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REVIEW ARTICLE

From sulfur to homoglutathione: thiol metabolism in soybean

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Abstract Sulfur is an essential plant nutrient and is metabolized into the sulfur-containing amino acids (cysteine and methionine) and into molecules that protect plants against oxidative and environmental stresses. Although studies of thiol metabolism in the model plant *Arabidopsis thaliana* (thale cress) have expanded our understanding of these dynamic processes, our knowledge of how sulfur is assimilated and metabolized in crop plants, such as soybean (*Glycine max*), remains limited in comparison. Soybean is a major crop used worldwide for food and animal feed. Although soybeans are protein-rich, they do not contain high levels of the sulfur-containing amino acids, cysteine and methionine. Ultimately, unraveling the fundamental steps and regulation of thiol metabolism in soybean is important for optimizing crop yield and quality. Here we review the pathways from sulfur uptake to glutathione and homoglutathione synthesis in soybean, the potential biotechnology benefits of understanding and modifying these pathways, and how information from the soybean genome may guide the next steps in exploring this biochemical system.

Keywords Soybean · Sulfur · Cysteine · Glutathione · Homoglutathione · Metabolism · Seed · Biotechnology · Food and feed

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Overview

Amino acid metabolism in plants not only provides basic metabolic building blocks for small molecules and proteins, but also is a critical determinant of both the nutritional composition of plants and the value of crops for food and feed purposes (Jez and Fukagawa 2008). For normal plant growth, sulfur, along with nitrogen, phosphorus, and potassium, is an essential nutrient. Moreover, metabolism of sulfur into thiol-containing compounds is critical for protecting plants from oxidative and environmental stresses. Studies in the model plant *Arabidopsis thaliana* (thale cress) provide significant insights on the biochemical processes and regulation of thiol metabolism (Bick and Leustek 1998; Leustek et al. 2000; Saito 2000; Rausch and Wachter 2005; Kopriva 2006; Meyer 2008; Höfgen and Hesse 2008; Kopriva et al. 2009; Yi et al. 2010); however, the depth of understanding these same pathways in various crop plants is somewhat limited. Progress toward unraveling thiol metabolism in major crops like soybean (*Glycine max*), corn (*Zea mays*), wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), and rice (*Oryza sativa*) is important for optimizing crop yield and quality. Here, we review the details of thiol metabolism in soybean, its potential biotechnology value, what is known about the pathways from sulfur uptake to glutathione synthesis in soybean, how the soybean genome is providing new insight

on these metabolic pathways, and what are the possible next steps in exploring this biochemical system.

Why focus on sulfur, cysteine, and homoglutathione in soybean?

A majority of cultivated crops produce seeds that are deficient, with respect to monogastric diets and rations, in one or more of the essential amino acids. In general, cereals are deficient in lysine, tryptophan, and threonine, while legumes contain inadequate amounts of methionine and cysteine (Shewry et al. 1995). The relative concentration of these essential amino acids in food and feed influences their nutritional and economic value because monogastric animals, including humans, cannot synthesize these essential amino acids. Consequently, to provide adequate sulfur amino acid levels, supplementation of animal feeds by addition of synthetic amino acids is required to promote optimal growth and development.

Soybean is an economically important legume with an estimated 2008 US crop value of $27.3 billion (http://www.soystats.com/2009/Default-frames.htm). It is the second most important cash crop in the US, next only to corn. In many food products, soy is a key ingredient. The two most important components of soybean are oil and protein. On average, soybeans contain 18% oil and 38% protein by weight. As a major source of vegetable oil, they account for 56% of world oil production. Because of their high protein content, soybeans are extensively used as a major ingredient in livestock feed with the majority of soybean meal produced in the US providing an amino acid and protein source in feed for poultry, pork, cattle, and other farm animals. In addition, an increasing amount of soybean meal is being used in fish food, a trend that is expected to increase in the future due to the scarcity and increasing cost of the currently used fish meal (http://www.soyaqua.org/researchtech.html).

Although soybeans already play a dominant role in the animal feed industry, improving its amino acid content would increase its nutritional value (Kerley and Allee 2003). Soybeans are an excellent source of high-quality protein, but the overall content of methionine and cysteine in soybean seed protein is not optimal for formulation of poultry or swine rations. To overcome this problem the animal industry supplements the soybean-based rations with synthetic methionine, a process that significantly adds to cost of the animal feed (Imsande 2001).

Concerted efforts using both traditional breeding and genetic engineering aim to increase sulfur-containing amino acid levels in soybean (Krishnan 2005, 2008). Mutagenesis and breeding yields soybean with modest increases in methionine and cysteine content (Imsande 2001; Panthee et al. 2006). Genetic engineering approaches to change amino acid content in soybean have used the expression of methionine-rich heterologous proteins, the expression of synthetic proteins containing a high percentage of sulfur-containing amino acids, or the expression of endogenous methionine-rich proteins (Townsend and Thomas 1994; Dinkins et al. 2001; Kim and Krishnan 2004; Li et al. 2005; Livingstone et al. 2007; Krishnan 2008). For example, expression of the 2S albumin from Brazil nut increased sulfur-amino acid content by accumulation of the heterologous protein, but at the expense of endogenous sulfur-rich proteins (Streit et al. 2001). This suggests that sulfur assimilation and cysteine biosynthesis do not supply sufficient metabolites in the engineered seeds to enhance sulfur-containing amino acid content.

