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REVIEW

Physiological importance and possible applications of β-substituted alanine synthase in plants

Kyung Hee Roh[1] · Byung Wook Kang[2] · Ho Bang Kim[3,4] · Sang Ho Lee[5] · Yoonkang Hur[2] · Hankuil Yi[2]

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Abstract Cysteine, as well as its precursors and derivatives, plays important roles in plant development and stress responses. In plants, a diverse range of reactions affecting cysteine content are catalyzed by the β-substituted alanine synthase (BSAS) enzyme family. Individual BSAS family members use similar reaction mechanisms involving pyridoxal phosphate cofactors and show catalytic preferences for biosynthesis, degradation, or modification of the cysteine amino acid. In *Arabidopsis thaliana* (Arabidopsis) of the *Brassicaceae* family, four distinct biochemical activities are characterized at the gene level, namely, *O*-acetylserine sulfhydrylase, β-cyanoalanine synthase, L-cysteine desulfhydrase, and *S*-sulfocysteine synthase activities. Reverse genetic approaches in Arabidopsis were used to elucidate the physiological roles of metabolites of cysteine metabolism (*O*-acetylserine, sulfide, cysteine, cyanide, and *S*-sulfocysteine) during the processes of root hair development, pollen tube germination, heavy metal tolerance, defense responses, stomatal closure, and autophagy. Key catalytic residues determining reaction specificities in different BSAS enzymes are being identified, along with the roles of macromolecular complexes involving BSAS. The biochemical properties of BSAS active sites are being investigated in various organisms, including plants, for possible application to the development of new biological materials and drugs. Systematic and comparative genomic studies of BSAS enzymes in *Brassica* plants, close relatives of Arabidopsis, requiring high cysteine production for optimum growth and disease resistance, will be useful for the future study of the diversification of BSAS and the biotechnological improvement of these important crop plants.

Keywords Abiotic stress · Biotic stress · β-Substituted alanine synthase (BSAS) · Cysteine · Non-natural amino acid · Sulfur metabolism

Yoonkang Hur and Hankuil Yi contributed equally to this work.

✉ Yoonkang Hur
ykhur@cnu.ac.kr

✉ Hankuil Yi
hankuil.yi@cnu.ac.kr

[1] Department of Agricultural Biotechnology, National Academy of Agricultural Science, Jeonju, Republic of Korea

[2] Department of Biological Sciences, Chungnam National University, Daejeon, Republic of Korea

[3] Life Sciences Research Institute, Biomedic Co., Ltd.,, Bucheon, Republic of Korea

[4] Faculty of Biotechnology, Jeju National University, Jeju, Republic of Korea

[5] Division of Biomedical Engineering & Health Science Management, Mokwon University, Daejeon, Republic of Korea

In plants, the biosynthesis of cysteine amino acids provides a primary entry point for organic sulfur. Cysteine is used as a building block for the production of low and high molecular weight polymers, such as proteins, glutathione, homoglutathione, and phytochelatin (Yi et al. 2010b; Ravilious and Jez 2012; Romero et al. 2014). Glutathione, a tripeptide redox buffer that is present in various bacteria and eukaryotes, is enzymatically synthesized from glutamate, cysteine, and glycine (Galant et al. 2011). Phytochelatin is a glutathione polymer that protects plants from heavy metal toxicity (Cobbett and Goldsbrough 2002). A recent study suggests that a balance between the biosynthesis of glutathione and phytochelatin is essential for optimum protection against heavy metal-induced oxidative stress (Cahoon et al. 2015). In addition, sulfur atoms,

which are first incorporated into the primary metabolite, the amino acid cysteine, are transferred to other sulfur-containing molecules such as methionine, iron-sulfur clusters, vitamin cofactors, sulfolipids, and many plant secondary metabolites (glucosinolates and phytoalexins among others) (Jez and Fukagawa 2008; Bednarek 2012).

Sulfur is a mineral macronutrient that is mainly absorbed as sulfate (SO_4^{2-}) from the soil (Amtmann and Armengaud 2009; Kopriva 2015). Sulfur atoms can be incorporated into plant metabolites through hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) gases in the atmosphere, too. Sulfur deficiency causes symptoms such as chlorosis, stunting of growth, anthocyanin accumulation, and the accumulation of large cellular inclusions in the root, which are similar to the symptoms observed when the nitrogen supply is limited (Taiz and Zeiger 2010; Jackson et al. 2015). Sulfur, which was once considered an unwelcome pollutant, became important for optimal plant growth due to reductions in the levels of air pollutants that acted as accidental sulfur fertilizers (Haneklaus et al. 2008). This change first became evident in *Brassica* species, which showed a significant accumulation of S-containing glucosinolates (Booth et al. 1991), although it also occurred in cereals and sugar beets (Haneklaus et al. 2005). Increased incidence of diseases related to sulfur deficiency is observed in many crops, including oilseed rape, grape, and potato, thereby supporting the notion of sulfur-induced resistance or sulfur-enhanced defense (Schnug et al. 1995; Klikocka et al. 2005; Rausch and Wachter 2005; Bloem et al. 2015). Therefore, identification and characterization of the enzymes involved in cysteine metabolism in crop plants could be beneficial for future breeding and biotechnological applications, as well as for the production of sulfur-containing bioactive molecules.

Cysteine production requires the coordination of metabolic reactions at the cellular and organism levels

A brief overview

Cysteine biosynthesis is an important contact point between sulfur metabolism and carbon/nitrogen metabolism: biosynthesis combines the backbone of a carbon- and nitrogen-containing amino acid derivative with reduced sulfur (Fig. 1). Cysteine production in plants can be divided into three stages as follows: (1) assimilatory reduction of inorganic sulfate, which is transported into plant cells from the soil; (2) generation of *O*-acetylserine (OAS), an activated form of a primary metabolite, the serine amino acid; and (3) production of cysteine, in which the acetate group in OAS is replaced with sulfide, a reduced sulfur moiety.

Reductive sulfate assimilation pathway

Adenosine triphosphate (ATP) + sulfate → adenosine 5′-phosphosulfate (APS) + PPi ATP sulfurylase (ATPS) catalyzes the first reaction of sequential assimilatory sulfate reduction. Sulfate, an oxidized form of sulfur, is the most abundant sulfur-containing metabolite in the soil and in plant cells. The uptake and mobilization of sulfate is mediated by multiple forms of H^+/sulfate co-transporters located on plant membrane systems (Gigolashvili and Kopriva 2014). Once transported from the soil into the root systems of plants, sulfate is moved to other plant parts via vascular systems and then distributed to various subcellular organelles. Nonetheless, sulfide production occurs exclusively in plastids at the subcellular level and starts with the activation of sulfate to form APS (Ravilious and Jez 2012; Schmidt and Trebst 1969). This first step, which generates a high-energy phosphosulfate mixed anhydride bond, is energetically unfavorable and requires much higher concentrations of substrates (sulfate and ATP) than those of products (APS and pyrophosphates) (Fig. 1). Both products of sulfate adenylation are inhibitors of ATPS. In this regard, the overall forward flux in the sulfate reduction pathway is maintained by the efficient consumption of APS in the subsequent reaction of cysteine biosynthesis, or by the addition of another phosphate group to generate 3′-phosphate-5′-adenosine phosphosulfate (Mugford et al. 2011; Kopriva et al. 2012). The sulfur moiety in 3′-phosphate-5′-adenosine phosphosulfate provides the additional sulfur atoms in glucosinolate.

