the *SERAT* genes in *Arabidopsis*. It is noteworthy that *SERAT2;1* and *SERAT2;2*, which encode plastidic and mitochondrial isoforms, respectively, were generated by a gene duplication event in *Arabidopsis*. This result suggests that *Arabidopsis* probably did not have independent *SERAT* members in plastids and mitochondria before the duplication event and that these compartment-specific isoforms were later generated by gene duplication events. Interestingly, plastidic OASTL (BSAS2;1) and mitochondrial OASTL (BSAS2;2) were also generated by a duplication event (see Supplemental Figure 4 online). This suggests that OAS and Cys syntheses in the three compartments resulted from gene duplication during the process of evolution and not from ancestors of organelles. Further analysis using the PGDD revealed cross-genome syntenic relationships in four gene pairs between Ptr: Pt05G0401/Vvi: GSVIVP00011752001, Ptr: Pt01G2684/Vvi: GSVIVP0003340001, Osa: Os03g0133900/Sbi: Sb01g048040, and Osa: Os03g0196600/Sbi: Sb01g044050 (Figure 1A; see Supplemental Figure 3B online). These evolutionary studies indicated the importance of the existence of all

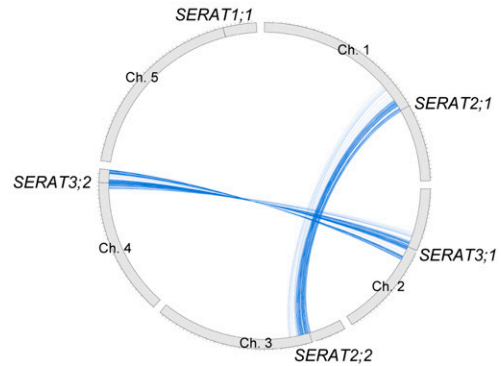
**A Phylogenetic relationships and genomic organizations of plant SERAT gene family**



**B Number and size distribution of exons of SERAT genes in group III**

Ath:AT2G17640 SERAT3;1	141	198	60	96	25	95	167	74	60	58
Ath:AT4G35640 SERAT3;2	252	198	60	96	25	95	167	74	60	41
Osa:Os03g0133900	273	198	60	96	25	95	167	74	60	17
Sbi:Sb01g048040	261	198	60	96	25	95	167	74	47	
Ptr:Pt05G0401	258	198	60	96	25	95	167	74	67	148
Vvi:GSVIVP00008446001	207	60	96	25	95	167	74	62		
Vvi:GSVIVP00011752001	147	198	60	96	25	95	167	136		

**C Gene duplications of SERAT genes in Arabidopsis**



**Figure 1.** Evolutional Studies of the SERAT Gene Family in Higher Plants.

**(A)** Left: Phylogenetic relationships of the SERAT proteins in higher plants. The phylogenetic tree was constructed from an alignment (see Supplemental Data Set 1 online) of deduced amino acid sequences from *Arabidopsis* (Ath), *P. trichocarpa* (Ptr), *V. vinifera* (Vvi), *O. sativa* (Osa), and *S. bicolor* (Sbi). Arrows indicate paralogous gene pairs generated by gene duplications within a species. Dashed arrows indicate the gene pairs in terms of cross-genome syntenic relationships. The bootstrap values from 1000 replicates are given at each node. Right: Exon-intron structures of the SERAT genes. The untranslated region (UTR) sequences are shown when available. Open boxes represent UTRs; solid boxes, coding regions; and solid lines, introns.

**(B)** Number and size distribution of exons within the coding sequence of SERAT members in group III. Exons are represented by boxes with the label showing the number of base pairs in the exon.

**(C)** Chromosome locations and duplication events for the regions surrounding the SERAT genes in the *Arabidopsis* genome. Connecting lines mark specific cases in which there is a strong correlation between duplicated genomic regions and the presence of SERAT genes in the same family.

*SERAT* isoforms in groups I, II, and III. Thus, a comprehensive reverse genetic study of all *SERAT* genes in *Arabidopsis* would be essential for understanding the function of the *SERAT* gene family in plants in general.

### No Visible Phenotypic Changes in Single Knockout Mutants

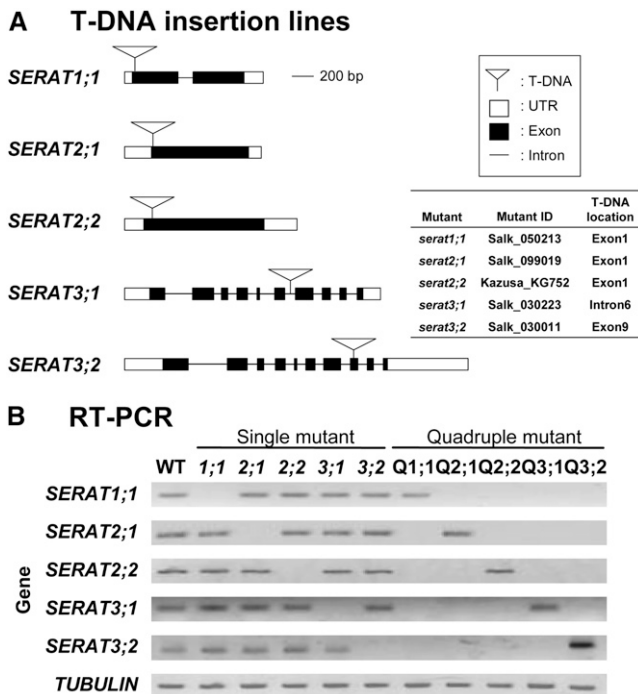
To define the *in vivo* function of individual *SERAT* isoforms, we analyzed *Arabidopsis* mutants deficient in *SERAT* genes. T-DNA insertion mutants for *serat1;1*, *serat2;1*, *serat3;1*, and *serat3;2* were identified by searching the Salk Institute Insertional Mutation Database (Alonso et al., 2003), and a T-DNA insertion mutant for *serat2;2* was identified using the screening system developed at the Kazusa DNA Research Institute. The *serat* mutants of Columbia (Col-0) background were identified in a PCR-based screening of the various T-DNA-transformed populations of *Arabidopsis* for those bearing insertions in the *SERAT* locus (Figure 2A). The DNA gel blot analysis and the genotype/phenotype relationships in *serat* mutants are presented in Supplemental Figure 5 and Supplemental Table 1 online. After selection of the homozygous T-DNA insertion mutants, RT-PCR analysis was performed for *SERAT* transcripts in 2-week-old wild-type plants and *serat* mutants (Figure 2B). In all single *serat* mutants, the

expression of the targeted *SERAT* genes for knockout mutation was repressed, but the expression levels of the other *SERAT* genes were not remarkably changed when compared with those of the wild type. In addition, no visible phenotypic changes were observed in any of the single *serat* mutants compared with the wild type under normal growth conditions or under stress conditions such as sulfur deficiency or cadmium treatment (see Supplemental Table 2 online). These findings suggest that there is a redundancy of function between *SERAT* isoforms.