Several studies demonstrate the influence of sulfur nutrient availability on soybean protein quality (Holowach et al. 1984; Gayler and Sykes 1985; Sexton et al. 1998; Kim et al. 1999). Ample supply of reduced sulfur during seed filling promotes soybean protein quality by reducing the accumulation of sulfur-poor 7S β-conglycinins (Gayler and Sykes 1985; Sexton et al. 1998). Likewise, providing an exogenous source of methionine to soybean plants results in 23 and 31% increases in methionine and cysteine content, respectively (Grabau et al. 1986). This observation suggests if one could increase the concentration of cysteine/methionine in developing seeds, then it should be feasible to accumulate sulfur-rich heterologous proteins to a level sufficient to meet the nutritional requirement of livestock and poultry (Jez and Krishnan 2009).

The general pathways of thiol metabolism in plants begin with a series of enzymatic reactions that reduce sulfate to sulfide, which then combines with *O*-acetylserine to yield cysteine (Fig. 1). Even though sulfur reduction and cysteine biosynthesis occur mainly in leaves (Saito 2000), several enzymes involved in thiol metabolism are active in developing soybean seeds (Sexton and Shibles 1999; Chronis and Krishnan 2003, 2004; Phartiyal et al. 2006, 2008). Cysteine is the metabolic precursor for cellular components containing reduced sulfur, including methionine, glutathione, homoglutathione, iron–sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites (Hell and Hillebrand 2001). Typically, cysteine and methionine in proteins account for about 80% of the organic sulfur found in plants with the rest present as either free amino acids or as low molecular weight thiols, such as glutathione or homoglutathione (Anderson 1990). In soybeans, homoglutathione acts as a major storage form of reduced sulfur and anti-oxidant (Matamoros et al. 1999). Since sulfur plays a vital role in numerous metabolic pathways and impacts plant productivity and nutritional quality, a thorough understanding of the biochemical pathways involved in thiol metabolism is

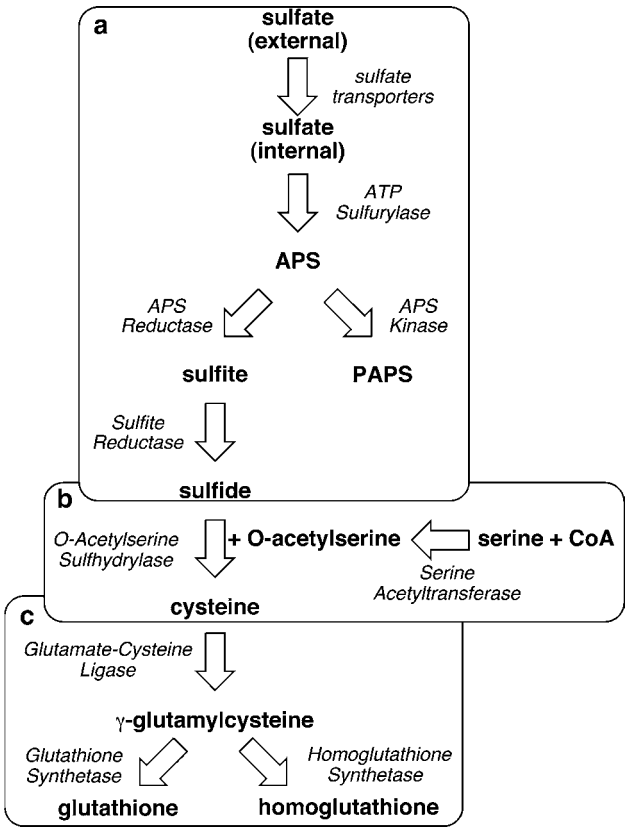

Fig. 1 Overview of thiol metabolism in soybean. Each *box* corresponds to the different components of thiol metabolism, as follows: **a** sulfur assimilation; **b** cysteine biosynthesis; **c** glutathione/homoglutathione biosynthesis. Metabolites are indicated in *bold* and proteins in *italics*

crucial for metabolic engineering of enhanced cysteine/methionine production in soybean.

The sulfur assimilation pathway in soybean

Interest in increasing the cysteine and methionine content of soybean has led to studies of sulfur assimilation in this crop. During vegetative growth, sulfur moves from older to younger tissues in the form of sulfate (Sunarpi and Anderson 1996). Under conditions of limited sulfur supply, soybean pods act as sinks for sulfate. Grain growth triggers the assimilation of sulfate and its incorporation into homoglutathione (Sunarpi and Anderson 1997). The sulfur assimilation pathway involves the uptake of sulfate from the environment and its chemical reduction to sulfide (Fig. 1a). Although these studies, combined with the completion of *G. max* genome project (http://www.phytozome.net/soybean.php; Schmutz et al. 2010), begin to provide a better understanding of the sulfur assimilation pathway in this crop, many aspects of sulfur assimilation in soybean remain unexplored.

The most abundant environmental source of sulfur is sulfate (SO_4^{2-}), a chemically inert molecule. Utilizing this essential nutrient requires the enzymatic conversion of SO_4^{2-} into a chemical species that is energetically favorable to reduce. Sulfate reduction occurs within plastids (Schmidt and Trebst 1969). This requires the uptake of sulfate from the environment, transport into the vascular system, and distribution to cells and subcellular compartments throughout the plant using multiple tissue-specific isoforms of sulfate transporters (Smith et al. 1995; Takahashi et al. 2000; Kataoka et al. 2004; Rouached et al. 2009). Studies in model plants establish the importance of this system, but to date there are no studies on sulfate transporters from *G. max*.