APS + glutathione (reduced) → adenosine monophosphate AMP + sulfite (SO_3^{2-}) + glutathione (oxidized) In the next reaction of the assimilatory sulfate reduction pathway, APS is further reduced by APS reductase (APR) at the cost of reduced glutathione (Fig. 1). Similar to the bacterial enzyme, plant APR contains an iron-sulfur $[4F2–4S]^{2+}$ cluster and is composed of an N-terminal reductase domain and a C-terminal glutaredoxin domain (Gutierrez-Marcos et al. 1996; Setya et al. 1996; Kopriva et al. 2001). APR activity in seed plants serves as an important regulating point in sulfur assimilation that is driven by the demand for reduced thiols (Tsakraklides et al. 2002; Vauclare et al. 2002; Loudet et al. 2007). The observation that the regulation of APR activity in a model moss, *Physcomitrella patens*, is independent from the availability of glutathione suggests that the regulation of sulfur assimilation by APR in seed plants evolved after the separation of the bryophytes (Hermsen et al. 2010).

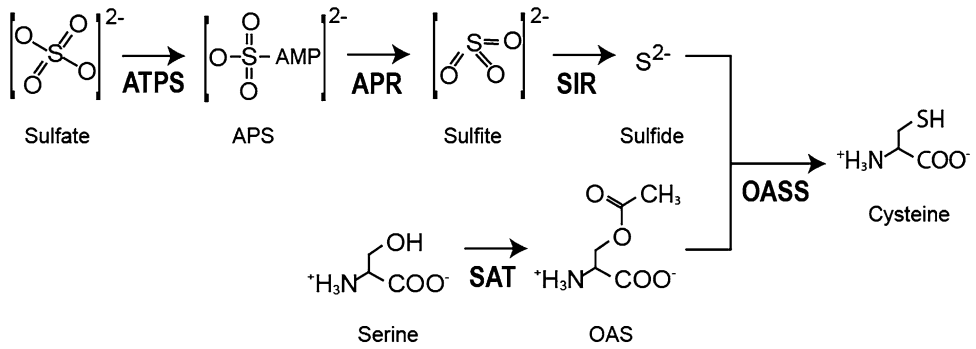

Fig. 1 Main metabolites in the cysteine biosynthesis pathway in plants. Only sulfur-containing molecules in the assimilatory sulfate reduction pathway are shown in the *upper row*, while serine and its derivative providing the carbon backbone of cysteine are shown in the *lower row*. Enzymes involved in each step are indicated in *bold*. *AMP* adenosine monophosphate, *APR* APS reductase, *APS* adenosine 5′-phosphosulfate, *ATPS* ATP sulfurylase, *OAS O*-acetylserine, *OASS O*-acetylserine sulfhydrylase, *SAT* serine acetyltransferase, *SIR* sulfite reductase

Sulfite (SO_3^{2-}) + *6 ferredoxin (reduced)* → *sulfide* (S^{2-}) + *6 ferredoxin (oxidized)* The final step in assimilatory sulfate reduction is catalyzed by sulfite reductase (SIR) using an iron-sulfur cluster and a siroheme (Yonekura-Sakakibara et al. 1998, 2000) (Fig. 1). Mutant analyses of SIR in *Arabidopsis thaliana* (Arabidopsis) and *P. patens* demonstrated that a compromise in SIR activity can cause growth retardation and developmental defects in plants, even in the absence of obvious differences in the amount of sulfur-containing metabolites (Khan et al. 2010; Wiedemann et al. 2010). This could be related to the fact that both SIR and sulfite oxidase play important roles in protecting plants from the toxic effects of sulfur dioxide and sulfite in Arabidopsis and tomato (Brychkova et al. 2007; Yarmolinsky et al. 2013).

Preparation of the cysteine carbon backbone

Serine + acetyl CoA → OAS + CoA The nitrogen- and carbon-containing backbone structure of cysteine is provided by OAS, which is generated by serine acetyltransferase (SAT or SERAT) (Fig. 1). SAT activity is a limiting factor in cysteine biosynthesis (Saito et al. 1995; Kawashima et al. 2005). As a result, transgenic overexpression of SAT generally increases the content of cysteine amino acids and thiol compounds in plants (Sirko et al. 2004; Tabe et al. 2010). The notion that SAT activity is the bottleneck in cysteine biosynthesis is further supported by the observation that OAS concentrations in various subcellular compartments of plant cells are much lower than the *Km* value of the enzymes involved in the subsequent steps of cysteine biosynthesis (Droux 2003; Wirtz et al. 2004; Krueger et al. 2009). As expected for an enzyme in a major regulating step, most SATs in plants can be feedback inhibited by cysteine, the final product of cysteine biosynthesis (Saito et al. 1995; Kumaran et al. 2009; Wirtz et al. 2012; Tavares et al. 2015). Structural studies using bacterial and plant SATs indicate that feedback inhibition is mediated by the competitive binding of cysteine to the serine substrate binding site (Olsen et al. 2004; Kai et al. 2006; Yi et al. 2013). Similar to their bacterial counterparts, plant SATs have amino acid residues important for cysteine feedback regulation in the C-terminus (Inoue et al. 1999). The modulation of cysteine sensitivity by changes in the phosphorylation status of the C-terminal region was previously reported for a soybean SAT isoform (Liu et al. 2006).

Formation of cysteine

OAS + sulfide → cysteine + acetate The last reaction in the biosynthesis of cysteine, the first stable reduced sulfur metabolite in the cell, is a substitution reaction in which the acetate group in OAS in the β-position of alanine is replaced with sulfide (Fig. 1). Because of this, the enzyme responsible for cysteine production is also called β-substituted alanine synthase (BSAS), in addition to *O*-acetylserine sulfhydrylase (OASS), *O*-acetylserine(thiol)lyase, and cysteine synthase (CS or CSase) (Droux et al. 1992; Hatzfeld et al. 2000; Jost et al. 2000; References in Romero et al. 2014). Similar to the bacterial enzymes involved in cysteine biosynthesis, plant BSAS enzymes are rather promiscuous in terms of substrate binding and can produce multiple products that are structurally related (Ikegami and Murakoshi 1994; Maier 2003). Throughout this review, the term OASS will be used to indicate the BSAS enzyme with a higher activity for cysteine synthesis than for other reactions. The term BSAS will also be used for OASS when an OASS enzyme is considered as a member of the BSAS enzyme family.

Members of the BSAS family enzymes in plants were initially thought to preferentially perform two physiologically relevant β-substitution reactions using the pyridoxal

phosphate (PLP) cofactor: namely, cysteine biosynthesis and detoxification of cyanide using a cysteine substrate (Wurtele et al. 1985; Hatzfeld et al. 2000; Warrilow and Hawkesford 2000; Heeg et al. 2008; Watanabe et al. 2008a). The BSAS enzyme responsible for cyanide detoxification is more frequently called β-cyanoalanine synthase (CAS or CASase) (Hatzfeld et al. 2000). However, recent findings indicated that other BSAS enzymes that were initially thought to have weak OASS activity catalyze distinct reactions, likely using mechanisms and pathways similar to those observed in cysteine biosynthesis (Bonner et al. 2005). Both OASS- and CAS-catalyzed reactions occur in two independent steps: (1) the formation of α-aminoacrylate from OAS or cysteine (Fig. 2) and (2) the β-substitution of acetate or sulfide (Yi et al. 2012). Whether all BSAS members, especially those generating final products without β-substitution, use the same reaction mechanism remains to be experimentally determined.

Two post-translational modifications of OASS enzymes have been reported: tyrosine nitration and N-terminal acetylation. Tyrosine nitration results in the inhibition of OASS, possibly by altering the spatial arrangement of the active site residues and PLP cofactor (Álvarez et al. 2011). This modification can provide a rapid local mechanism to reduce cysteine production capacity, which can be useful in signal transduction by reactive oxygen species (ROS). Although protein acetylation seems to be widespread in Arabidopsis and soybean OASS enzymes, the exact roles of these modifications in cysteine biosynthesis await careful molecular characterization (Wirtz et al. 2010; Yi and Jez 2012). The modulation of protein stability by N-terminal acetylation was recently reported (Gibbs 2015).