### The Survival of Five Quadruple Mutants and the Embryo-Lethal Phenotype of the Quintuple Mutant Indicate That All *SERAT* Isoforms Are Functional

To clarify the capacity and contribution of each *SERAT* isoform for OAS synthesis *in vivo*, the multiple mutant approach was used. Multiple mutants could be obtained according to Mendel's law since each of the five *SERAT* genes of *Arabidopsis* is located on different chromosomes (Figure 1C). All combinations of crossing for multiple mutants are listed in Supplemental Table 3 online. In crossing, five quadruple mutants were obtained, in which only one *SERAT* gene among the five *SERAT* genes was retained, suggesting that all five isoforms are functional *SERAT* *in vivo*. We named these five quadruple mutants according to the *SERAT* gene retained in each mutant: Q1;1 (*serat2;1 serat2;2 serat3;1 serat3;2*), Q2;1 (*serat1;1 serat2;2 serat3;1 serat3;2*), Q2;2 (*serat1;1 serat2;1 serat3;1 serat3;2*), Q3;1 (*serat1;1 serat2;1 serat2;2 serat3;2*), and Q3;2 (*serat1;1 serat2;1 serat2;2 serat3;1*).

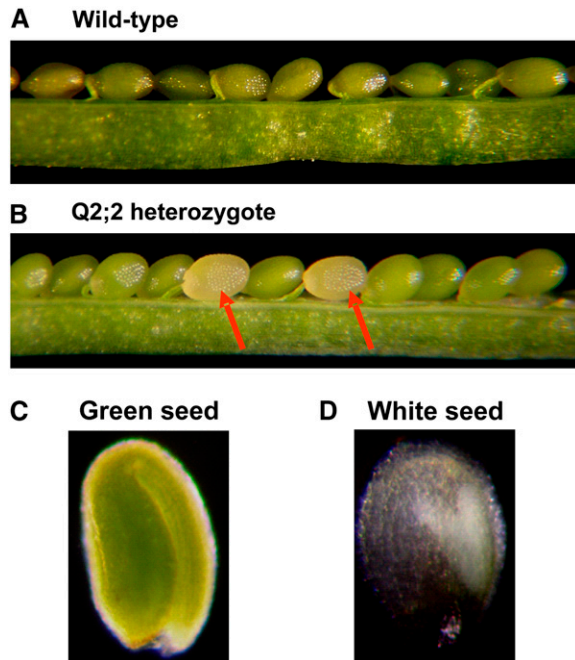
To examine whether all five isoforms are functional *SERAT* and whether any other pathway operates for Cys formation, we attempted to isolate quintuple mutants from the Q2;2 heterozygote. Among the seeds of the self-fertilized Q2;2 heterozygote, ~25% seeds failed to germinate. The ratio of germinated seeds: nongerminated seeds was 59:21 ( $\chi^2 = 0.1$ ;  $P > 0.8$ ; an expected ratio was 3:1). Among the 59 germinated seeds, no quintuple mutant was detected by genotype analysis using PCR. The homozygous Q2;2:heterozygous Q2;2 ratio was 20:39 ( $\chi^2 = 0.01$ ;  $P > 0.9$ ; an expected ratio was 1:2). This suggested that the quintuple mutant conferred an embryo-lethal phenotype. Siliques of Q2;2 heterozygotes contained ~25% immature seeds that were white rather than green (Figures 3A and 3B). The green:white ratio was 277:85 ( $\chi^2 = 0.45$ ;  $P > 0.5$ ; an expected ratio was 3:1). Microscopy observation of the embryos in transparent green seeds and white seeds at 9 d after fertilization indicated that embryos in green seeds were at the mature stage (Figure 3C), whereas embryos in white seeds were almost all at the torpedo stage (Figure 3D). This result demonstrated that the quintuple mutant is lethal at the torpedo stage of embryo development. A preliminary trial of rescuing the embryos by adding OAS and Cys failed. Based on these results, the following conclusions can be drawn: (1) the *SERAT* gene is essential for embryo maturation, (2) the above-mentioned five isoforms are the only functional *SERAT* in *Arabidopsis*, and (3) no alternative pathway exists for Cys production from OAS formed by *SERAT* in this developmental stage. Since the quintuple mutant is embryonically lethal but the five quadruple mutants are not lethal, the five *SERAT* isoforms, including *SERAT3;1* and *SERAT3;2*, are functional *SERAT* *in vivo* and are partially redundant. These



**Figure 2.** Genomic Organization of *serat* T-DNA Insertion Lines.

(A) Schematic diagram of insertions in *serat* mutants. Open boxes represent UTRs; solid boxes, coding regions; and solid lines, introns. The T-DNA insertion point in each *serat* mutant is shown.

(B) *SERAT* transcripts in the *serat* mutants by RT-PCR analysis. Total RNA was extracted from 2-week-old homozygous *serat* mutants and the wild type. RT-PCR was performed with specific primers for the *SERAT* genes, and the  $\alpha$ -*TUBULIN* gene was used as the control.



**Figure 3.** Embryo Development in Seeds from the Self-Fertilized Q2;2 Heterozygote.

Siliques of wild type (A) and Q2;2 heterozygote (B) were opened 7 to 9 d after self-fertilization. Approximately 25% seeds in the siliques of the Q2;2 heterozygote were white rather than green. Arrowheads indicate white seeds. Green seeds (C) and white seeds (D) from self-fertilized Q2;2 heterozygote 9 d after fertilization were cleared using chloral hydrate/glycerol/water (8:2:1). Embryos in green seeds were at the mature stage. Embryos in white seeds were at the torpedo stage.

results also suggest that OAS, Cys, or its derivatives must be able to transfer across the compartments to compensate for the loss of OAS and Cys in each subcellular compartment of the mutants.