Following uptake of sulfate, the first enzymatic reaction in the sulfur assimilation pathway is the non-reductive adenylation of SO_4^{2-} catalyzed by ATP sulfurylase (ATPS) to yield adenosine 5′-phosphosulfate (APS) (Osslund et al. 1982), as follows:

$$ATP + SO_4^{2-} \leftrightarrow APS + PP_i$$

Transfer of SO_4^{2-} generates a high-energy phosphosulfate mixed anhydride bond, which drives the subsequent metabolic steps in the pathway. Studies of the genomic organization and biochemical activity of a soybean ATPS reveal similar attributes to the enzyme from other plants (Phartiyal et al. 2006). The isolated cDNA clone encoded a predicted plastid-localized ATPS, which was shown to function as a homodimer with steady-state kinetic parameters similar to the corresponding enzyme isolated from *A. thaliana* and *Penicillium chrysogenum* (Seubert et al. 1983; Murillo and Leustek 1995). Southern blot analysis of soybean genomic DNA probed with the ATPS cDNA showed two to four genes encode ATPS isoforms in this crop. This is consistent with the presence of multiple genes encoding cytosolic and plastid/chloroplast forms, as shown in *Arabidopsis*, *Brassica juncea* (Indian mustard), and potato (Leustek et al. 1994; Klonus et al. 1994; Logan et al. 1996; Heiss et al. 1999; Hatzfeld et al. 2000a). Analysis of the soybean genome indicates the presence of four ATPS isoforms (Table 1), only one of which has been biochemically examined (Phartiyal et al. 2006). The localization, developmental expression, and biochemical functions of the different ATPS in soybean are unclear.

APS, the product of the first enzyme in the pathway, is a substrate for two different enzymes—APS reductase (APSR), which commits the sulfur in the compound to further reduction (Suter et al. 2000), and APS kinase (APSK), which phosphorylates APS to provide a sulfur donor involved in the synthesis of glucosinolates and flavonoids (Kopriva 2006). This branch point partitions sulfur flux between two competing pathways (Mugford et al. 2009), as described below.

Table 1 Summary of putative ATPS, APSR, APSK, and SIR isoforms in the soybean genome

Gene identification number	Isoform	Protein length/MW (kDa)	Predicted localization	cDNA expression/enzyme activity
Glyma10g38760	GmATPS1	465 aa/51	Plastidic	Yes/yes[a]
Glyma20g28980	GmATPS2	467 aa/51	Plastidic	ND/ND
Glyma13g06940	GmATPS3	488 aa/54	Plastidic	ND/ND
Glyma19g05020	GmATPS4	553 aa/62	Plastidic	ND/ND
Glyma11g05140	GmAPSK1	297 aa/32	Secretory	ND/ND
Glyma05g22440	GmAPSK2	207 aa/23	Cytosolic	ND/ND
Glyma16g04530	GmAPSK3	207 aa/23	Cytosolic	ND/ND
Glyma19g28900	GmAPSK4	207 aa/23	Cytosolic	ND/ND
Glyma17g17430	GmAPSK5	207 aa/23	Cytosolic	ND/ND
Glyma09g00670	GmAPSR1	470 aa/52	Plastidic	Yes/yes[b]
Glyma15g11540	GmAPSR2	472 aa/52	Plastidic	ND/ND
Glyma07g39130	GmAPSR3	466 aa/52	Plastidic	ND/ND
Glyma11g09890	GmSIR1	687 aa/77	Plastidic	ND/ND
Glyma12g02200	GmSIR2	688 aa/77	Plastidic	ND/ND

Molecular weight was calculated based on the complete amino acid sequence using Protparam (http://www.expasy.ch/tools/protparam.html). Subcellular localization was analyzed using TargetP (http://www.cbs.dtu.dk/services/TargetP/). When a clear localization is not predicted, a "–" is shown. Confirmation of either cDNA expression or enzyme activity is noted, as follows: yes or *ND* not determined

[a] Phartiyal et al. 2006

[b] Phartiyal et al. 2008

In the reductive assimilatory pathway, APSR catalyzes the reduction of APS to sulfite (SO_3^{2-}) and AMP, utilizing glutathione as an electron donor (Setya et al. 1996; Bick et al. 1998), as follows:

$$\text{APS} + \text{glutathione} \leftrightarrow \text{AMP} + SO_3^{2-}$$

Like the bacterial version of the enzyme, the plant APSR require a $[4Fe\text{-}4S]^{2+}$ cluster for activity and contain an N-terminal reductase domain and a C-terminal glutaredoxin-like domain (Setya et al. 1996; Gutierrez-Marcos et al. 1996; Kopriva et al. 2001). In soybean, expression of a predicted chloroplast-localized isoform lacking the predicted N-terminal plastidic targeting sequence yielded a homodimeric enzyme that catalyzed the glutathione-dependent reduction of APS to SO_3^{2-} (Phartiyal et al. 2008). Based on Southern blot analysis, soybean encodes at least three APSR isoforms (Phartiyal et al. 2008). Sequence analysis suggests the presence of three APSR isoforms in soybean, only one of which has been functionally confirmed (Phartiyal et al. 2008) (Table 1).

Metabolically, APSR is a predominant regulator of sulfate flux through the sulfur assimilatory pathway in plants (Tsakraklides et al. 2002; Vauclare et al. 2002), and expression of APSR is responsive to changes in nutrient demand (Phartiyal et al. 2008). Transcript levels of APSR in soybean decrease significantly in the absence of nitrogen and increase under sulfur-deprivation conditions. The coordinated regulation of nitrogen and sulfur use is a common feature in plants and provides a system to manage nutrient levels in response to protein synthesis demands (Reuveny et al. 1980; Brunold et al. 1987; Sunarpi and Anderson 1996; Takahashi et al. 1997; Yamaguchi et al. 1999; Koprivova et al. 2000; Hawkesford 2000). Likewise, expression of both ATPS and APSR undergo concomitant changes in both gene expression and enzyme activity across developmental stages (Phartiyal et al. 2006, 2008). In general, demand on the sulfur assimilation pathway appears greater in young leaf and seed tissues (Adams and Rinne 1969; Sexton and Shibles 1999), as these are sites of metabolic activity in the growing organism.