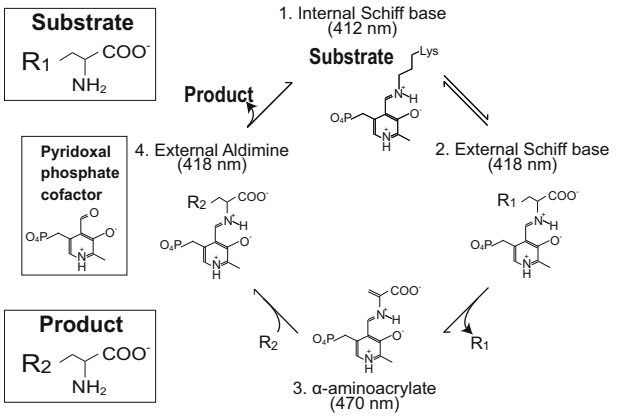

Fig. 2 Reaction mechanism of BSAS enzymes with pyridoxal phosphate (PLP). The PLP cofactor is covalently linked to an active site lysine residue to form an internal Schiff base. By generating an α-aminoacrylate reaction intermediate, BSAS in general carries out the β-substitution of R_1 by R_2. For OASS (*O*-acetylserine sulfhydrylase) in cysteine biosynthesis, R_1 and R_2 are acetate and sulfide, respectively. The peak absorption wavelength of BSAS enzymes depends on the chemical moiety linked to the PLP cofactor

Interplay of the plant subcellular compartments in cysteine biosynthesis

In Arabidopsis and other plants studied to date, both SATs and BSASs with confirmed OASS activity are encoded by multiple members of two small gene families. In addition to plastids, the enzymes are targeted to the cytosol and mitochondria, where exclusive assimilatory sulfate reduction occurs (Kawashima et al. 2005; Heeg et al. 2008; Watanabe et al. 2008a; Yi and Jez 2012; Table 1 in Romero et al. 2014; Tavares et al. 2015). For example, the soybean genome has eight putative SATs, including two biochemically confirmed SAT enzymes found in the cytosol and chloroplasts (Chronis and Krishnan 2004; Liu et al. 2006), of which one is targeted dually to the cytoplasm and chloroplasts (Liu et al. 2006). In addition, 16 BSAS members were identified in soybean by genome-wide sequence analysis, and further studies showed that at least five of them have biochemical characteristics comparable to those reported for other plant OASS enzymes (Chronis and Krishnan 2003; Yi et al. 2010b; Yi and Jez 2012). Among the five soybean OASS enzymes characterized, two contain putative signal peptides for plastid targeting; however, experimental validation for the exact subcellular localization is necessary. Designation of individual SAT or BSAS isoforms is based on phylogenetic analyses, which group SATs into three clades and BSAS into five clades (Hatzfeld et al. 2000; Kawashima et al. 2005; Yi et al. 2010b). Two numbers separated by a semicolon are added as a suffix to the abbreviations of enzyme names, such as SERAT1;1 for SAT and BSAS3;1 for BSAS. In Arabidopsis, the first numbers, '1, 2, and 3' for SAT, provide information on the subcellular localization: '1' or '3' is used for cytosolic localization, whereas '2' indicates chloroplast or mitochondrial localization. For BSASs, the numbering system is somewhat different in that '3' designates mitochondrial BSASs with a cyanide detoxification function, and '4' and '5' are used for BSAS isoforms in the cytosol and chloroplast, respectively.

The relative contributions of each subcellular compartment to cysteine biosynthesis are diverse among different plant species. Detailed studies of Arabidopsis with five SAT enzymes and eight functional BSAS enzymes revealed that the cytosol, plastids, and mitochondria all have their own SAT and OASS isozyme(s), and these isozymes are encoded by different members in each gene family (Watanabe et al. 2008a, b). Compared to *Chlamydomonas reinhardtii*, in which the entire cysteine biosynthesis pathway is confined to the chloroplast, OASS

enzyme activity in *P. patens* is also found in the cytosol (Birke et al. 2012b). In spinach (*Spinacea oleracea*), purified mitochondrial extracts do not show SAT activity (Brunold and Suter 1982), and it is unclear whether spinach has mitochondrial OASSs (Lunn et al. 1990; Warrilow and Hawkesford 2000). OASS activity initially observed in spinach mitochondria seems to be the side activity of a BSAS enzyme for cyanide detoxification. The unequal contribution of different subcellular compartments to cysteine biosynthesis was best established in Arabidopsis, the model land plant. Enrichment of subcellular compartments using non-aqueous fractionation, followed by enzyme assays and metabolite analyses, strongly suggests that cysteine formation by OASS predominantly takes place in the cytosol, while the penultimate reaction by SAT mainly happens in mitochondria (Krueger et al. 2009). The distribution of OASS activity and the content of cysteine highly resemble those of cytosolic protein markers. Consistent with this observation, phenotypic analysis of Arabidopsis T-DNA insertional mutants revealed that only the loss of major cytosolic OASSs causes a significant decrease in the content of cysteine and glutathione (Heeg et al. 2008; Watanabe et al. 2008a). Studies of transgenic and mutant plants with compromised SAT activities in specific compartment(s) showed that mitochondrial SAT plays a major part in cysteine biosynthesis, while the contribution of chloroplast SAT to cysteine metabolism is limited (Haas et al. 2008; Watanabe et al. 2008b; Krueger et al. 2009). In quadruple Arabidopsis mutants with only one functional SAT, growth retardation and a huge decrease (less than 50 % of wild-type plant levels) of sulfate levels were observed when both cytosolic and mitochondrial SAT mutations were included among the four SAT mutations (Watanabe et al. 2010). Taken together, these results indicate that (1) cysteine biosynthesis is a coordinated process involving various subcellular compartments, and (2) substrates and products of OASS can be redistributed relatively easily across organellar membranes.

The cysteine regulatory complex: a bienzyme complex

SAT and OASS, the two enzymes involved in the last two consecutive steps of cysteine biosynthesis, form bienzyme complexes. Early studies in which SAT or OASS enzymes from various plant species were purified and characterized showed that these enzymes can be present as parts of a macromolecular complex in addition to the corresponding free forms (Droux et al. 1992; Bogdanova and Hell 1997; Droux et al. 1998; Zhu et al. 1998; Jost et al. 2000). Later experiments, including heterologous expression of plant enzymes using *Escherichia coli*, yeast two-hybrid screening, and in vitro assembly of SAT/OASS hetero-oligomeric complexes, further revealed that even the SAT and OASS enzymes originating from different sources can form complexes. Complex formation was observed for OASSs and SATs from different eukaryotic species, such as between unicellular green algae (*Chlorella sorokiniana*) and Arabidopsis (Salbitani et al. 2014), *P. patens* and Arabidopsis (Birke et al. 2012b), bacteria (*Escherichia coli*), and soybean (Yi and Jez 2012), as well as SATs and OASSs targeted to different subcellular organelles of Arabidopsis and soybean (Heeg et al. 2008; Yi and Jez 2012). As with their bacterial counterparts, the functional units of SAT and OASS seem to be trimers and dimers, respectively (Bonner et al. 2005; Kumaran et al. 2009). In plants, SAT exists as a trimer or hexamer (dimer of trimers), with its three active sites located at the interfaces between two neighboring SAT monomers (Yi et al. 2013). Size exclusion chromatography and analytical ultracentrifugation using Arabidopsis mitochondrial SAT and soybean cytosolic SAT demonstrated that SATs can be found as hexamers or trimers, raising the possibility that the quaternary structure of SAT can be dynamic and affected by solution conditions and SAT:OASS ratios (Feldman-Salit et al. 2009; Kumaran et al. 2009; Wirtz et al. 2010). Although a maximum of six OASS dimers can bind to a SAT hexamer, the initial binding of an OASS dimer interferes with subsequent OASS binding through negative cooperativity (Feldman-Salit et al. 2009; Kumaran et al. 2009; Wirtz et al. 2010). As a result, the stoichiometry of two OASS dimers per SAT hexamer (most possibly one OASS dimer per SAT trimer) is preferred. In the cell, OASS is present in excess with respect to SAT, and maximum cysteine synthesis in vitro is achieved only when OASS is in excess along with SAT (Ruffet et al. 1994; Droux et al. 1998). In the cell, most of the SAT enzymes are likely present in the complex, while most of the OASSs function in free forms.