#### Quadruple Mutants Q2;1, Q3;1, and Q3;2 Showed a Dwarf Phenotype

To investigate the roles of individual *SERAT* isoforms in vivo, single mutants and quadruple mutants were analyzed simultaneously. No remarkable phenotypic changes were observed in any of the five single mutants and in two quadruple mutants Q1;1 and Q2;2. By contrast, three quadruple mutants Q2;1, Q3;1, and Q3;2, showed dwarf phenotypes under normal growth conditions (Figure 4A). To evaluate plant biomass production, fresh weights of single and quadruple mutants were measured. Biomass production represented by the fresh weights of leaves in Q2;1, Q3;1, and Q3;2 decreased to 74, 54, and 56%, respectively, compared with that of the 2-week-old wild type (Figure 4B). These growth retardations and dwarf phenotypes were observed during the entire plant development. The quadruple mutants Q2;1, Q3;1, and Q3;2 never attained the size of the wild type, but they showed normal seed setting and flowering patterns. These results suggest that *SERAT*1;1 and *SERAT*2;2 play predominant roles in OAS formation in vivo and that even

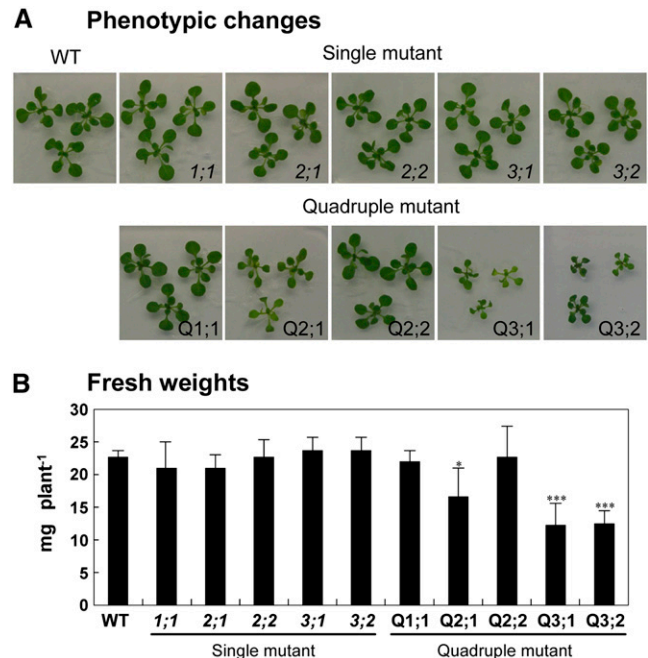
*SERAT*2;1, *SERAT*3;1, and *SERAT*3;2 have the capacity to form OAS for the entire plant life cycle despite a limited extent.

#### The Expression of *SERAT*3;2 Is Stress Inducible

RT-PCR analysis was performed for *SERAT* transcripts in 2-week-old quadruple mutants (Figure 2B). In Q1;1, Q2;1, Q2;2, and Q3;1, the expression level of each *SERAT* gene that was retained in each quadruple mutant was not remarkably changed compared with that of the wild type. By contrast, the expression level of *SERAT*3;2 in Q3;2 was apparently increased. Real-time RT-PCR revealed that the expression of *SERAT*3;2 in Q3;2 was increased to 3.4-fold compared with that of the wild type. The expression of the *SERAT*3;2 gene was reported to be induced in the wild-type plant under stress conditions such as sulfur deficiency and cadmium treatment (Kawashima et al., 2005). The induction in Q3;2 was presumably caused by stress, most likely by the decrease in Cys and GSH contents with less formation of OAS in the mutant (see below).

#### Mitochondrial *SERAT*2;2 Predominantly Contributes to the Cellular *SERAT* Activity

To investigate the contribution of each *SERAT* isoform to cellular *SERAT* activity, the *SERAT* activities in the leaves, roots, stems,



**Figure 4.** Growth Phenotypes of *serat* Mutants.

(A) Phenotypic changes of *serat* mutants. Plants were grown for 2 weeks on germination medium (GM) with agar. Q2;1, Q3;1, and Q3;2 showed dwarf phenotype.

(B) Fresh weights of leaves of 2-week-old plants. Plants were grown on GM agar medium. Data represent the mean ( $\pm$ SD) of three experiments, with 15 plants in each experiment. Differences between the wild type and *serat* mutants analyzed using Student's *t* test were statistically significant where indicated (\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

and siliques of the *serat* mutants were determined using crude protein extracts of the mutants and compared with those of the wild type (Figure 5). In the leaf and stem, significant reductions in the SERAT activity were observed in one single mutant, *serat2;2*, and all quadruple mutants except Q2;2. SERAT activities in the leaf and stem decreased to 21 and 12% in *serat2;2*, 9 and 7% in Q1;1, 13 and 1% in Q2;1, 5 and 1% in Q3;1, and 2 and 0.8% in Q3;2, respectively. In the root, SERAT activities in two single mutants, *serat1;1* and *serat2;2*, decreased to approximately half that of the wild type. Significant reductions in SERAT activities were also observed in four quadruple mutants, Q1;1, Q2;1, Q3;1, and Q3;2, in which the activities were decreased to 27, 5, 0.1, and 0.4%, respectively. In the silique, all five quadruple mutants, but no single mutants, showed significant reductions in SERAT activities. The SERAT activities in siliques decreased to 62, 40, 68, 48, and 29% in Q1;1, Q2;1, Q2;2, Q3;1, and Q3;2, respectively. These results indicated that mitochondrial SERAT2;2 showed predominant SERAT activity in the leaf and stem and moderate activity in the root. After SERAT2;2, cytosolic SERAT1;1 showed the most abundant activity in the root. In the silique, the dominance of SERAT2;2 activity in the plant cell was reduced, resulting in the contribution of activities of other isoforms for OAS formation.

#### The Cellular OAS and Thiol Levels in *serat* Mutants

To determine the contribution of each SERAT isoform to OAS formation and subsequent Cys synthesis *in vivo*, OAS and thiol levels were measured in the leaves, roots, stems, and siliques of the *serat* mutants and compared with those of the wild type (Figure 5). Absolute values of thiol contents are available in Supplemental Table 4 online. Among the single mutants, the *serat2;2* mutant alone showed significant reduction in the OAS content in the leaf, root, and stem but not in the silique. Among the quadruple mutants, significant reductions in OAS and thiol contents in the leaf were observed in Q1;1, Q2;2, and the dwarf mutant Q3;2 but not in dwarf mutants Q2;1 and Q3;1. In the root, the OAS content decreased remarkably in all of the quadruple mutants except Q2;2, and the thiol content significantly decreased in both Q3;1 and Q3;2, in which the OAS content decreased to undetectable levels. In the stem, significant reductions in the OAS content were observed in four quadruple mutants Q1;1, Q2;1, Q3;1, and Q3;2, in which the OAS levels decreased to 38, 14, 21, and 12%, respectively. All five quadruple mutants showed reductions in the thiol content of the stem, although the GSH content in Q1;1 and Q2;2 remained unchanged compared with that of the wild type. In the silique, all five quadruple mutants, including Q2;2, showed a decrease in the OAS content to approximately half of that of the wild type, with a significant decrease in the thiol content. The Cys and GSH levels were in the following order: Q1;1 > Q2;2, Q3;1 > Q3;2 > Q2;1. These results indicated that mitochondrial SERAT2;2, which showed the highest SERAT activity in the leaf, root, and stem, was mainly responsible for OAS formation in these tissues. By contrast, since the activity of SERAT2;2 was decreased in the silique, the contribution of SERAT2;2 to OAS formation in the silique was less than that in the other tissues. Instead of SERAT2;2, cytosolic SERAT1;1, SERAT3;1, and SERAT3;2 presumably play significant roles in OAS formation in the silique.