In the competing pathway, APSK phosphorylates APS at the 3′-ribose position to yield 3′-phosphate-5′-adenosine phosphosulfate (PAPS) (Varin et al. 1997; Lee and Leustek 1998), as follows:

$$\text{APS} + \text{ATP} \leftrightarrow \text{PAPS} + \text{ADP}$$

PAPS provides a sulfur-donor for various sulfotransferases involved in the synthesis of plant hormones, sulfolipids, flavonoids, and glucosinolates (Rouleau et al. 1999; Varin et al. 1997). To date, no biochemical data is available on APSK from soybean, but the genome encodes five APSK isoforms (Table 1). Biochemical studies of APSK from Arabidopsis and *P. chrysogenum* show that APS can bind to the enzyme forming an inhibitor complex (MacRae and Segel 1999; Lillig et al. 2001); this renders the enzyme more sensitive to intracellular APS concentrations

than either ATPS or APSR, which are not inhibited by this compound. Functional analysis reveals multiple APSK isoforms in Arabidopsis (Lee and Leustek 1998; Leustek et al. 2000; Mugford et al. 2009). Interestingly, T-DNA insertional knockout lines of APSK-1 and -2 in Arabidopsis resulted in a dwarfed phenotype and a 450% increase in cysteine content (Mugford et al. 2009), suggesting that partitioning of sulfate flux between the reductive assimilatory and APS phosphorylation pathways is important for growth and development.

Sulfite reductase (SIR) catalyzes the final reaction in the assimilation of sulfur by converting sulfite to sulfide, as follows:

$$SO_3^{2-} + 6\,\text{ferredoxin}_{red} \leftrightarrow S^{2-} + 6\,\text{ferredoxin}_{ox}$$

The enzyme contains a siroheme and a [4Fe-4S] cluster as the catalytically active redox centers and catalyzes the six-electron reductions of sulfite using electrons donated from ferredoxin (Yonekura-Sakakibara et al. 1998, 2000; Nakayama et al. 2000; Hirasawa et al. 2004). Previously, Chi-Ham et al. (2002) identified a DNA-compacting protein in soybean as SIR based on N-terminal sequencing and UV/Vis spectroscopy; however, the enzymatic activity of this protein remains unconfirmed. In Arabidopsis and tobacco, one and two genes, respectively, encode the enzyme (Bork et al. 1998; Yonekura-Sakakibara et al. 1998). In soybean, there appears to be two SIR isoforms (Table 1). No other information on this enzyme in soybean is available.

Connecting sulfur to amino acids: cysteine biosynthesis

Cysteine biosynthesis provides the entry point for sulfur into organic molecules containing thiol groups (Fig. 1b). The two steps in cysteine biosynthesis involve the acetylation of serine by acetyl-CoA generating *O*-acetylserine followed by β-replacement of the acetyl group in *O*-acetylserine with sulfide, producing cysteine and acetate, as follows:

$$\text{SAT} : \text{serine} + \text{acetyl-CoA} \leftrightarrow \text{O-acetylserine} + \text{CoA}$$

$$\text{OASS} : \text{O-acetylserine} + S^{2-} \leftrightarrow \text{cysteine} + \text{acetate}$$

Serine acetyltransferase (SAT; also abbreviated as SERAT in the gene nomenclature) catalyzes the first reaction and *O*-acetylserine sulfhydrylase [OASS; also known as *O*-acetylserine(thiol)lyase] performs the second reaction. OASS is part of the β-substituted alanine synthase (BSAS) family of enzymes, along with enzymes producing β-cyanoalanine from cysteine and cyanide (Hatzfeld et al. 2000b). A recent report indicates that enzyme in BSAS family is also capable of catalyzing the desulfuration of cysteine into pyruvate, ammonia, and sulfide; this expands the catalytic diversity of this enzyme family in plants (Burandt et al. 2001; Alvarez et al. 2010).

Multiple experimental approaches indicate that SAT catalyzes the limiting step in cysteine biosynthesis. Calculated *O*-acetylserine concentration in various subcellular compartments is well below the K_m value for OASS under sulfur sufficient conditions (Wirtz et al. 2004; Krueger et al. 2009). Moreover, OASS activity in many plant species exceeds that of SAT (Ruffet et al., 1995; Droux 2003; Heeg et al. 2008). Consistently, transgenic overexpression of SAT generally increases cysteine and glutathione content, but overexpression of OASS results in relatively small changes in thiol levels (Reviewed in Sirko et al. 2004). The effect of OASS overexpression on the production of thiol compounds in tobacco was significant only when OAS was additionally applied (Saito et al. 1994); however, increased tolerance of transgenic plants overexpressing OASS in stress conditions demonstrates that OASS activity also becomes limited when demand for cysteine supply is high (Noji et al. 2001; Dominguez-Solis et al. 2004).

In all plant species studied so far, small nuclear multigene families encode SAT and OASS (Lunn et al. 1990; Rolland et al. 1992; Ruffet et al. 1995; Noji et al. 1998; Hesse et al. 1999; Droux 2003). For example, the Arabidopsis genome contains five genes for SAT and eight functional genes for OASS (Summarized in Watanabe et al. 2008a, b). Available genomic sequence data for other species also predicts multiple isoforms for SAT and OASS, suggesting that Arabidopsis represents a general situation from algae to higher plants (Kopriva et al. 2009). Phylogenetic analysis of these enzymes using amino acid sequences shows that a group of either SAT or OASS from a species cluster together with those from different species, possibly reflecting conserved targeting and function in different compartments in the cell, as well as their evolutionary origins (Hatzfeld et al. 2000b; Jost et al. 2000; Kawashima et al. 2005; Watanabe et al. 2008b).