As opposed to what is expected for metabolic channeling, bienzyme complex formation between SAT and OASS enhances SAT activity in the preceding step of cysteine biosynthesis and inactivates OASS in the subsequent step. Metabolic channeling is a phenomenon in which the formation of a complex between enzymes involved in the sequential steps of a biochemical pathway facilitates the transfer of reaction intermediates between two or more active sites (Winkel 2004). Contrary to the overall increase in productivity observed for metabolic channeling, complex formation between SAT and OASS in bacteria and plants reduces the flux during cysteine biosynthesis (Kredich et al. 1969; Droux et al. 1998). The mechanism of OASS inactivation was explained by the involvement of amino acid residues in the OASS active site during complex formation (Huang et al. 2005; Francois et al. 2006). In plant SATs, the interaction domain is located in the

C-terminus, which can affect the biochemical activity of SATs (Mino et al. 2000; Wirtz et al. 2001). Co-crystallization experiments showed that the C-terminal ten amino acids of Arabidopsis SAT can be physically inserted into the active site of the *Arabidopsis* OASS, thereby blocking the access of substrates involved in OASS reactions (Francois et al. 2006). The C-terminal Ile in SAT is positioned at the site of interaction between the OAS substrate and the PLP cofactor of OASS. Isothermal titration calorimetry using the C-terminal ten amino acids of SAT and OASS suggests that hydrophobic interactions play an important role (Kumaran and Jez 2007). Consistent with the involvement of the active site in bienzyme complex formation, mutation of the key catalytic residues of OASS reduces enzyme activities and binding affinities between the mutant OASS enzyme and SAT (Francois et al. 2006). Although the process of OASS inactivation during complex formation is conserved between bacteria and plants, the enhancement of SAT activity, which was reported in plant complexes, is not observed in the bacterial SAT–OASS complex (Droux et al. 1998; Mino et al. 2000; Kumaran et al. 2009; Wirtz et al. 2010). The mechanism responsible for the enhancement of SAT activity upon complex formation is not yet well understood. However, recent studies showed that CSC (cysteine synthase complex) formation significantly increases the catalytic efficiency of SAT for acetyl CoA and identified an amino acid residue that may be repositioned in the complex to stabilize acetyl CoA binding (Kumaran et al. 2009; Yi et al. 2013).

Complex formation between SAT and OASS enzymes can provide an adequate amount of cysteine to the cell and responds to the availability of substrates and the demand for the cysteine-containing molecules. The interaction between SAT and OASS is reversible, and several metabolites in cysteine biosynthesis affect the assembly and dissociation of the complex. OAS, the product of SAT and a substrate of OASS, can dissociate the bienzyme complex by competing with the C-terminus of SAT for the OASS active site (Droux et al. 1998). Bimolecular interaction analyses indicate that the physiological range of OAS concentrations effectively controls complex formation (Berkowitz et al. 2002; Krueger et al. 2009). In addition, sulfide, another substrate of OASS, stabilizes the complex, while cysteine, the product of OASS, has the opposite effect and dissociates the complex (Droux 2003; Wirtz and Hell 2006). At the protein complex level, these features can modulate the activity of SAT and OASS, thereby maintaining the homeostasis of sulfur metabolism. As proposed by Hell and Hillebrand, most SAT enzymes in the cell are expected to interact with OASS and show enhanced activity through complex formation when the sulfide supply is sufficient (Hell and Hillebrand 2001). This occurs because of the excess amount of OASS enzyme compared to SAT and allows sufficient cysteine production using free OASS enzymes outside bienzyme complexes. If the sulfur supply is limited, OAS cannot be consumed efficiently for cysteine production and accumulates, dissociating SAT from the complex and reprogramming gene expression to overcome the sulfur-deficient condition at the transcriptional level. OAS treatment or conditional *in planta* OAS accumulation using transgenic SAT plants induces APR and other genes, such as sulfur transporters in Arabidopsis, although the role of OAS as a signaling molecule is not generally accepted or controversial (Hirai et al. 2003; Hopkins et al. 2005; Hubberten et al. 2012). The resulting enhanced uptake or utilization of sulfate is expected to reduce the cellular OAS concentration and create favorable conditions for SAT–OASS interactions. According to Hell and Hillebrand, cysteine and sulfide affect the equilibrium between bienzyme complex assembly and dissociation (Hell and Hillebrand 2001). Sulfide-mediated stabilization of the complex, which provides extra OAS, could be useful for the rapid conversion of cytotoxic sulfide into stable cysteine amino acids (Wirtz and Hell 2006). Regulation of cysteine biosynthesis at the protein complex level is supported by a rather constitutive transcription of SAT and OASS in general, with a few exceptions under stress conditions (Barroso et al. 1995, 1999; Hesse et al. 1999; Yamaguchi et al. 2000; Domínguez-Solís et al. 2001; Kawashima et al. 2005). Taken together, these data suggest that the SAT–OASS complex functions as a macromolecular sensor, which re-balances fluxes in cysteine biosynthesis. To reflect its function, the name "cysteine regulatory complex (CRC)" was adopted to describe the SAT–OASS bienzyme complex (Yi et al. 2010a). However, lack of mitochondrial CRC in moss and spinach leads one to question how widely macromolecular complex formation modulates subcellular cysteine synthesis in land plants (Lunn et al. 1990; Birke et al. 2012b; Warrilow and Hawkesford 2000).

CRC formation protects the SAT enzyme under conditions leading to the inhibition and inactivation of the enzyme. The effect of high cysteine concentrations on cysteine biosynthesis are two-fold in the cell, leading to the feedback inhibition of free SAT and the abolishment of enhanced SAT activity through the dissociation of the complex (Kumaran et al. 2009). CRC formation increases the catalytic capacity of the plant SAT enzyme (Droux et al. 1998; Kumaran et al. 2009; Wirtz et al. 2010). Recent observations expanded the role of CRC formation. While free SATs outside the CRC complex are in general sensitive to feedback inhibition by cysteine, CRC formation can significantly mitigate the inhibitory effect of cysteine on SAT activity (Saito et al. 1995; Kumaran et al. 2009; Wirtz et al. 2012). For example, the K_I for cysteine is approximately 70 μM for soybean SAT in CRC and 2 μM for free SAT (Kumaran et al. 2009). Considering that the

organellar concentrations of cysteine in plant cells are in the ranges capable of inhibiting free SAT enzymes, CRC formation seems to contribute to the efficient production of OAS (Krueger et al. 2009). CRC formation also abolishes the substrate inhibition observed for free SAT with serine, which is present at the millimolar level in plant cells (Kumaran et al. 2009). CRC formation may reposition and reshape the C-terminus of SAT, thereby preventing its stable interaction with cysteine and serine. The OASS-interacting region of SAT is located close to the amino acid residues that make physical contact with the cysteine feedback inhibitor (Inoue et al. 1999; Olsen et al. 2004). In addition, CRC formation protects plant SATs from so-called "cold inactivation" (Yi et al. 2013). Although the catalytic activity of the free SAT enzyme incubated at low temperatures (0–4 °C) for 60 min decreases significantly, approximately 20 % compared to that of the SAT enzyme at 25 °C, that of SAT in CRC remains unchanged at low temperatures. A similar phenomenon was reported previously for a bacterial SAT from *E. coli* (Mino et al. 2000). Physical interactions with OASS may prevent SAT from acquiring a conformation that is catalytically less efficient and preferred at low temperatures.