#### Reorganization of Amino Acid Metabolism in the Leaf of Dwarf Quadruple Mutants

In the quadruple mutants Q2;1 and Q3;1 exhibiting dwarf phenotypes, the total formation of OAS and thiols per plant was considerably lower than that in the wild type because of reduced biomass production; however, the concentrations of these metabolites in the leaf of Q2;1 and Q3;1 were not changed when compared with those in the wild type. Analysis of the amino acid content of quadruple mutants revealed that the content of some amino acids, such as Ser, Gly, and Gln, in these mutants was increased remarkably (Table 1). These amino acid contents were reported to change in the wild-type plant under sulfur deficiency (Nikiforova et al., 2006; Hoefgen and Nikiforova, 2008). Therefore, these mutants reorganize amino acid metabolism in response to constant deficiency of OAS and Cys formation in plant cells; thus, no alteration was apparently observed in OAS and thiol contents in these mutants. Absolute values of amino acid contents are available in Supplemental Table 5 online.

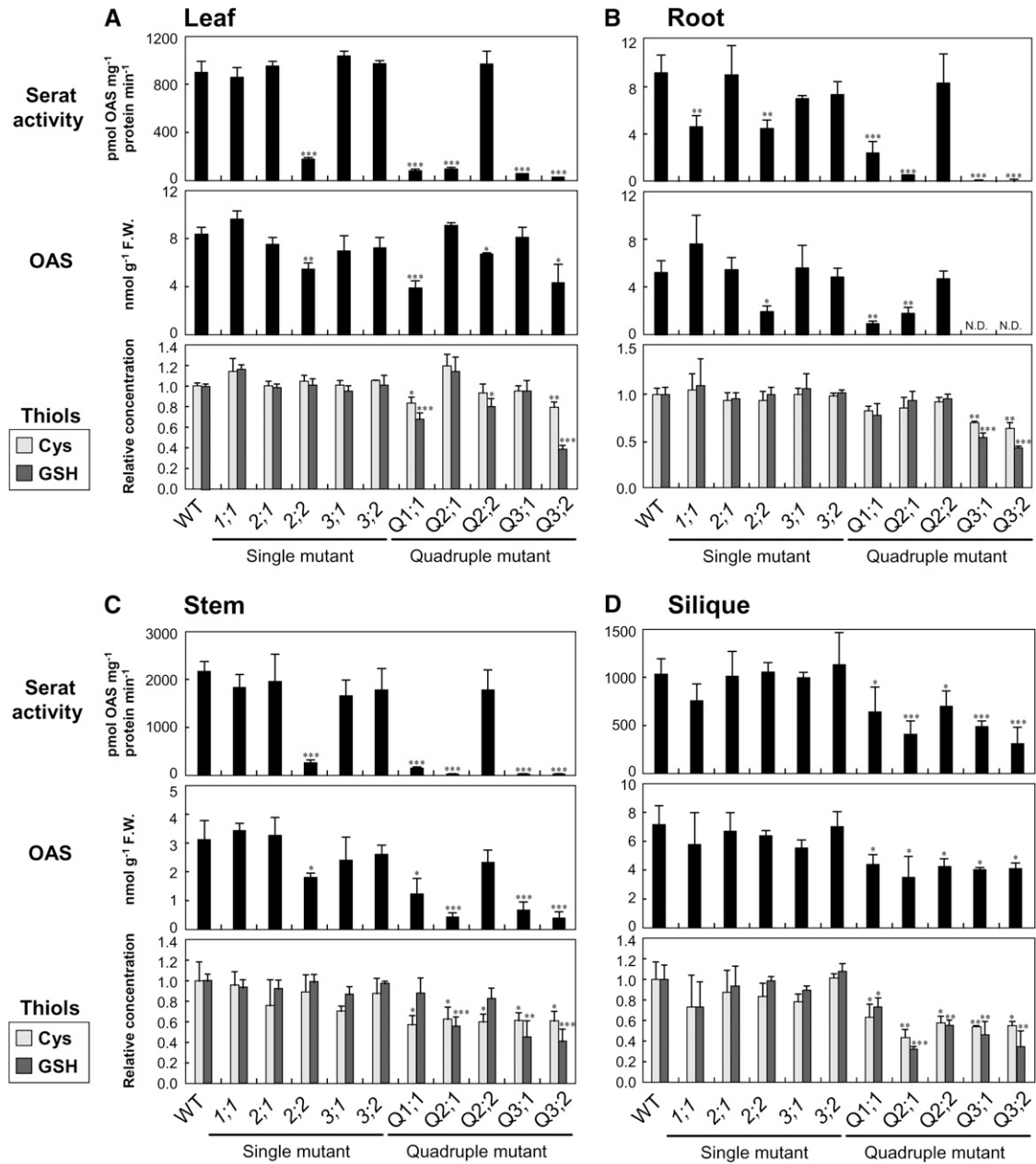
## DISCUSSION

#### Evolution of the *SERAT* Gene Family in *Arabidopsis* and Other Plant Species

The present comparative genomics study indicates that the three subfamilies of *SERAT* are evolutionally conserved in all plant species investigated, with the conservation of exon-intron structures. This clearly suggests that the three subfamilies are functionally indispensable for regular cellular processes in plants. The *SERAT* gene family in *Arabidopsis* has evolved by duplications of chromosomal segments, which occurred several times during the evolution of *Arabidopsis* (Figures 1 and 6). With an increase in the number of genes in the family, the *SERAT* family might have acquired a great adaptive advantage by allowing differential regulation of each gene family member in response to different endogenous (subcellular localization, tissue, and developmental) and exogenous (e.g., environmental) stimulation (Figure 6). The complex evolution of the reduction enzymes in the sulfate assimilation pathway in eukaryotes was also reported (Patron et al., 2008). In *Arabidopsis*, all members of the *SERAT* family cooperate for OAS formation in the plant cell; thus, cellular Cys homeostasis may be maintained efficiently and robustly. In addition, gene redundancy might serve as an important source of evolutionary innovations on the biochemical level and simultaneously exert a protective effect against deleterious mutations.