Studies in Arabidopsis suggest that the relative contribution of specific SAT and OASS isoforms to metabolism depends on organ type and growth conditions. The mitochondrial SAT isoform (SERAT2;2) contributes most of the cellular SAT activity in Arabidopsis leaves, but the cytosolic isoform (SERAT1;1) provides about half of SAT activity in roots, as much as the mitochondrial SAT form (Haas et al. 2008; Watanabe et al. 2008b). Intriguingly, specific down-regulation of mitochondrial SAT using an artificial microRNA resulted in severe growth retardation, but no growth defect was observed in T-DNA insertional RNA null mutant of the mitochondrial SAT (Watanabe et al. 2008b; Krueger et al. 2009). Distinct growth conditions (e.g., light regime and nutrient condition) used in these two studies may account for the discrepancy in the relative importance of cytosolic isoform and the growth

phenotype observed in plants deficient in the mitochondrial SAT (Haas et al. 2008; Watanabe et al. 2008b; Krueger et al. 2009). Similarly, the differential impact on total OASS activity and growth retardation incurred by deficiency of specific isoforms was also reported for the major Arabidopsis OASS isoforms found in the cytosol, plastid, and mitochondria (i.e., BSAS1;1, BSAS2;1, and BSAS2;2, respectively) (Heeg et al. 2008; Lopez-Martin et al. 2008; Watanabe et al. 2008a). Despite some differences, these studies demonstrate overlapping functions for the SAT and OASS isoforms, and the transport of metabolites produced from these enzymes between cellular compartments.

A central control feature of cysteine biosynthesis is the formation of a macromolecular complex containing both SAT and OASS. Historically, cysteine synthase complex was coined to describe this multienzyme assembly (Kredich et al. 1969); however, cysteine synthase is also a common name for OASS (Masada et al. 1975). We suggest that the complex formed by SAT and OASS be termed the *cysteine regulatory complex* (*CRC*) (Yi et al. 2010), as it more accurately reflects the proposed biological function of this protein assembly.

Multiple lines of evidence suggest a critical role for formation of the CRC in plants. At the mRNA level, SAT and OASS are constitutively expressed, although levels of some isoforms increase under nutritional and environmental stress conditions (Barroso et al. 1999; Hesse et al. 1999; Yamaguchi et al. 2000; Dominguez-Solis et al. 2001; Kawashima et al. 2005). Moreover, the observation that SAT and OASS expression does not show compensation at either the mRNA or protein level in mutants, which lack one or two isoforms, further supports that transcriptional control plays a limited role in regulating expression of cysteine synthesis (Haas et al. 2008; Heeg et al. 2008; Watanabe et al. 2008a, b). Alternatively, interaction between SAT and OASS, which is mediated by C-terminal tail of SAT and active site pocket of OASS, appears to provide an effective regulatory mechanism that readily responds to cellular concentration of sulfide and *O*-acetylserine (Bogdanova and Hell 1997; Hell and Hillebrand 2001; Bonner et al. 2005; Francois et al. 2006; Kumaran and Jez 2007; Kumaran et al. 2009).

According to the model proposed by Hell and Hillebrand (2001), SAT activity in the CRC increases while completely blocking OASS activity when low *O*-acetylserine and high sulfide concentration in the cell facilitates CRC formation (Droux et al. 1998; Berkowitz et al. 2002; Droux 2003; Wirtz et al. 2004). This model further predicts that accumulation of *O*-acetylserine in the cell under sulfur-deficient condition dissociates CRC into the components, to decrease flux through SAT (Wirtz et al. 2004). High concentrations of *O*-acetylserine and/or low levels of thiol compounds in the cell are likely signals that induce high-affinity sulfate transporter and restore the conditions favoring formation of the CRC (Hirai et al. 2003; Hopkins et al. 2005). In addition to increasing SAT activity, CRC formation can alleviate the inhibitory effect of cysteine on SAT activity (Kumaran et al. 2009).

Cysteine biosynthesis in soybean

The soybean genome contains 8 putative SAT and 15 putative BSAS (OASS plus related enzymes) genes. In phylogenetic trees constructed with a neighbor-joining method, these putative SAT or BSAS isoforms in soybean form distinct branches with the corresponding enzymes from different species (Figs. 2, 3) (Saito et al. 1992, 1993; Hell et al. 1994; Barroso et al. 1995; Noji et al. 1998; Hatzfeld et al. 2000b; Jost et al. 2000; Yamaguchi et al. 2000; Lai et al. 2009). These groupings seem to reflect the evolutionary origin and possible subcellular localization of the predicted soybean proteins, as discussed for other species (Hatzfeld et al. 2000b; Jost et al. 2000; Kawashima et al. 2005; Watanabe et al. 2008b). The SAT phylogenetic tree suggests grouping by subcellular localization (Fig. 2). The presence of soybean pairs showing the highest sequence similarity in these phylogenetic trees likely reflects the recent whole genome duplication in soybean (Shoemaker et al. 2006; Van et al. 2008; Gill et al. 2009). Similar to the SAT isoforms, the cytosolic and organellar BSASes with predominant OASS activity (BSAS1 and BSAS2, respectively) form two different clades with predicted soybean proteins, separate from yet another clade that includes BSAS3 with strong CAS activity (Hatzfeld et al. 2000b; Lai et al. 2009) (Fig. 3). The presence of multiple SAT and OASS isoforms suggests that different forms are expressed at specific developmental stages or under different environmental conditions to tailor cysteine synthesis.