In the model plant Arabidopsis, individual BSAS members have overlapping but distinct roles in cysteine metabolism that are important for plant growth and development

OASS and other-related BSAS enzymes with PLP cofactors are involved in various aspects of cysteine metabolism, including cysteine production, conversion of cysteine into β-cyanoalanine, and cysteine breakdown (Yi and Jez 2012). Reaction intermediates and products in these processes (OAS, sulfide, cysteine, cyanide, and *S*-sulfocysteine) are used as metabolites and signaling molecules for plant responses and development (Romero et al. 2014).

OASS for growth, stress tolerance, and pollen development

OASS: OAS + sulfide → cysteine + acetate

In Arabidopsis, BSAS members catalyzing cysteine production in different subcellular compartments have partially overlapping roles. It is assumed that only the BSAS members with OASS activity can interact with SAT to form CRC (Jost et al. 2000). The partial genetic redundancies revealed by reverse genetic approaches clearly demonstrate that knock-out of any individual BSAS member does not lead to seedling lethality (Heeg et al. 2008; Watanabe et al. 2008a). It seems that cysteine can be exchanged easily across subcellular membrane barriers. Multiple facilitated mechanisms that mainly depend on the pH gradient for cysteine transport are present in mitochondrial membranes (Lee et al. 2014). Nonetheless, BSAS isoforms with a catalytic preference for cysteine production show differential contributions to specific physiological processes.

The most abundant cytoplasmic OASS, *AtBSAS1;1* (*AT4G14880* or *AtOASA1*), is required for tolerance to abiotic and biotic stresses. *AtBSAS1;1* is responsible for the synthesis of approximately 50 % of the cysteine in leaves and 75 % of the cysteine in roots (Heeg et al. 2008; López-Martín et al. 2008; Watanabe et al. 2008a), and is induced under salt and heavy metal stress conditions (Barroso et al. 1999; Domínguez-Solís et al. 2001). The role of AtBSAS1;1 in heavy metal stress responses is confirmed by the higher tolerance of transgenic plants overexpressing *AtBSAS1;1* and the hypersensitivity of two independent knock-out mutants on a cadmium-supplemented medium (Domínguez-Solís et al. 2001; López-Martín et al. 2008). In addition, *atbsas1;1* mutants show higher susceptibility to both biotrophic and necrotrophic pathogens than wild-type plants (Álvarez et al. 2012a; Tahir et al. 2013). External application of cysteine at the site of pathogen infection restores the hypersensitive response in *atbsas1;1*. Two independent T-DNA insertional knock-out mutants of *atbsas1;1* show no obvious growth phenotypes under standard growth conditions, although increased production of H_2O_2 and ROS is evident at the cellular level (Heeg et al. 2008; López-Martín et al. 2008; Watanabe et al. 2008a). Intriguingly, a point mutation in AtBSAS1;1 that substitutes glycine 162 with glutamate causes necrosis in the leaves in a semidominant manner, which differs from the phenotypes of knock-out mutants (Shirzadian-Khorramabad et al. 2010). The mutant *old3* (*onset of leaf death 3*), which carries this substitution mutation, displays its phenotype depending on temperature and genomic background, while the mutant protein has no detectable OASS activity. The genome-dependent leaf death phenotype of *old3* requires a specific *RPP1*-like plant disease resistance gene that may or may not be present in different Arabidopsis accessions (Tahir et al. 2013). It was proposed that specific forms of RPP1-like proteins monitor possible modification(s) in AtBSAS1;1 by unknown virulence factors and then activate defense responses. These results indicate that AtBSAS1;1 plays a major part in antioxidative protection during biotic and abiotic stress responses in Arabidopsis.

Alterations in the mitochondrial OASS, *AtBSAS2;2* (*AT3G59760* or *AtOASC*), lead to defects in growth and root hair development. Although *AtBSAS2;2* contributes to only approximately 5 % of the total cysteine production in leaves, and the phenotypic outcome is apparently

influenced by yet-to-be-specified environmental or nutritional conditions, mutation of *AtBSAS2;2* can result in a dramatic decrease in biomass (by approximately 50 % dry weight) of the wild type (Heeg et al. 2008; Álvarez et al. 2012c). The *atbsas2;2* mutant shows a significant accumulation of cyanide and sulfide (Álvarez et al. 2012c). These changes may explain the short root hair length observed in *atbsas2;2*. Both cyanide and sulfide can inhibit cytochrome c oxidase for electron transport in mitochondria. A significant decrease in cytochrome c oxidase activity, along with the increase in alternative oxidase transcription and respiration rate, was also detected in the *atbsas2;2* mutant (Álvarez et al. 2012c; Birke et al. 2012a).

Arabidopsis mutants defective in more than one *BSAS* member show different phenotypes depending on the specific combination of *BSAS* mutations. Although *atbsas2;1*, a mutant of plastidic OASS (*AT2G43750* or *AtOASB*), along with the *atbass1;1* single mutant, does not exhibit any visible phenotypes under normal conditions, the *atbass1;1 atbsas2;1* double mutant shows stunted morphology, which is even stronger than that of the *atbsas2;2* mutant (Heeg et al. 2008). OAS, cysteine, and glutathione content are higher in *atbass1;1 atbsas2;1* double mutants than in wild-type plants, despite the severe growth retardation. Unlike *atbass1;1 atbsas2;1*, no visible phenotypes have been reported for *atbass2;1 atbsas2;2* double mutants, which lack major OASS enzymes in chloroplasts and mitochondria (Birke et al. 2013). Similarly, no obvious growth alteration is observed for the *atbass1;1 atbsas2;2* double mutant during the vegetative stage. However, silique length, which reflects the degree of successful fertilization, is significantly reduced in this double mutant (Birke et al. 2013). Reciprocal crosses between mutants defective in combinations of major BSAS genes (*AtBSAS1;1*, *AtBSAS2;1*, and *AtBSAS2;2*) revealed that problems in male gametophytes or pollens from *atbass1;1 atbsas2;2* plants cause reduced fertility. The pollen phenotype is *BSAS* gene dose-dependent and associated with the amount of maternally supplied glutathione. *In vitro* pollen germination, in the absence or presence of glutathione, shows that external application of glutathione can restore the germination frequency of affected pollens. The *pad2* mutant, which has approximately 20 % of the glutathione observed in the wild type, also shows a pollen germination problem (Zechmann et al. 2011). Triple mutation of three major *OASS* genes (*AtBSAS1;1*, *AtBSAS2;1*, and *AtBSAS2;2*) in three compartments leads to male gametophyte lethality, indicating that loss of combined activity cannot be compensated by other BSAS members, at least in pollen (Birke et al. 2013).

CAS for root hair development and plant defense responses

CAS: cysteine + cyanide → β-cyanoalanine

Using a reaction mechanism similar to that catalyzed by OASS, the CAS enzyme catalyzes a reaction that removes cyanide by producing β-cyanoalanine (Yi et al. 2012; García et al. 2014) (Fig. 3). In monocot and dicot plants, CAS enzymes are found inside mitochondria, where cyanide can inhibit cytochrome C oxidase in the electron transport chain (Hatzfeld et al. 2000; Lai et al. 2009). In plants, cyanide, a gaseous defense chemical, is produced during the biosynthesis of ethylene and camalexin, which are involved in defense responses (Yip and Yang 1988; Chivasa and Carr 1998; Böttcher et al. 2009; Seo et al. 2011). β-Cyanoalanine produced during the detoxification process is used by nitrilase to synthesize asparagine and aspartate; nitrilase has a remarkably high hydratase activity (Piotrowski and Volmer 2006).