#### OAS Formation by *SERAT* Is Indispensable for Plant Viability

The lethal phenotype of the quintuple mutant indicated the indispensability of the *SERAT* gene family and the absence of alternative pathway for Cys synthesis. The embryo of the quintuple *serat* mutant was arrested at the torpedo stage of embryo development (Figure 3D). The embryo-lethal phenotype was also observed in the embryo homozygous for T-DNA insertion mutations of GSH1 (Glu-Cys ligase; EC 6.3.2.2), which is responsible for the synthesis of  $\gamma$ -glutamylcysteine ( $\gamma$ -EC) from Cys (Cairns et al., 2006). The embryo of the *gsh1* mutant, however, could



**Figure 5.** SERAT Activity and Accumulation of OAS and Thiols in *serat* Mutants.

(A) Leaves of 2-week-old plants were analyzed.

(B) Roots of 2-week-old plants were analyzed.

(C) Stems of 8-week-old plants were analyzed.

(D) Siliques (5 to 10 d after flowering) of 8-week-old plants were analyzed.

SERAT activities were determined by measuring the formation of OAS from Ser and acetyl-CoA using total extracts of soluble proteins prepared from plant materials. OAS and thiol contents were determined by HPLC analysis. Data represent the mean ( $\pm$ SD) of three experiments, with 15 plants in each experiment. Where indicated differences between the wild type and *serat* mutants analyzed using Student's *t* test were statistically significant for the analysis of the leaf and root (\*,  $P < 0.01$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ ) and stem and silique (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ). N.D., not detected.



**Table 1.** Changes in the Amino Acid Contents of the Leaf of 2-Week-Old *serat* Mutants

Single Mutant						Quadruple Mutant				
	1:1	2:1	2:2	3:1	3:2	Q1:1	Q2:1	Q2:2	Q3:1	Q3:2
Ala	1.27	0.99	1.09	0.95	0.97	1.06	2.09*	1.04	2.60*	1.44
Asn	1.29	1.23	1.17	1.11	1.09	1.32	2.27*	1.13	2.41*	1.98
Asp	1.25	1.07	1.11	1.08	0.99	1.09	1.10	1.04	1.28	1.47
Gln	1.81	1.46	1.46	1.22	1.14	1.26	4.61*	1.33	5.69*	3.23*
Glu	1.30	1.06	1.10	1.03	0.94	1.12	1.66	1.08	2.18*	1.80
Gly	1.26	1.16	1.20	1.05	0.91	1.12	3.03*	1.00	4.14*	2.05*
His	1.58	1.40	1.43	1.21	1.18	1.16	3.34*	1.32	3.29*	1.91
Ile	1.28	1.07	1.48	1.05	1.04	1.00	1.77	1.20	1.47	1.31
Leu	0.77	1.00	1.01	0.67	1.01	0.91	2.39	0.54	1.58	0.63
Lys	1.26	1.22	1.31	1.15	1.06	1.34	2.89*	1.17	2.43*	1.93
Met	1.12	0.88	1.01	1.02	0.95	0.81	1.13	0.88	1.06	0.73
Ser	1.61	1.29	1.32	1.16	1.04	1.19	3.53*	1.25	5.02*	2.56*
Thr	1.19	1.10	1.19	1.07	0.99	1.19	1.48	1.18	1.52	1.82
Trp	1.08	0.99	1.21	1.00	1.02	1.02	0.87	1.12	1.22	1.38
Tyr	1.82	1.49	1.49	1.20	1.45	1.22	1.42	1.63	1.93	1.26
Val	1.42	1.17	1.38	1.08	1.04	0.93	2.10*	1.07	1.90	1.28

Contents of amino acids were determined by HPLC analysis with O-phthalaldehyde (Kim et al., 1997). Fold changes relative to the wild type are shown. Differences between the wild type and *serat* mutants analyzed using Student's *t* test were statistically significant where indicated (\*,  $P < 0.01$ ).

grow to the mature stage despite bleaching during embryo development similar to a quintuple *serat* mutant. In addition, the embryo homozygous for T-DNA insertion mutations of GSH2 (glutathione synthetase; EC 6.3.2.3), which is responsible for the synthesis of GSH from  $\gamma$ -EC, showed a seedling-lethal phenotype and not embryo-lethal phenotype (Pasternak et al., 2008). These findings suggested that OAS and Cys itself, not  $\gamma$ -EC and GSH, formed autonomously within the developing embryo were indispensable for embryo maturation to a fully mature stage and that the embryo could not receive sufficient supply of OAS or Cys from the maternal tissues in late stages of development. The lethal phenotype in the mutants for synthesis of sulfur-containing

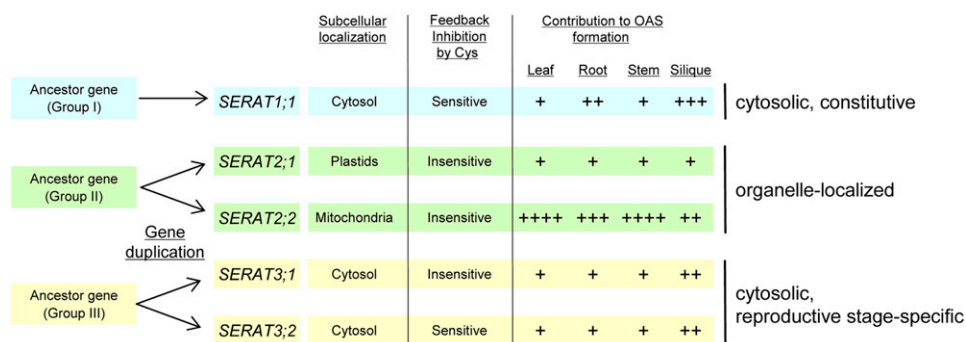
compounds such as Met (Levin et al., 2000) and biotin (Schneider et al., 1989) was also reported. This also indicates the importance of OAS formation by SERAT, subsequently converted to Cys, in embryo maturation.

### Sulfate Reduction and Cys Synthesis in the Plastid

The plastids have been conceived to be important sites for the biosynthesis of Cys since sulfide is produced only in plastids and the first step of GSH synthesis is also localized in only plastids (Wachter et al., 2005). Therefore, plastidic SERAT has also been thought to be important for Cys synthesis in plants. In *serat2;1*, however, no obvious changes were observed in OAS accumulation and thiol contents compared with the wild type (Figure 5). In addition, Q2;1 showed a dwarf phenotype (Figure 4). These results suggested that Cys synthesis does not predominantly occur in plastids, in contrast with the previous belief, and that significant amounts of sulfide might be exported from plastids to the cytosol. This idea was consistent with the results of the studies of OASTL mutants, which demonstrated that the mutants of plastidic OASTL showed smaller changes in Cys and GSH contents compared with the mutants of cytosolic and mitochondrial OASTL (Heeg et al., 2008; Watanabe et al., 2008). In *Arabidopsis*, the important role of plastids is probably to supply sulfide to the cytosol, and Cys synthesized in the cytosol and mitochondria could be transferred to plastids for adequate GSH synthesis (Figure 7).