Among the putative SAT isoforms in soybean (Fig. 2; Table 2), two SATs are functionally characterized and demonstrate commonalities and differences in their expression, biochemical properties, and regulation of activity. A screen for soybean expressed sequence tags (EST) showing sequence similarity to known SATs identified SSAT1 (GLYMA16G03080) and an interaction screen isolated GmSerat2;1 (GLYMA18G08910) as a substrate for a calcium-dependent protein kinase (CDPK) (Chronis and Krishnan 2004; Liu et al. 2006). Although up-regulation of SSAT1 during seed development and GmSerat2;1 under oxidative stress was reported, the expression pattern of these isoforms over developmental stage and under different growth conditions awaits further investigation. SSAT1 lacks an N-terminal signal peptide and is assumed to be localized in the cytosol, consistent with its

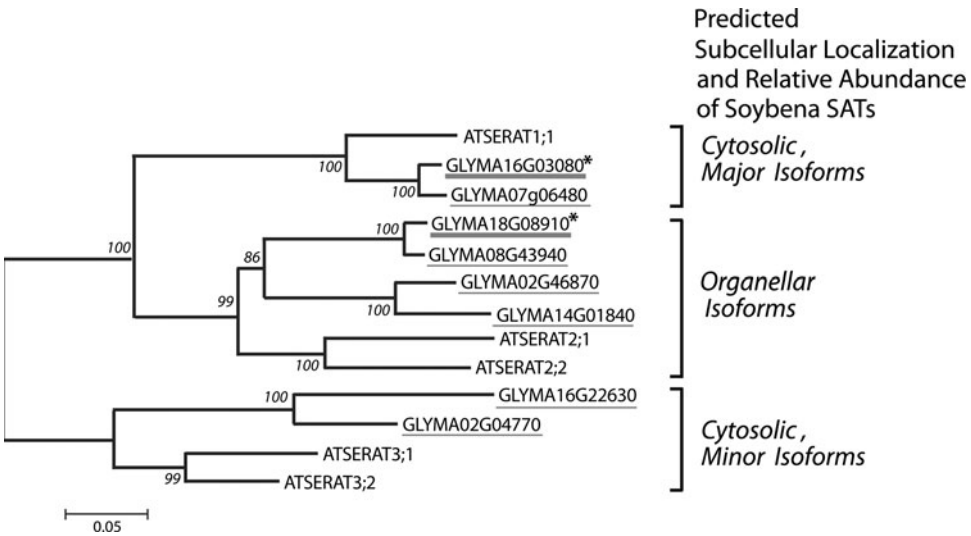

Fig. 2 Evolutionary relationships of SAT isoforms in soybean and Arabidopsis. Phylogenetic analyses were conducted in MEGA4 using the neighbor-joining method (Saitou and Nei 1987; Tamura et al. 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset. Prediction of subcellular localization and abundance was based on the data available for Arabidopsis genes. The isoforms with reported EST or full-length cDNAs are *underlined with thin* and *thick lines*, respectively. Functionally characterized soybean isoforms, GLYMA16G03080 (SSAT1) and GLYMA18G08910 (GmSerat2;1), are indicated with *asterisks* (Chronis and Krishnan 2004; Liu et al. 2006). *Scale bar* shows the distance between two proteins with 5% amino acid sequence difference. Abbreviations for non-soybean SAT correspond to the following accession numbers: ATSERAT1;1—NP_200487.1; ATSERAT2;1—NP_175988.1; ATSERAT2;2—NP_187918.1; ATSERAT3;1—NP_565421.1; and ATSERAT3;2—NP_195289.3

grouping with cytosolic SAT isoforms, including ATSERAT1;1 (Chronis and Krishnan 2004). Transient expression of GmSerat2;1 fused with green fluorescent protein revealed its dual targeting to cytosol and plastid (Liu et al. 2006). ATSERAT2;1 is also found in both cytosol and plastid in the later developmental stage, but is exclusively targeted to the plastid in the earlier stage of Arabidopsis (Noji et al. 1998).

Both characterized soybean SAT isoforms are sensitive to feedback inhibition by cysteine but to varying degrees (Chronis and Krishnan 2004; Liu et al. 2006). Nonetheless, GmSerat2;1 lacking the N-terminal localization sequence becomes insensitive to cysteine when it is phosphorylated by CDPK at a site close to the C-terminus by CDPK (Liu et al. 2006). The finding that full-length GmSerat2;1 does not display similar phosphorylation-dependent sensitivity to cysteine suggests that a combination of subcellular localization and phosphorylation determines the effect of feedback inhibition. In the case of SSAT1, which does not have a putative CDPK-phosphorylation site at its C terminus, CRC formation with OASS provides a similar protecti on to SSAT1 against cysteine (Kumaran et al. 2009). Whereas none of five Arabidopsis SAT isoforms contain a putative CDPK phosphorylation site, GmSerat2;1 and four other SAT isoforms in soybean carry potential CDPK-dependent phosphorylation sites (*B-X-X-S/T*: where *B* is a basic residue lysine or arginine, *X* is any residue, and *S/T* is serine or threonine) near the C-terminus (Liu et al. 2006). Given that putative CDPK-phosphorylation sites are also found near the C-terminus in the SAT from other plants, including tobacco, sunflower, and poplar (Liu et al. 2006), it needs to be determined whether C-terminal phosphorylation of SAT affects CRC formation and whether this post-translation modification is more widely used to modulate feedback inhibition by cysteine.

