Review article

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Current status of research on *o*-acetylserine (thiol) lyase and β-cyanoalanine synthase, two enzymes of plant cysteine biosynthesis- a review

Annabelle U. Novero

Department of Biology, College of Science and Mathematics
University of the Philippines Mindanao, Davao City 8022. The Philippines

E-mail: anovero@upmin.edu.ph

Abstract

The production of cysteine is the first major significant event in the plant sulfate assimilation process. Cysteine is a precursor of many important biological molecules. Two of the enzymes involved in cysteine biosynthesis are β-cyanoalanine synthase (CAS) and *o*-acetylserine (thiol) lyase, members of the Beta-subsituted alanine synthase (Bsas) gene family. There were about forty Bsas genes cloned and characterized from a wide range of plant families in a span of about two decades. Some information on the molecular and biochemical characteristics, as well as subcellular localization of the Bsas genes were recorded. Several reports concurred that the Bsas genes are regulated by external factors such as sulfur and nitrogen availability. More research work toward the elucidation of as many cysteine biosynthesis genes as possible is needed because the biochemical functions of majority of such genes deposited in the databases have not yet been proven. The results will have direct applications in sulfur-related genetic engineering of plants such as breeding for higher protein content.

Keywords: sulfate assimilation; cysteine synthase; gene expression

Abbreviations: APR-adenosine phosphosulfate reductase; Bsas-β-substituted alanine synthase; CAS-β-cyanoalanine synthase; OAS-TL-O-acetylserine (thiol) lyase; SAT-serine acetyltransferase

Introduction

Sulfur is an important macroelement that is vital to life. It is much less abundant in nature than carbon and nitrogen because it is not a major structural component of biomolecules. Instead, sulfur is a major catalytic and electro- chemical component essential for numerous biological functions (Leustek *et al*., 2000). Plants take up sulfur from the soil in the form of sulfate (SO_4^{2-}; Saito, 2004). Biological functions in plants that are mediated by sulfur include electron transport in Fe/S clusters in photosynthesis and respiration, catalytic centers and protein-disulfide bridges (Hell, 1997). Long-distance translocation of sulfate plays a significant role in sulfur assimilation although little is known about the specificity of this transport (Hell, 1997). It is presumed that in the plant cell, the vacuole is the main storage center for sulfate (Cram, 1990).

Unlike plants, animals do not synthesize sulfur from sulfate and are dependent on plants for their sulfur nutrition. Animals take up sulfur in the form of methionine. Cysteine is the sulfur donor for methionine and other sulfur-containing secondary products (Saito, 2004). Hence, cysteine, methionine and other secondary products that contain sulfur such as thiamine and biotin are essential for human nutrition. In plants, sulfate is activated to adenosine 5'-phosphosulfate (APS), reduced to sulfite (SO_3^{2-}), and then to sulfide (S^{2-}). Cysteine is formed when sulfide is coupled with O-acetylserine (OAS), which is formed from serine and O-acetyl coenzyme A. There are two biochemical pathways involved in the formation of cysteine from sulfur in plants. The first occurs when sulfate (SO_4^{2-}) is reduced to sulfide (S^{2-}). The second is the production of cysteine. Cysteine is

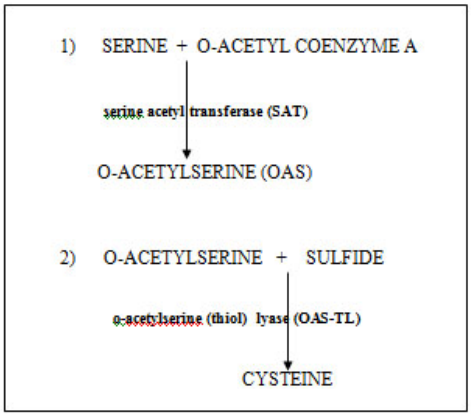

Fig 1. The two-step process of cysteine synthesis in plants.