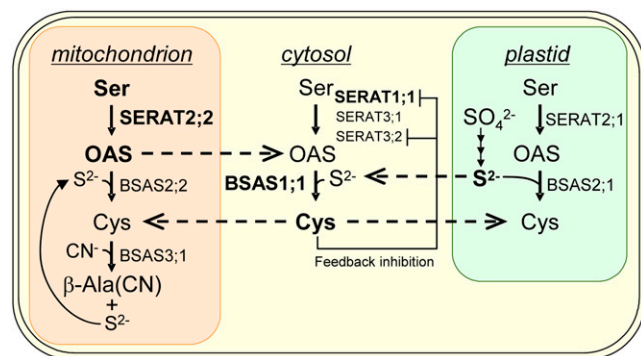
### Mitochondrial SERAT2;2 Plays a Predominant Role in OAS Formation in *Arabidopsis*

Mitochondrial SERAT2;2 is mainly responsible for OAS formation in *Arabidopsis* (Figure 5). High activity of OAS-producing SERAT was also found in the mitochondria of *Pisum sativum* (Ruffet et al., 1995). In *Arabidopsis*, Ser is biosynthesized by two different pathways: a photorespiratory pathway via Gly in the mitochondria and a plastidic pathway via the phosphorylated metabolites from 3-phosphoglycerate (Ho et al., 1999; Ho and Saito, 2001). Expression analysis of the genes involved in both pathways and recent study on the mutant (Voll et al., 2006) suggested that Ser

**Figure 6.** The Evolutional and Functional Divergence of the *SERAT* Gene Family in *Arabidopsis*.

Differentiation of the *Arabidopsis* *SERAT* genes in terms of subcellular localization, sensitivity of feedback inhibition by Cys, and the contribution to OAS formation in several tissues. The number of plus signs indicates the relative extent of contribution to OAS formation.





**Figure 7.** Schematic Representation of Cys Metabolism in *Arabidopsis*.

Schematic representation of five SERAT isoforms and four BSAS isoforms mainly involved in the Cys metabolism in the cytosol, plastids, and mitochondria of *Arabidopsis*. Relative significance of each isoform and relative production of each metabolite are indicated by different font sizes. Solid arrows indicate enzymatic reactions. Dashed arrows indicate the proposed schemes for metabolite transfer between the subcellular compartments.  $\beta$ -Ala(CN),  $\beta$ -cyanoalanine;  $\text{SO}_4^{2-}$ , sulfate;  $\text{S}^{2-}$ , sulfide;  $\text{CN}^-$ , cyanide.

production in the mitochondria seems to be the major source of Ser supply in photosynthetic tissues. By contrast, the plastidic pathway seems to play an important role in nonphotosynthetic tissues (Ho et al., 1999; Ho and Saito, 2001). The activity of mitochondrial SERAT in the photosynthetic leaf and stem, where sulfate assimilation might be more efficient and SERAT activity was collectively higher than that in the root, might be dominant to use sufficient Ser efficiently in the compartment (Figure 7). In addition, sulfide is known to bind to cytochrome aa3 in the mitochondria (Bagarinao, 1992). A high production of OAS in the mitochondria might be required to use sulfide immediately for Cys synthesis and thereby maintain a low level of the toxic sulfide. However, despite the abundant OAS supply in the mitochondria, a low OASTL activity in the mitochondria of various plant species, including *Arabidopsis*, was found (Lunn et al., 1990; Kuske et al., 1996; Droux, 2003; Heeg et al., 2008; Watanabe et al., 2008). The low OASTL/SERAT activity ratio in the mitochondria suggests an insufficient conversion of OAS to Cys since a high OASTL/SERAT activity ratio is required for complete conversion (Droux et al., 1998; Heeg et al., 2008). Therefore, a significant amount of OAS formed in the mitochondria might be transferred to the cytosol where the activity of OASTL is high (Heeg et al., 2008; Watanabe et al., 2008) (Figure 7).

#### Cytosolic Isoforms Might Play an Important Role during Seed Development

In the silique, cytosolic forms SERAT1;1, SERAT3;1, and SERAT3;2 might be responsible for OAS formation rather than mitochondrial SERAT2;2 (Figure 5). This is consistent with their gene expression patterns. A search in the public transcriptome database Genevestigator (<https://www.genevestigator.ethz.ch>) and gene expression analysis by real-time RT-PCR (Kawashima et al., 2005) showed that gene expression levels of SERAT2;2

and SHM1 (Ser hydroxymethyltransferase; EC 2.1.2.1), which is mainly responsible for the synthesis of Ser from Gly in the mitochondria (Voll et al., 2006), were decreased in seed development (see Supplemental Figure 6 online); this suggested that OAS formation in the mitochondria might be decreased. By contrast, gene expression levels of SERAT3;1 (see Supplemental Figure 6A online) and SERAT3;2 (Kawashima et al., 2005) were increased in the reproductive stage. In the silique, especially in the developing seed, the cytosol may be an important site for OAS formation. The decrease in SERAT2;2 and SHM1 expression and the increase in SERAT3;1 expression are also observed in the germinated seed (see Supplemental Figure 6 online); this suggests that the seed-specific regulation of OAS and Cys synthesis probably exists in *Arabidopsis*.

#### Cytosolic SERAT1;1 Maintains the Cellular Cys Level

The contents of OAS, Cys, and GSH in *serat1;1* were not changed when compared with those in the wild type; however, Q1;1 could grow normally, similar to the wild-type plant, although the contents of OAS and thiols were decreased in the former (Figures 4 and 5). These results indicated that despite the presence of SERAT1;1 enzyme, the activity might be inhibited by Cys in the wild type during plant development, except under particular conditions such as seed development. An in vitro assay suggested that the activity of SERAT1;1 was regulated by feedback inhibition by Cys at physiological concentrations (Noji et al., 1998). This idea is supported by the result that although a significant reduction of the SERAT activity in *serat1;1* was observed in the root, no alteration was observed in OAS and thiol contents in the mutant. SERAT1;1 might be constitutively available in plants to enable OAS formation as rapidly as possible during emergency requirement for OAS and Cys. In addition, although the SERAT activity in Q1;1 was considerably lower than that in Q2;2, Q1;1 could provide OAS for normal plant growth (Figure 4). This result also implies that cytosolic SERAT1;1 might be an efficient SERAT or that the cytosol might be a good environment for the production of OAS. Since the cytosolic OASTL BSAS1;1 was abundant in the leaf and root in *Arabidopsis* (Heeg et al., 2008; Watanabe et al., 2008), Cys concentration in the plant cell might be maintained by strictly regulating SERAT1;1 activity or OAS formation in the cytosol. In the cytosol, Cys might be mainly formed using free sulfide released from plastids and sufficient OAS released from the mitochondria (Figure 7).