A T-DNA insertional mutation of the *CAS* gene in Arabidopsis (*AtBSAS3;1*, *AT3G61440*, *CYSC1*) shows alterations in root hair development. The *atbsas3;1* mutant with significantly reduced CAS activity (approximately 23 % of that in wild-type plants) shows abnormal root hair elongation, but is otherwise similar to wild-type plants in its development (García et al. 2010). Root hair development in the mutant, which begins normally, is soon aborted without much elongation, resulting in the production of small bulges. The mutant is characterized by a high-cyanide content in the root, which is associated with the decreased production of ROS in the root hair tip. The role of cyanide in root hair defects was further confirmed by the following observations: (1) cyanide treatment induces an *atbsas3;1*-like phenotype in wild-type roots, and (2) treatment with hydroxocobalamin, a cyanide antidote, restores normal root hair development in the *atbsas3;1* mutant (García et al. 2010). A similar root hair phenotype is observed in the *atbsas2;2* mutant, which is defective in mitochondrial OASS (Álvarez et al. 2012c). Consistent with the phenotypic outcome, cyanide accumulation is increased in the *atbsas2;2* mutant. The mitochondrial OASS was suggested to play an important role in the detoxification of sulfide, which is generated during the cyanide detoxification process by the mitochondrial CAS enzyme. The cytotoxic effect of hydrogen sulfide gas, which acts on cytochrome C oxidase, is well established in mammals (Cooper and Brown 2008; Birke et al. 2012a).

In addition to its role in root hair development, CAS is involved in defense responses. A genome-wide analysis of genes differentially expressed in the *atbsas3;1* mutant and wild-type plants showed that the expression of biotic stress-

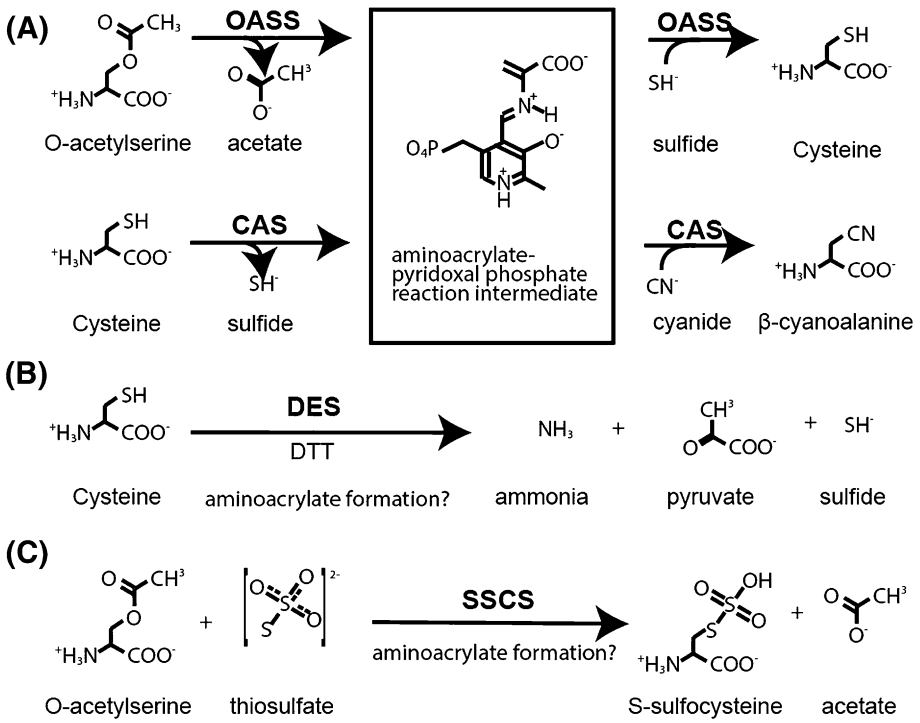

Fig. 3 Reactions catalyzed by various BSAS members in plants. (**A**) OASS (*O*-acetylserine sulfhydrylase) and CAS (β-cyanoalanine synthase) use an α-aminoacrylate-pyridoxal phosphate reaction intermediate to produce cysteine and β-cyanoalanine from *O*-acetylserine and cysteine, respectively. Sulfide and cyanide, as well as acetate and sulfide, in the products, are located in the β-position in the amino acid derivatives. The abbreviated names of the enzymes are shown in *bold*. (**B**, **C**) DES (cysteine desulfhydrase) degrades cysteine into ammonia, pyruvate, and sulfide in the presence of reducing agents, such as DTT (dithiothreitol). SSCS (*S*-sulfocysteine synthase) uses *O*-acetylserine and thiosulfate to produce *S*-sulfocysteine and acetate. It has not been demonstrated experimentally that the aminoacrylate intermediate is produced during the reactions catalyzed by SSCS and DES. The abbreviated names of the enzymes are shown in *bold*

responsive genes correlates with the endogenous cyanide content (García et al. 2013). The predicted role of *ATBSAS3;1* in plant defense was confirmed by an infection experiment using various pathogens. In brief, *atbsas3;1* displays higher resistance to the avirulent biotrophic pathogens *Pseudomonas syringae* and geminivirus (beet curly top virus), whereas it shows increased susceptibility to the necrotrophic pathogen *Botrytis cinerea*. The elevated ROS level and constitutive expression of *PR-1*, a proxy of defense signaling activation, suggest that *atbsas3;1* activates SA-dependent defense signaling even in the absence of pathogen infection.

DES (cysteine desulfhydrase) for immune response and autophagy

DES: Cysteine → pyruvate + ammonia + sulfide

BSAS4;3 (*AT5G28030* or *DES1*) counteracts *BSAS1;1* and maintains cysteine homeostasis in the cytosol. *BSAS4;3* encodes a cytosolic enzyme with L-cysteine desulfhydrase activity that degrades cysteine (Álvarez et al. 2010) (Fig. 3). The enzyme also catalyzes cysteine synthesis, although to a much lesser extent than its L-cysteine desulfhydrase activity. The *bsas4;3* mutant still has approximately 80 % of the wild-type desulfhydrase activity.

In line with the main catalytic activity of BSAS4;3 during cysteine degradation, slightly higher cysteine and glutathione concentrations are observed in the *bsas4;3* mutants than in wild-type plants. Similar to BSAS1;1-overexpressing transgenic plants with a high cysteine content, *bsas4;3* mutants show higher tolerance to oxidative stress imposed by cadmium or H_2O_2 treatment than wild-type plants (Domínguez-Solís et al. 2001; Álvarez et al. 2010). In addition, *bsas4;3* mutants show early flowering and early onset of senescence-associated gene expression (Álvarez et al. 2010). Similar to *atbsas3;1*, *bsas4;3* mutants behave as plants with constitutive, systemic acquired resistance, showing high levels of SA accumulation and constitutive upregulation of *PR-1*. However, *bsas4;3* mutants are resistant to both necrotrophic (*B. cinerea*) and biotrophic pathogens (virulent *P.*

syringae) (Álvarez et al. 2012a). Contrary to the phenotypes of *bsas4;3* mutants, *bsas1;1* mutants with decreased cysteine show high susceptibility to both biotrophic and necrotrophic pathogens, as well as hypersensitivity to cadmium treatment (López-Martín et al. 2008; Álvarez et al. 2012a).

The phenotypic outcome of the *bsas4;3* mutation is partially attributable to the decrease in sulfide, which is one of the degradation products of cysteine. Senescence-associated vacuole formation and autophagy-associated ATG8 accumulation/lipidation, which appear precociously in the *bsas4;3* mutant, can be rescued by sulfide treatment (Álvarez et al. 2012b). Transcriptional changes reflecting early senescence can be reversed by sulfide treatment. In addition, abscisic acid (ABA)-induced stomatal closure requires functional *BSAS4;3* to generate sulfide as a signaling molecule (Scuffi et al. 2014). Failure of stomatal closure in the *bsas4;3* mutant can be rescued by hydrogen sulfide treatment. Transcriptional regulation of *BSAS4;3* suggests that sulfide signaling, at least in Arabidopsis, interacts with hormone signaling, namely, transcriptional upregulation and repression are induced by ABA and auxin, respectively (Laureano-Marín et al. 2014).