synthesized in a two-step process (Figure 1; Hell *et al.,* 2002). The two major enzymes involved in the production of cysteine from sulfate are serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). O-acetylserine is produced by SAT during catalysis of the reaction between serine and acetyl coenzyme A. In the presence of OAS-TL, cysteine is generated by the reaction between OAS and free sulfide. Serine acetyltrans- ferase and OAS-TL together form a complex known as the SAT/OAS-TL bi-enzyme complex (also called "cysteine synthase complex") that regulates cysteine synthesis (Leustek *et al*., 2000). Both enzymes are present in the chloroplast, cytosol and mitochondrion (Hofgen *et al*., 2001). Cysteine formation is the final step in the pathway of sulfur assimilation in plants and its importance is comparable to the synthesis of glutamine in the nitrogen assimilation pathway. Cysteine and other metabolites that are derived from the sulfur pathway, that carry free sulfhydryl groups are called thiols (Hofgen *et al*., 2001).

Droux (2003) suggested that the cysteine synthase complex is the center of the cellular-metabolite sensing model. The complex is found in both plants and bacteria, suggesting an efficient metabolic channeling that prevents loss of intermediate OAS (Bogdanova and Hell, 1997). The complex itself is not responsible for efficient synthesis of cysteine since OAS-TL is mainly active in the free form and almost inactive when bound to SAT. However, the cooperativity between SAT and OAS-TL alters the kinetic behavior of SAT towards the substrate (OAS) when it is associated with the complex (Droux *et al.,* 1998). This interaction is significant for cysteine formation because the concentration of acetyl CoA in plastids is limiting (Roughan, 1997). The amount of SAT bound to the complex determines the rate of OAS formation, directly affecting cysteine formation (Berkowitz *et al*., 2002).

Although OAS-TL occupies a central position in the final step of the sulfur assimilation pathway, it was earlier assumed not to have a major role in the regulation of cysteine synthesis (Hofgen *et al*., 2001). This is because over-expression of OAS-TL was not found to affect the cysteine content in transgenic tobacco plants (Saito *et al*., 1994). However, Wirtz *et al*. (2004 studied the kinetic properties of both free and bound OAS-TL and found that fluctuations of OAS in the cell controls the binding and dissociation of OAS to OAS-TL. Therefore, OAS-TL was shown to control the rate of cysteine synthesis, establishing its integral role in the cysteine regulatory circuit (Wirtz *et al*., 2004).

The formation of β-cyanoalanine from cysteine

β-cyanoalanine is a non-protein amino acid that is formed when the enzyme β-cyanoalanine synthase (CAS) catalyzes the reaction between cysteine and cyanide (Blumenthal *et al*., 1968). Cyanide is a by-product of ethylene biosynthesis formed during cellular respiration. The CAS enzyme plays a major role in cyanide detoxification. For this reason, cyanide does not accumulate in non-cyanogenic plants even if the rate of ethylene biosynthesis is high (Miller and Conn, 1980; Poulton, 1990).

Two classes of CAS have been proposed to exist in plants on the basis of amino acid composition and protein structure: 1) a monomeric enzyme that was first detected in blue lupin (Akopyan *et al*., 1975), and; 2) a homodimeric enzyme that was first detected in spinach and *Lathyrus latifolius* (Ikegami *et al*., 1988a, 1988b). The second class is similar in structure to OAS-TL (Droux *et al*., 1992). CAS was first fully characterized by Blumenthal-Goldschmidt *et al*. (1963) following the hypothesis by Ressler (1962) that CAS and hydrolase are involved in the synthesis and degradation of β-cyanoalanine in plants. Hendrickson and Conn (1969) subsequently showed that CAS was distinct from all cysteine biosynthetic enzymes. In several species, both β-cyanoalanine and γ-glutamyl-β-cyanoalanine, a compound synthesized from β-cyanoalanine by γ-glutamyl transferase, were shown to be readily metabolized into asparagines by the enzyme asparaginase (Blumenthal *et al*., 1968). However, Ressler *et al*. (1969) found that the β-cyanoalanine route to asparagines in several accessions of *V. sativa* was inactive, leading to a buildup of the neurotoxin compound in plant tissues (Figure 2). The CAS enzyme has been purified from