#### Characteristic Features of SERAT3;1 and SERAT3;2 Isoforms

In *serat3;1* and *serat3;2*, no obvious changes were observed in the SERAT activity, OAS accumulation, and thiol contents compared with those in the wild type, and the quadruple mutants Q3;1 and Q3;2 showed dwarfism (Figures 4 and 5). These results suggested that SERAT3;1 and SERAT3;2 had less capacity to supply OAS in vivo than other isoforms. Both of these isoforms, however, might play significant roles in the reproductive stage (Kawashima et al., 2005) (Figure 5; see Supplemental Figure 6 online). Seemingly, SERAT3;1 and SERAT3;2, which were generated by duplication, play similar roles in vivo. However,

SERAT3;2 is sensitive to feedback inhibition by Cys, and the expression of *SERAT3;2* was remarkably induced under stress conditions (i.e., sulfur deficiency and cadmium stress) (Kawashima et al., 2005) (Figure 6); this suggests that each isoform plays a distinct role, although the contribution of both isoforms for OAS formation might still be small. In our study, compared with the wild type, the *serat3;2* mutant showed normal growth and did not suffer any growth retardation due to sulfur deficiency or heavy metal treatment (see Supplemental Table 2 online). Indeed, SERAT3;1 and SERAT3;2 have the capacity for OAS production in vivo, but the low substrate affinities of both isoforms still raises the possibility that the OAS-producing activities of these isoforms might be the side activities of these proteins. They may have another unknown function(s) similar to OASTL and  $\beta$ -cyanoalanine synthase in the *BSAS* gene family (Hatzfeld et al., 2000; Warrilow and Hawkesford, 2000; Watanabe et al., 2008).

## METHODS

### Phylogenetic Analysis

Homologous sequences of *Arabidopsis thaliana* SERAT proteins were obtained using homologous protein cluster data consisting of protein sequences of *Arabidopsis* (The Arabidopsis Information Resource [TAIR]; <ftp://ftp.Arabidopsis.org/home/tair/Sequences/>), *Oryza sativa* (Rice Annotation Project Database; <http://rapdb.dna.affrc.go.jp/rapdownload/>), *Sorghum bicolor* (Joint Genome Initiative [JGI]; [ftp://ftp.jgi-psf.org/pub/JGI\\_data/Sorghum\\_bicolor/](ftp://ftp.jgi-psf.org/pub/JGI_data/Sorghum_bicolor/)), *Populus trichocarpa* (JGI; [http://genome.jgi-psf.org/Poptr1\\_1/Poptr1\\_1.download.ftp.html](http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.download.ftp.html)), and *Vitis vinifera* (Genoscope; [http://www.genoscope.cns.fr/externe/Download/Projets/Projet\\_ML/data/annotation/](http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ML/data/annotation/)). The homologous protein cluster data were constructed using PSI-CD-HIT of the CD-HIT program (<http://cd-hit.org>; Li and Godzik, 2006) with a threshold identity  $\geq 30\%$  and word size = 2. A phylogenetic tree was constructed with the aligned SERAT protein sequences by MEGA (version 3.0; <http://www.megasoftware.net/index.html>; Kumar et al., 2004) using the neighbor-joining method with the following parameters: Poisson correction, complete deletion, and bootstrap (1000 replicates, random seed). SERAT protein sequences were aligned by ClustalW implemented in MEGA. The alignment data are available in Supplemental Data Set 1 online.

### Gene Duplication

Segmental duplications of each *SERAT* gene in *Arabidopsis*, *O. sativa*, *S. bicolor*, *P. trichocarpa*, and *V. vinifera* genomes were searched in the PGDD (<http://chibba.agtec.uga.edu/duplication/>). Segmental duplication regions corresponding to each *SERAT* gene of the *Arabidopsis* genome were retrieved from the duplicated block data downloaded from the PGDD with a threshold of score  $\geq 300$ . Segmental duplication maps of the *Arabidopsis SERAT* gene (Figure 1C) were visualized using Circos (version 0.31; <http://mkweb.bcgsc.ca/circos/>) with a map data of annotated *Arabidopsis* gene released by TAIR.

### Plant Materials and Growth Conditions

*Arabidopsis* (ecotype Columbia) plants were used as the wild type in this study. For the analysis of the leaf and root, plants were cultured on GM (Valvekens et al., 1988) containing 1% sucrose with 0.8% agar in a growth chamber at 22°C under 16 h/8 h light (~2500 lux)/dark cycles for 2 weeks. For the analysis of the stem and silique, plants were germinated and

cultured on GM agar medium in a growth chamber at 22°C under 16 h/8 h light/dark cycles for 3 weeks before transfer to a standard greenhouse. The plants were grown for an additional 5 weeks in a greenhouse at 22°C in 16 h/8 h light/dark cycles. The plants were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until use. The plant materials were powdered using a multi-beads shaker (Yasui Kikai). Identical plant materials were analyzed for their gene expression levels, enzyme activities, and metabolite profiles.

### Screening of T-DNA Mutants

T-DNA-inserted mutants of *Arabidopsis*, namely, line 050213 (*serat1;1*), line 099019 (*serat2;1*), line 030223 (*serat3;1*), and line 030011 (*serat3;2*), were obtained from the Salk Institute. A T-DNA-inserted mutant of *Arabidopsis*, line Kazusa\_KG752 (*serat2;2*), was obtained from the Kazusa DNA Research Institute. Homozygous mutants were identified using a PCR method (Alonso et al., 2003) according to the protocol described at the Salk Insertional Mutant Database. The primers used for the screening of homozygous *serat* mutants are listed in Supplemental Tables 6 and 7 online.

### RT-PCR Analysis

Total RNA of the wild type and each *serat* mutant was extracted with the RNeasy Plant Mini Kit (Qiagen), and cDNA was synthesized with SuperScript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The primers used for RT-PCR are listed in Supplemental Table 8 online. The primers for  $\alpha$ -*TUBULIN* were designed as described by Ludwig et al. (1987). The PCR program for amplification was as follows: 94°C for 3 min; 24 to 30 cycles of 94°C for 30 s, 55 to 60°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 10 min.