SSCS (*S*-sulfocysteine synthase) for proper chloroplast development and growth

SSCS: OAS + thiosulfate → S-sulfocysteine + acetate

Unlike BSAS2;1, a BSAS targeted to plastids to produce cysteine, and BSAS5;1 (AT3G03630 or CS26) generates *S*-sulfocysteine in the chloroplast (Fig. 3). Arabidopsis BSAS5;1 is similar to bacterial CYSM in terms of a deletion of approximately 10 amino acid residues between the β8A and β9A domains, which are conserved among plant BSAS and bacterial CYSK with OASS activities (Bermúdez et al. 2010). The *bsas5;1* mutant shows light regime-dependent growth phenotypes associated with photosynthetic capacity. Under long-day (LD) conditions, *bsas5;1* mutants develop small and pale-green leaves when compared with wild-type plants; however, the mutants are indistinguishable from the wild type under short-day (SD) conditions (Bermúdez et al. 2010). The mutants show a higher production of ROS than wild-type plants only when plants are grown under LD conditions. A significant reduction of most photosynthetic parameters and an increase in nonphotochemical quenching, which can generate excess electrons resulting in higher ROS production, are observed in the mutants (Bermúdez et al. 2012). *S*-sulfocysteine, which is produced by BSAS5;1 in the thylakoid lumen, was suggested to act as a mild oxidant that functions as a signaling molecule to dissipate excess light energy absorbed in the form of heat (Gotor and Romero 2013). Thiosulfate accumulation increases dramatically (approximately 1000-fold) when the sulfide supply is sufficient in Arabidopsis (Birke et al. 2015). It is possible that *S*-sulfocysteine is used as a precursor of cysteine in plants, given that cysteine can be generated from *S*-sulfocysteine in *E. coli* if enough reducing power is provided (Nakatani et al. 2012).

Genome-wide study of BSAS enzymes in *Brassica* plants offers tools for fine-tuning cysteine homeostasis in desired plant phenotypes

Investigation of the exact role of each BSAS member in various plants, especially in *Brassica* crop plants, will be informative for developing plant varieties with enhanced production of sulfur-containing molecules such as glucosinolates. Studies using Arabidopsis showed that at least four different biochemical activities are attributable to individual enzymes in the eight-member BSAS family (Watanabe et al. 2008a; Álvarez et al. 2010; Bermúdez et al. 2012). Research using mutants also revealed that each member has distinct but somewhat overlapping roles in Arabidopsis growth, development, and various biotic and abiotic stress responses (Romero et al. 2014). Based on these results, several substrates or products of BSAS enzymes (OAS, sulfide, cysteine, cyanide, and *S*-sulfocysteine) have been proposed and considered as signaling molecules in Arabidopsis (Gotor and Romero 2013; García et al. 2014; Romero et al. 2014). Although much progress has been made on the biochemical and physiological roles of BSAS in the model plant Arabidopsis, no genome-wide functional study of BSAS members has been performed in plant species other than soybean (*Glycine max*). Sixteen putative BSAS genes were identified in the soybean genome and three biochemical activities have been confirmed for approximately half of these 16 genes as follows: cysteine synthesis in five genes, cysteine degradation in one gene, and cyanide detoxification in one gene (Yi and Jez 2012). However, *S*-sulfocysteine synthesis activity was not detected when one candidate gene was tested. Whether BSAS enzymes in other plants also display the four distinct biochemical activities identified in Arabidopsis remains unclear; neither is it clear which biochemical reaction is preferred by individual BSAS enzymes. Only some *BSAS* genes responsible for OASS, CAS, or DES activities have been identified in apple, spinach, potato, rapeseed, and other plants (Hatzfeld et al. 2000; Maruyama et al. 2000; Han et al. 2007; Xie et al. 2013). The subcellular localization and relative contribution of a specific isoform to a distinct BSAS activity have not been investigated in plants other than Arabidopsis. A systematic study of BSAS members in

diverse plants may provide answers to these questions. Such a study will also provide a basis to identify the key active site amino acid residues responsible for the apparent biochemical preferences among different BSAS enzymes, as done for the soybean CAS enzyme (Yi et al. 2012).

A genome-wide study of BSAS members in *Brassica* crop plants, which are closely related to Arabidopsis in the *Brassicaceae* family, would be beneficial. Although the importance of sulfur nutrition in stress responses was first identified more than 20 years ago, information on basic cysteine metabolism in *Brassica* species, which provides the entry point of organic sulfur, remains largely unknown at the gene level (Booth et al. 1991; Bloem et al. 2015). Moreover, most existing physiological studies on sulfur metabolism have focused on *Brassica napus*, with little research in other *Brassica* plants. Knowledge of Arabidopsis BSAS enzymes will facilitate the prediction of putative *BSAS* members in many *Brassica* plants with different agricultural traits. In particular, biochemical characterization will help us understand how cysteine homeostasis is modulated by the coordinated regulation of various BSAS members in these species with a high cysteine demand for glucosinolate production and a proper defense response (Schnug et al. 1995; Bloem et al. 2004). Furthermore, information collected for *Brassica* BSAS enzymes can be used in targeted breeding to achieve the desired sulfur metabolism, as well as for biotechnological improvement. The information will broaden our understanding of how individual BSAS members have evolved and diverged. To date, the genome sequences of five different *Brassica* species have been reported, namely *B. rapa* ssp. *pekinensis* (Wang et al. 2011), *B. rapa FPsc* (*Brassica rapa FPsc* v1.3, DOE-JGI, http://phytozome.jgi.doe.gov/), *B. oleracea* var. *capitata* (Liu et al. 2014), *B. oleracea* var. *oleracea* (Parkin et al. 2014), and *B. napus* 'Darmor-*bzh*' (Chalhoub et al. 2014). A genome-wide survey of putative BSAS enzymes in *B. rapa* ssp. *pekinensis* line Chiifu-401-42 identified 12 different putative BSAS members localized in the cytosol, mitochondria, or chloroplasts (Table 1). Furthermore, phylogenetic analysis of Chiifu BSAS protein sequences in comparison with those in Arabidopsis and soybean revealed that Chiifu BSAS members are found in five different clades, which seems to reflect functional diversification (Fig. 4). Previously, a similar approach was successfully used to predict the biochemical specialization of soybean BSAS enzymes (Yi and Jez 2012). According to the study, BSAS enzymes in clades I and III are OASSs, while those in clades V are CASs. Others in clade II include DES enzymes. The complete identification and biochemical characterization of *BSAS* genes in other *Brassica* species will be necessary to fully understand cysteine biosynthesis in *Brassica* plants.

Extensive information on BSAS enzymes with various plant origins and activities will be useful for the production of unnatural amino acids and drugs for biomedical purposes

A detailed understanding of the substrate specificity and exact reaction mechanisms of BSAS enzymes in various plants will lead to the development of efficient semisynthetic methods for producing unnatural amino acids for biomedical purposes. The reactions catalyzed by OASS enzymes start with the formation of α-aminoacrylate, which is likely to be conserved among other BSAS members (Fig. 2). β-Replacement, the second half of the BSAS reaction, involves a nucleophile substrate and shows relaxed substrate specificity. Plants and other organisms contain various S-substituted cysteine derivatives and heterocyclic β-substituted alanine, such as L-mimosine, and these and artificial amino acids can be produced by OASS enzymes (Ikegami and Murakoshi 1994; Maier 2003). Amino acids with unnatural side chains have markedly different physicochemical properties and provide remarkable opportunities for the development of new biomedical materials for use as drugs and reactants, and for other uses (Stevenazzi et al. 2014). Amino acids with unnatural side chains in the protein can vastly expand the number of post-translational modifications and increase the stability of proteins in biological contexts. One example is Trastuzumab, an antibody–drug conjugate in which the auristatin toxin is ligated to *para*-acetylphenylalanine in the light chain of a monoclonal antibody (Axup et al. 2012). Investigation of the exact role of the substrate tunnel, which seems to modulate the size, shape, and properties of incoming nucleophiles for the second half of the BSAS reaction, and the subsequent engineering of it and other parts of BSASs is likely to provide a possible semisynthetic route to produce desirable unnatural amino acids (Renata et al. 2015). In addition, a genome-wide approach to the study of BSAS enzymes in various plant species may identify enzymes that catalyze new β-substitution reactions, which have not been reported in Arabidopsis.