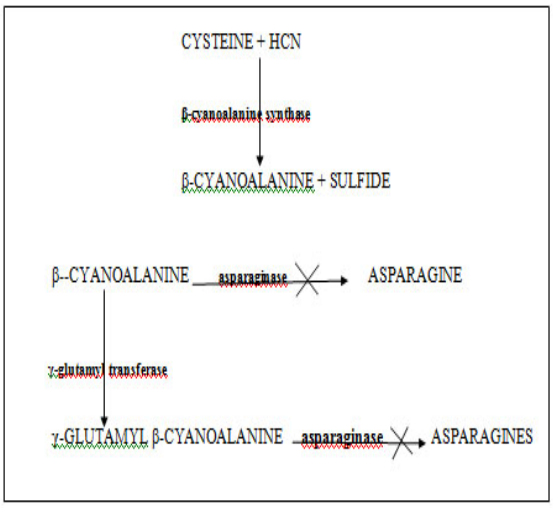

Fig 2. Pathways of β-cyanoalanine production and blockage of the asparaginase step in several accessions of *V. sativa*. (Adapted from Ressler *et al.*, 1969).

plants such as spinach, *Lathyrus* and *Vicia* (Akopyan *et al*., 1975; Ikegami *et al*., 1988a, 1988b, 1989) and is thought to exist exclusively in the mitochondria, due to its major role in the detoxification of hydrogen cyanide (HCN) during cellular respiration (Meyers and Ahmad, 1991). O-acetylserine (thiol) lyase and CAS are structurally and chemically similar, suggesting that the genes encoding them may have a common ancestor (Ikegami *et al*., 1989). Many studies have documented the high degree of structural and functional similarity between the two enzymes (Ikegami *et al*., 1989; Maruyama *et al*., 1998; Warrilow and Hawkesford 1998; Hatzfeld *et al.,* 2000). Comparison of the gene structures of four OAS-TL genes from *Arabidopsis* revealed close relatedness, which was presumed to be due to a common ancestor that gave rise to subsequent gene duplications (Jost *et al*., 2000). O-acetylserine (thiol) lyase A, B and C from *Arabidopsis* were found to have a bi-functional OAS-TL/CAS activity *in vitro* but function only as OAS-TL under natural physiological conditions (Jost *et al*., 2000).

β-cyanoalanine synthase was shown to catalyze the synthesis of cysteine and cyanoalanine reactions (Warrilow and Hawkesford, 2002). Some cysteine synthases have been found to catalyze the formation of β-cyanoalanine from OAS and HCN as an additional activity (Ikegami *et al*., 1989, Ikegami and Murakoshi 1994). High activities of both enzymes were detected in cyanogenic and non-cyanogenic plants including *Vicia angustifolia* (Ikegami *et al*., 1989). Studies have shown that in *Arabidopsis*, this dual activity is attributed to an evolutionary event that might have created a CAS gene via intrachromosomal duplication owing to its close position to OAS C on chromosome 3 (Hatzfeld *et al*., 2000).

The β-substituted alanine synthase gene family

β-substituted alanines are non-protein amino acids that are synthesized in plants as secondary metabolites (Ikegami and Murakoshi, 1994). The β-substituted alanine synthase (Bsas) gene family includes OAS-TL and CAS. They belong to a large family of genes that encode the pyridoxal phosphate-dependent enzymes (Yamaguchi *et al*., 2000). Pyridoxal phosphate (PLP) is a cofactor for OAS-TL activity and binds with its lysine residue to form a Schiff base (Hayashi 1995). The PLP enzymes are classified into α, β and γ subfamilies, in reference to how the protein products are folded (Yamaguchi *et al*., 2000). Pyridoxal phosphate-dependent enzymes are vitamin B_6 derivatives that are versatile organic cofactors used by many enzymes in biological reactions. Over 600 amino acid sequences that encode for approximately 60 B_6 enzymes from various plant species are present in published databases (Mehta and Christen, 1998). The B_6 enzymes link carbon and nitrogen metabolism and almost all are known to participate in amino acid biochemical pathways (Mehta and Christen, 1998).