Out of 15 putative BSAS isoforms present in the soybean genome, biochemical activity for cysteine biosynthesis has been shown for five isoforms isolated from two different cultivars, Williams 82 and NN99-10 (Fig. 3; Table 3) (Chronis and Krishnan 2003; Zhang et al. 2008b). A subsequent study using a pair of cytosolic OASS and SSAT1 (cytosolic SAT in soybean) demonstrated that CRC formation in soybean has a similar effect on the activity of SAT and OASS, as observed when the corresponding proteins from other plant species are used—an increase in SAT efficiency and a complete loss of OASS activity in the complex (Droux et al. 1998; Berkowitz et al. 2002; Droux 2003; Kumaran et al. 2009). The work also revealed that formation of the CRC can prevent cysteine inhibition of SAT, possibly by sequestering the C-terminus of SAT from the exposure to cysteine (Kumaran and Jez 2007; Kumaran et al. 2009). When bound to SAT, cysteine contacts amino acids in the C-terminal region, which is located close to the sequence determinants of OASS interaction (Inoue et al. 1999; Olsen et al. 2004).

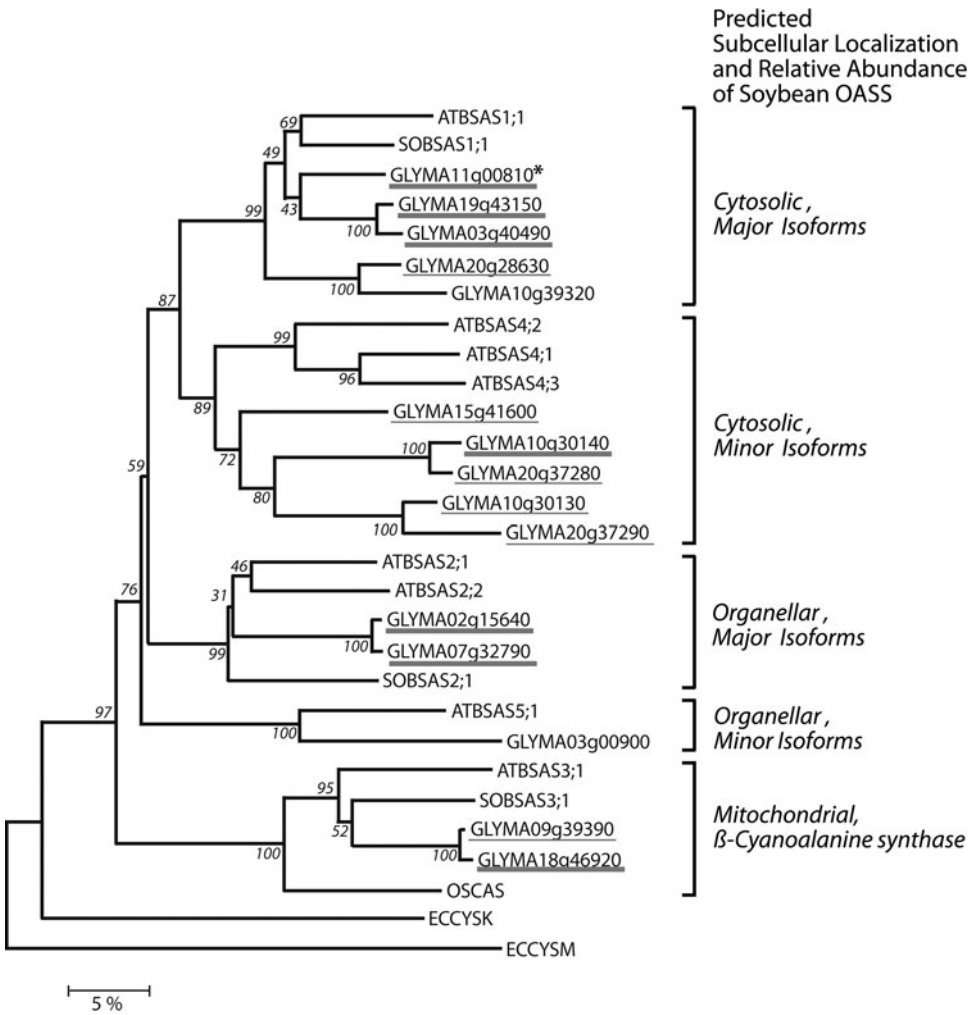

Fig. 3 Evolutionary relationships of BSAS isoforms in various plant species and *E. coli*. Phylogenetic analyses were conducted in MEGA4 using the neighbor-joining method (Saitou and Nei 1987; Tamura et al. 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset. Prediction of subcellular localization and abundance was based on the data available for Arabidopsis genes. Isoforms with reported EST or full-length cDNAs are *underlined with thin* and *thick lines*, respectively. The soybean isoforms, whose cysteine synthase activity are biochemically confirmed, are marked with an *asterisk* (Chronis and Krishnan 2003; Zhang et al. 2008b). *Scale bar* shows the distance between two proteins with 5% amino acid sequence difference. ATBSAS and SOBSAS are BSAS proteins from *Arabidopsis thaliana* and *Spinacia oleracea*, respectively. OSCAS is included in the tree because of its confirmed activity as β-cyanoalanine synthase (Lai et al. 2009). ECCYSK and ECCYSM are two proteins in *E. coli* for cysteine biosynthesis. Abbreviations for non-soybean BSAS correspond to the following accession numbers: ATBSAS1;1—NP_849386.1; ATBSAS2;1—NP_181903.1; ATBSAS2;2—NP_851023.1; ATBSAS3;1—NP_191703.1; ATBSAS4;1—NP_001078628.1; ATBSAS4;2—NP_566243.1; ATBSAS4;3—NP_974843.1; ATBSAS5;1—NP_187013.1; SOBSAS1;1—BAA01279.1; SOBSAS2;1—BAD08329.1; SOBSAS3;1—BAA07177.1; OsCAS—AAV48542.1; ECCYSK—AP003008.1; and ECCYSM—AP003015.1