Extensive knowledge of the mechanism and forces responsible for the OASS/SAT interaction may offer valuable information for the development of antibacterial drugs. Animals do not require the OASS enzyme for cysteine biosynthesis, as cysteine in animals is produced using methionine as a starting material and homocysteine as a reaction intermediate in a process called "reverse transsulfuration." Unlike animals, but similar to plants, microorganisms, including human pathogens such as *Mycobacterium tuberculosis* and *Salmonella typhimurium*, rely on an OASS-dependent cysteine supply for antibiotic resistance and the antioxidation process, which are essential for

Table 1 List of putative BSAS members in *Brassica rapa* ssp. *pekinensis* line Chiifu-401-42

Identifier	Chromosome: coordination	Predicted localization iPSORT/TargetP 1.1	Protein length (amino acids)/ molecular weight (kDa)	Note
Bra036910	A01: 11965137–11967125	Chloroplast/cytosol	319/33.53	Clade I
Bra039708	A08: 6553205–6555085	Cytosol/cytosol	322/33.85	Clade I
Bra036115	A09: 2409285–2411150	Cytosol/cytosol	323/34.39	Clade II
Bra020605	A02: 23711526–23716079	Possibly cytosol (mitochondria/mitochondria)	434/47.25	Clade II
Bra036114	A09: 2412752–2414449	Cytosol/cytosol	342/36.83	Clade II
Bra001131	A03: 14920698–14924088	Possibly cytosol (mitochondria/mitochondria)	400/43.27	Clade II
Bra004781	A05: 1807522–1809777	Chloroplast/chloroplast	396/42.04	Clade III
Bra037682	A04: 18304741–18307197	Chloroplast/chloroplast	389/41.29	Clade III
Bra014529	A04: 1250650–1253526	Mitochondria/mitochondria	422/45.05	Clade III
Bra036448	A01: 26246912–26248806	Chloroplast/chloroplast	393/41.89	Clade IV
Bra007604	A09: 31496716–31499336	Mitochondria/mitochondria	368/39.66	Clade V
Bra014436	A04: 645518–647426	Mitochondria/mitochondria	368/39.81	Clade V

NA not applicable

The identifier and chromosomal location of each gene were obtained from the "chromosome v.1.5" in BRAD (http://brassicadb.org/cgi-bin/gbrowse/Brassica/). Possible subcellular locations of proteins were predicted using two web-based analysis tools, iPSORT(http://ipsort.hgc.jp/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Bannai et al. 2002; Emanuelsson et al. 2007). Clade numbers were designated based on previous grouping, which reflects sequence similarity, subcellular localization, and biochemical functions in *Arabidopsis* (Yi and Jez 2012)

Bra020605 and *Bra001131* are N-terminally fused to parts of ribosomal proteins. Predicted locations by web-based tools are indicated in parenthesis.

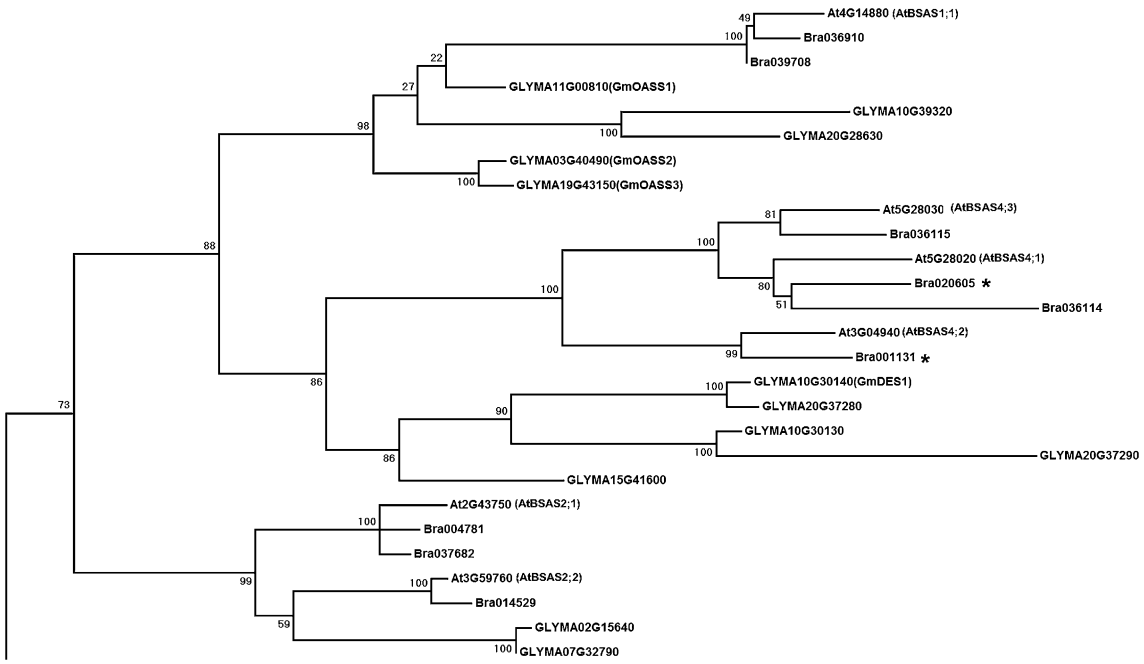

Fig. 4 Molecular phylogenetic analysis of BSAS in several model plants using the Maximum likelihood method. The evolutionary history was inferred using the Maximum likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (−6044.4367) is shown. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths determined by the number of substitutions per site. All positions with less than 95 % site coverage were eliminated. Evolutionary analyses were conducted using MEGA6 (Tamura et al. 2013)

the survival and virulence of these pathogens during infection (Bhave et al. 2007; Turnbull and Surette 2008). This clear difference in cysteine metabolism has made OASS a promising target for antimicrobial molecules (Salsi et al. 2010). In addition, the finding that OASS activity is inhibited by complex formation with SAT, and that inhibition is

mediated by the physical insertion of SAT C-terminal peptides into the OASS active site, raises the possibility that peptidic or nonpeptidic inhibitors mimicking the behaviors of SAT C-termini can work as potent drugs targeting OASS (Mino et al. 2000; Huang et al. 2005; Salsi et al. 2010). Docking simulations of pentapeptides inside an OASS active site, followed by experimental determination of binding affinities, proved the feasibility of this approach and helped define a pharmacophoric scaffold for peptidomimetic inhibitor drugs (Salsi et al. 2010). A follow-up study revealed that interactions between the OASS active site and pentapeptides are heavily affected by subtle changes in the active sites (Spyrakis et al. 2013). The binding specificity and strength are largely determined by peptide sequences in the P1–P2 and P3–P4–P5 positions, respectively. Further research on SAT–OASS interactions in various organisms, including plant enzymes, will broaden our understanding of the interaction mechanism and provide a basis to design specific drugs targeting the cysteine biosynthesis pathway in pathogens.

Concluding remarks

In Arabidopsis, genome-wide identification, biochemical studies, and mutant analyses of individual BSAS enzymes expanded our understanding of cysteine metabolism and its important roles in various aspects of plant life. The accumulated information about the model plant and other plant species will enable comparative genomic studies of BSAS enzymes in crop plants. The comparative genomic approach will be especially useful for answering the following questions: (1) which reaction is catalyzed by an individual BSAS enzyme in the plant of interest (2) how have BSAS enzymes evolved to obtain different biochemical specificities, and (3) how are BSAS enzyme activities for cysteine homeostasis modulated in coordination to achieve optimum plant growth and stress responses in different plants? The diversity of *Brassica* plants, their high demand for cysteine, and their close phylogenetic relationship with Arabidopsis make them perfect materials for this approach. Future research on BSAS enzymes in *Brassica* plants will provide information that can be applied to breeding for desired phenotypes and to the development of BSAS enzymes with preferred biochemical properties.

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