Hatzfeld *et al.* (2000) proposed the classification of OAS and CAS genes into six subfamilies within the Bsas gene family. Table 1 shows a summary of the OAS-TL and CAS genes currently available in the databases. Based on kinetic analysis, proteins encoded by the Bsas family are classified into two groups, true OAS-TL and OAS-TL-like proteins (Warrilow and Hawkesford, 2000; Hatzfeld *et al.,* 2000; Jost *et al.,* 2000). True OAS-TL proteins exhibit both OAS-TL function and CAS activity but not cyanide detoxification (Jost *et al* 2000). Further, true OAS-TL proteins exhibit a side reaction by releasing sulfide from cysteine in the presence of thiols (Burandt *et al*., 2002). OAS-TL-like proteins catalyzed the formation of β-cyanoalanine in spinach and *Arabidopsis* (Hatzfeld *et al*., 2000; Maruyama *et al*., 2000).

Subcellular localization of OAS-TL genes

The gene encoding the OAS-TL enzyme was the first cloned gene in the plant sulfur assimilation pathway (Romer *et al*., 1992; Saito *et al*., 1992). The protein isoforms from the cytosol and plastids of spinach have subsequently been well-characterized (Droux *et al*., 1992; Rolland *et al*., 1993; Warrilow and Hawkesford, 1998). There are at least three isoforms of OAS-TL in higher plants. Lunn *et al*. (1990) suggested that the presence of multiple isoforms in

Table 1. Designation and subcellular localization of β-substituted alanine synthase genes.

Gene Family	Product	Gene Designation	Accession Number	Plant Species	Location	References
Bsas 1	OAS-TL	*So CS-A*	D10476	Spinach, *Spinacea oleracea*	Cytosol	Saito et al 1992
		Ta Cys1	D13153	Wheat, *Triticum aestivum*	Cytosol	Youssefian *et al* 1993
		Cl	D28777	Watermelon, *Citrulus vulgaris* var. *lanatus*	Cytosol	Noji *et al* 1994
		Zm Mcysp	X85803	Maize, *Zea mays*	Cytosol	Brander *et al* 1995
		At cys-3A	X84097	Thale cress, *Arabidopsis*	Cytosol	Barroso *et al* 1995
		At Cys1	X81697	*Arabidopsis*	Cytosol	Hesse and Altmann 1995
		Atg14880 (Bsas 1;1)	BT025878	*Arabidopsis*	Undetermined	Hell et al 1994; Barroso et al 1995; Jost et al 2000
		At3g22460 (Bsas 1;2)	NM_113145	*Arabidopsis*	Cytosol	Jost et al 2000
		Bj OAS-TL6	Y10847	Indian mustard, *Brassica juncea*	Cytosol	Schafer *et al* 1998
		Car	AJ006024	Chickpea, *Cicer arietinum*	Cytosol	Dopico *et al* 1998
		St CS-A	AF044172	Potato	Cytosol	Hesse and Hofgen 1998
		Os rcs1	AF073695	Rice, *Oryza sativa*	Cytosol	Nakamura *et al* 1999
		Os rcs3	AF073697	Rice	Cytosol	Nakamura *et al* 1999
		PCS-1	AB029511	Potato	Cytosol	Maruyama and Ishizawa 2001
Bsas 2	OAS-TL	*Can*	CAA46086	Pepper,*Capsicum annum*	Plastid	Romer *et al* 1992
		At cpACS1	X81698	*Arabidopsis*	Plastid	Hesse and Altmann 1995
		At mtACS1	X81973	*Arabidopsis*	Plastid	Hesse and Altmann 1995
		At3g22460 (Bsas 2;1)	AY065375	*Arabidopsis*	Plastid	Barroso et al 1995; Hesse et al 1999; Jost et al 2000
		At3g59760 (Bsas 2;2)	AY128885	*Arabidopsis*	Mitochondrion	Hesse et al 1999; Jost et al 2000
		PCS-2	AB029512	Potato, *Solanum tuberosum*	Plastid	Maruyama *et al* 2001
Bsas3	CAS	*So cys-C*	D37963	Spinach	Mitochondrion	Saito *et al* 1994
		At OAS5	AJ010505	*Arabidopsis*	Mitochondrion	Hatzfeld *et al* 2000
		Atg61440 (Bsas3;1)	Y128782	*Arabidopsis*	Mitochondrion	Hatzfeld et al 2000; Yamaguchi et al 2000
		PCAS-1	AB027000	Potato	Mitochondrion	Maruyama *et al* 2001
		PCAS-2	AB029338	Potato	Mitochondrion	Maruyama *et al* 2001
Bsas4	OAS-TL	*Bj OAS-TL5*	Y10846	Indian mustard	Cytosol	Schafer *et al* 1998
		At OAS3 (cysD1)	AJ011603	*Arabidopsis*	Cytosol	Hatzfeld and Saito 1999
		At OAS6 (cysD2)	AB024283	*Arabidopsis*	Cytosol	Yamaguchi *et al* 2000
		At5g28020 (Bsas 4;1)	AK317505	*Arabidopsis*	Cytosol	Hatzfeld et al 2000; Yamaguchi et al 2000
		At3g04940 (Bsas 4;2)	BT008721	*Arabidopsis*	Cytosol	Hatzfeld et al 2000; Yamaguchi et al 2000
		At5g28030 (Bsas 4;3)	BT2002155	*Arabidopsis*	Plastid	Nakamura *et al* 1997
Bsas5		*At CS26*	NM_111234.3	*Arabidopsis*	Cytosol	Nakamura *et al* 1997
		At3g03630 (Bsas 5;1)	BT002155	*Arabidopsis*	Plastid	Nakamura *et al* 1997
		Voas-tl5	DQ456491	Vetch, *Vicia sativa*	Cytosol	Novero et al 2008
Bsas6		*Os rcs4*	AF073698	Rice	Cytosol	Nakamura *et al* 1999
Unclassified		Soybean OAS-TL	AF452451	Soybean, *Glycine max*	Cytosol	Chronis and Khrishnan 2003
		Poplar OAS-TL	AY781280	Poplar, *Populus alba* x *Populus tremula*	Undetermined	Herschbach et al 2005
		Vicia sativa OAS-TL	EF193211	Vetch	Undetermined	Pajuelo et al 2007