### Real-Time RT-PCR Analysis

Total RNA of the wild type and Q3;2 mutant was extracted with the RNeasy Plant mini kit, and cDNA was synthesized with SuperScript II RNase H<sup>-</sup> reverse transcriptase according to the manufacturer's instructions. One hundred nanograms of cDNA was used for real-time RT-PCR analysis. The primers used for real-time RT-PCR are listed in Supplemental Table 9 online. The primers for *ACTIN2* were designed as described by Himanen et al. (2002). SYBR green real-time RT-PCR Master Mix (Toyobo) was used for amplification according to the protocols provided by the supplier. Real-time RT-PCR was conducted in a 96-well plate with an ABI 5700 sequence detection system (Applied Biosystems).

### Crossing of *serat* Mutants

The following crosses are also listed in Supplemental Tables 1 and 3 online. Single mutants *serat1;1* and *serat2;1*, *serat2;1* and *serat2;2*, and *serat3;1* and *serat3;2* were crossed to generate double mutants *serat1;1 serat2;1*, *serat2;1 serat2;2*, and *serat3;1 serat3;2*, respectively. Single mutant *serat1;1* and double mutant *serat2;1 serat2;2* were crossed to generate triple mutant *serat1;1 serat2;1 serat2;2*. Single mutant *serat1;1* and double mutant *serat3;1 serat3;2* were crossed to generate triple mutant *serat1;1 serat3;1 serat3;2*. Double mutants *serat2;1 serat2;2* and *serat3;1 serat3;2* were crossed to generate quadruple mutant Q1;1 (*serat2;1 serat2;2 serat3;1 serat3;2*). Triple mutants *serat1;1 serat2;1 serat2;2* and *serat1;1 serat3;1 serat3;2* were crossed to generate quadruple mutants Q2;1 (*serat1;1 serat2;2 serat3;1 serat3;2*), Q2;2 (*serat1;1 serat2;1 serat3;1 serat3;2*), Q3;1 (*serat1;1 serat2;1 serat2;2 serat3;2*), and Q3;2 (*serat1;1 serat2;1 serat2;2 serat3;1*). Q2;2 heterozygote was also obtained from this crossing process. Insertions were confirmed by

genomic PCR with gene-specific and T-DNA border primers (see Supplemental Tables 6 and 7 online).

#### Visualization of Quintuple Mutant Seeds

Seeds were cleared prior to microscopy using a mixture of chloral hydrate/glycerol/water (8:2:1) and incubation at room temperature for at least 2 h. Microscopy was performed on an Olympus SZ-ST5 light microscope.

#### Assay of SERAT Enzymatic Activity

Plant materials were homogenized in 5 volumes (fresh weight basis) of extraction buffer containing 250 mM potassium phosphate, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid, and 10 mM 2-mercaptoethanol. The enzymatic activity of SERAT was determined in reaction mixtures (100  $\mu$ L) containing 50 mM Tris-HCl, pH 8.0, 1 mM acetyl-CoA, and 10 mM Ser. The reaction was performed at 30°C for 10 min and terminated by the addition of 10  $\mu$ L of 7.5% (w/v) trichloroacetic acid. SERAT activity was determined for the production of OAS, which was derivatized with O-phthalaldehyde using HPLC according to Kim et al. (1997).

#### Determination of OAS and Amino Acid Contents by HPLC

Plant materials were homogenized in 3 volumes of 0.1 M HCl (fresh weight basis) using the mixer mill MM 300 (Qiagen). OAS and amino acids were detected by fluorescence spectrophotometry after a postcolumn reaction with O-phthalaldehyde using HPLC according to Kim et al. (1997).

#### Determination of Cys and GSH Contents

Quantitative analyses of reduced forms of Cys and GSH were performed by a combination of monobromobimane fluorescent labeling and HPLC (Anderson, 1985; Fahey and Newton, 1987). Plant materials were homogenized in 3 volumes of 0.1 M HCl (fresh weight basis) using the mixer mill MM 300. A mixture of 20  $\mu$ L of extract and 40  $\mu$ L of 25  $\mu$ M *N*-acetyl-Cys as the internal standard was allowed to react with 5  $\mu$ L of 30 mM monobromobimane in acetonitrile and 10  $\mu$ L of 8.5 mM *N*-ethylmorpholine for 20 min at 37°C in the dark. The labeling reaction was terminated by the addition of 10  $\mu$ L of acetic acid, and the resulting solution was then subjected to HPLC analysis. HPLC was performed as described previously (Saito et al., 1994).

#### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under accession numbers NM\_125059 (*SERAT1;1*), NM\_104470 (*SERAT2;1*), NM\_112150 (*SERAT2;2*), NM\_127318 (*SERAT3;1*), and NM\_119729 (*SERAT3;2*).

#### Supplemental Data

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

**Supplemental Figure 1.** Alignment of Deduced Amino Acid Sequences of the *SERAT* Genes in Different Plants.

**Supplemental Figure 2.** Evolutional Studies of the *SERAT* Gene Family in Higher and Lower Plants.

**Supplemental Figure 3.** Gene Duplications and Syntenic Relationships in the *SERAT* Gene Family in Plants.

**Supplemental Figure 4.** Chromosome Locations and Duplication Events for the Regions Surrounding the *BSAS* Genes in the *Arabidopsis* Genome.

**Supplemental Figure 5.** DNA Gel Blot Analysis of *serat* Mutants.

**Supplemental Figure 6.** Gene Expression Patterns of the *SERAT* and *SHM* Gene Families during Development.

**Supplemental Table 1.** T-DNA Insertion Lines of *SERAT* Genes.

**Supplemental Table 2.** The Concentration of Sulfate and Heavy Metal Used for Stress.

**Supplemental Table 3.** All Combinations of Crossing for Multiple Mutants.

**Supplemental Table 4.** The Accumulation of Thiols in *serat* Mutants.

**Supplemental Table 5.** The Amino Acid Contents of the Leaf of 2-Week-Old *serat* Mutants.

**Supplemental Table 6.** Primers for Identification of T-DNA Insertion in *serat* Mutants.

**Supplemental Table 7.** Primers for Isolation of Homozygote *serat* Mutants.

**Supplemental Table 8.** Primers for RT-PCR Analysis of the *SERAT* Genes in *serat* Mutants.

**Supplemental Table 9.** Primers for Real-Time RT-PCR Analysis of the *SERAT3;2* Gene in the Q3;2 Mutant.

**Supplemental Data Set 1.** Text File of the Alignment Used to Generate the Phylogenetic Tree of *SERAT* Proteins in Figure 1.

**Supplemental Data Set 2.** Text File of the Alignment Used to Generate the Phylogenetic Tree of *SERAT* Proteins from Higher and Lower Plants Shown in Supplemental Figure 2 Online.

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