different compartments may be due to the inability of the compartments to transport the enzymes. In *Arabidopsis*, there are nine OAS-TL-like genes (Table 1). The presence of isoforms in multiple compartments was presumed necessary in the coordination of cysteine and β-cyanoalanine metabolism (Nakamura *et al*., 1999). In general, the compartmentalization of isoforms and their varying substrate specificities has made the progress towards elucidating the exact functions of plant OAS-TL enzymes slow (Wirtz *et al*., 2004). To date, the exact number of OAS-TL isoforms and their functions has not been determined fully for any plant species.

Since the formation of cysteine is the first occurrence of sulfur in a reduced, organic form in the cell, all compartments involved in protein biosynthesis appear to contain the OAS-TL and SAT genes. The cytosol, plastids and mitochondria of spinach and cauliflower had been found to contain these two enzymes (Lunn *et al*., 1990; Rolland *et al*., 1992). In spinach, CAS activity was found to be predominant in the mitochondria but was also detected in the chloroplast and cytosol (Warrilow and Hawkesford 1998).

Regulation of OAS-TL activity

In the two-step process of sulfur assimilation and cysteine formation in plants, the following enzymes are involved: sulfate transporter, ATP sulfurylase, APS kinase, 3'(2)'-phosphatase, APS reductase, sulfite reductase, OAS-TL and SAT (Saito *et al*., 2000). O-acetylserine (thiol) lyase serves as the terminal enzyme. Sulfur availability is one of the factors that regulate the activities of these genes including OAS-TL (Nakamura *et al*., 1999).

The response of OAS-TL genes to varying levels of sulfur has been studied in several plant species. In *Arabidopsis*, OAS-TL was found to respond differently to sulfur starvation at the cell and whole plant levels. At the cell level, Takahashi and Saito (1996) found no significant effect of sulfur starvation on OAS-TL mRNA activity. In whole seedlings, an increase in OAS-TL activity was noted (Hesse *et al*., 1997). Warrilow and Hawkesford (1998) found small increases in OAS-TL activity from root tissues but no increase in activity in leaf tissues. At the level of gene expression, regulation of OAS-TL was deemed minimal. In soybean cotyledons, Kim *et al*. (1999) found sulfur deficiency led to the accumulation of OAS. In the plant metabolic pathway, cysteine is the first molecule that contains both nitrogen and sulfur (Saito, 2004).

Reuveny *et al*. (1980) found earlier that sulfur and nitrogen metabolism are linked and the absence or reduction of one leads to the deprivation of the other. Plants, therefore, have to adjust their metabolism to compensate for changes in nitrogen and sulfur contents. Such changes can trigger molecular mechanisms that can alter other biosynthetic pathways like glutamine biosynthesis (Hesse *et al*., 2004). For example, barley plants starved of sulfur were found to have a depressed ability to take up nitrates and ammonium accompanied by increased capacity for sulfur intake (Clarkson *et al*., 1989). Sulfur assimilation is dependent on the supply of OAS which is a precursor of cysteine. The availability of OAS in turn is dependent on nitrogen and carbon availability (Kopriva *et al*., 2002). Reactions catalyzed by the OAS-TL/SAT bi-enzyme complex represent the major link between carbon/nitrogen and sulfate assimilation (see Section 2.3). O-acetylserine (thiol) lyase and SAT are both found to have several isoforms found in the chloroplast, cytosol and mitochondrion, suggesting that the ability to form cysteine in all these three compartments is important for compartments that need to produce proteins (Hoefgen *et al*., 2001). Since there is insufficient evidence to explain these intricate mechanisms, there is a need to isolate and characterize all OAS-TL genes of a given plant.

Nakamura *et al*. (1999) determined that the functions of four OAS-TL isoforms in *Oryza sativa* were distinct from one another and were regulated in a coordinated way by the availability of sulfur, nitrogen and light. Sulfur starvation induced the accumulation of the *rcs1* gene in roots and shoots; *rcs2* accumulated in shoots grown only in light; *rcs3* was abundant in roots and was reduced in dark conditions and under sulfur deprivation and *rcs4* was scarce in all organs. Transcriptome analyses of genes involved in sulfur metabolism (two sulfate transporters and one APR (adenosine phosphosulfate reductase) gene in *Arabidopsis,* indicated that the presence of nitrate could induce the genes to have an increased capacity for uptake (Hesse *et al*., 2004). Data from the same study also suggested that the absence of nitrate in the roots could down-regulate the capture and assimilation of sulfur by the roots. In soybean, the accumulation of seed storage proteins was regulated by nitrogen and sulfur availability (Kim *et al*., 1999); cysteine synthesis was found to be dependent on the availability of sulfur and OAS (Chronis and Krishnan, 2003). Transcription levels of *Vicia sativa* OAS-TL increased as a response to sulfur stress (Novero and Ford, 2009), establishing the functionality of said gene.

***Arabidopsis* as a model for understanding plant cysteine biosynthesis**

Out of around 40 Bsas genes cloned from various plant species, nine isoforms were reported from *Arabidopsis* (Arabidopsis Genome Inititative 2000; Watanabe et al 2008). Some were well-characterized in terms of enzymatic characteristics and cellular localization: cytosol (Bsas 1;1), plastid (Bsas 2;1) and mitochondrion (Bsas 2;2). Despite the published details on the biochemical nature of the Bsas isoforms of *Arabidopsis*, much remains to be discovered about the molecular mechanisms of the genes in vivo as well as their influence on the response of the whole plant to sulfur deprivation. Since OAS-TL is a key metabolite for cysteine synthesis, it is important to determine the response of plant OAS-TL to sulfur nutritional stimuli because it will give a better understanding of this gene's molecular mechanism.

Conclusion

The creation of crops that are better equipped to provide improved nutrition for humans and animals is a major goal of plant genetic engineering. One of the ways to achieve this is by increasing the content of essential amino acids such as cysteine, the first major organic product of the plant sulfate assimilation pathway. Unlike the nitrogen assimilation pathway, there are still many gaps to be filled in the understanding of the enzymes and pathways of sulfate assimilation. In the past two decades, considerable progress has been made in the understanding of these genes but reports on biochemical characterization are few and far between. Thus, further research on the roles of the Bsas genes (OAS-TL and CAS) in as many crops as possible, are valuable.

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