At the transcript level, BSAS isoforms show dynamic but partially overlapping expression pattern depending on organ types and developmental stages (Chronis and Krishnan 2003; Zhang et al. 2008b). In this regard, overall rate of cysteine synthesis at a certain time and location in soybean may be determined by interplay among BSAS isoforms expressed: some carry out cysteine biosynthesis while the others are more attuned for β-cyanoalanine synthesis and desulfuration using cysteine as substrate. A discrepancy in OASS activity and mRNA expression level of a BSAS isoform in wild soybean (*Glycine soja*) may be related to differential expression patterns of multiple isoforms and/or difference in preferred biochemical activity among BSAS isoforms (Zhang et al. 2008a). Although the mRNA expression level of BSAS isoforms studied so far largely corresponds to the total OASS activity during seed development, it is not clear exactly how many BSAS isoform are expressed during seed development and what is the in vivo function of the each enzyme expressed (Chronis and Krishnan 2003; Zhang et al. 2008b). It would be

Table 2 Summary of putative SAT isoforms in the soybean genome

Gene identification number	Isoform	Protein length/ MW (kDa)	Predicted or confirmed localization	cDNA expression/enzyme activity
Glyma16g03080	GmSERAT1;1	286 aa/30	Cytosolic	Yes/yes[a]
Glyma07g06480	GmSERAT1;2	286 aa/30	Cytosolic	Yes/ND
Glyma18g08910	GmSERAT2;1	391 aa/43	**Cytosolic/plastidic**	Yes/yes[b]
Glyma08g43940	GmSERAT2;2	387 aa/42	–	Yes/ND
Glyma02g46870	GmSERAT2;3	356 aa/39	–	Yes/ND
Glyma14g010840	GmSERAT2;4	351 aa/38	–	Yes/ND
Glyma16g22630	GmSERAT3;1	391 aa/44	Secretory	Yes/ND
Glyma02g04770	GmSERAT3;2	385 aa/42	Cytosolic	ND/ND

Isoform names are based on phylogenetic groupings, as in Fig. 2. Molecular weight was calculated based on the complete amino acid sequence using Protparam (http://www.expasy.ch/tools/protparam.html). Experimentally confirmed subcellular localization is indicated in *bold*. Subcellular localization was analyzed using TargetP (http://www.cbs.dtu.dk/services/TargetP/). When a clear localization is not predicted, a "–" is shown. For GmSERAT3;1, the predicted subcellular localization based on the N-terminal sequence differs from that inferred by phylogenetic analysis. Confirmation of either cDNA expression or enzyme activity is noted, as follows: yes or *ND* not determined

[a] Chronis and Krishnan 2004

[b] Liu et al. 2006

Table 3 Summary of putative BSAS isoforms in the soybean genome

Gene identification number	Isoform	Protein length/MW (kDa)	Predicted localization	cDNA expression/ enzyme activity
Glyma11g00810	GmBSAS1;1	325 aa/34	Cytosolic	Yes/yes[a]
Glyma19g43150	GmBSAS1;2	325 aa/34	Cytosolic	Yes/ND
Glyma03g40490	GmBSAS1;3	325 aa/34	Cytosolic	Yes/yes[b]
Glyma20g28630	GmBSAS1;4	315 aa/33	Cytosolic	Yes/ND
Glyma10g39320	GmBSAS1;5	286 aa/30	Cytosolic	ND/ND
Glyma02g15640	GmBSAS2;1	394 aa/42	Plastidic	Yes/ND
Glyma07g32790	GmBSAS2;2	389 aa/41	Plastidic	Yes/yes[b]
Glyma09g39390	GmBSAS3;1	373 aa/40	–	ND/ND
Glyma18g46920	GmBSAS3;2	372 aa/40	–	Yes/yes[b]
Glyma15g41600	GmBSAS4;1	321 aa/34	Cytosolic	Yes/ND
Glyma10g30140	GmBSAS4;2	324 aa/35	Cytosolic	Yes/ND
Glyma20g37280	GmBSAS4;3	323 aa/35	Cytosolic	Yes/ND
Glyma10g30130	GmBSAS4;4	323 aa/34	Cytosolic	Yes/yes[b]
Glyma20g37290	GmBSAS4;5	295 aa/32	Cytosolic	ND/ND
Glyma03g00900	GmBSAS5;1	320 aa/35	Plastidic	ND/ND

Isoform names are based on phylogenetic groupings, as in Fig. 3. Molecular weight was calculated based on the complete amino acid sequence using Protparam (http://www.expasy.ch/tools/protparam.html). Subcellular localization was analyzed using TargetP (http://www.cbs.dtu.dk/services/TargetP/). When a clear localization is not predicted, a "–" is shown. Confirmation of either cDNA expression or enzyme activity is noted, as follows: Yes or *ND* not determined

[a] Chronis and Krishnan 2003

[b] Zhang et al. 2008b

informative to systematically determine which OASS and SAT isoforms are expressed at different developmental stages in various tissues, which biochemical reaction is favored by a specific BSAS isoform, and whether there is a preference in the interaction between the co-expressed SAT and BSAS isoforms.

Synthesis of thiol peptides: glutathione and homoglutathione

In plants, the thiol-containing tripeptide glutathione is a major regulator of cellular redox state as well as an essential contributor to processes such as the detoxification

of xenobiotics, the sequestration of heavy metals, the storage of excess sulfur in the form of cysteine, and as a substrate for APSR in sulfur assimilation (Setya et al. 1996; Bick et al. 1998; Noctor and Foyer 1998; Mullineaux and Rausch 2005; Meyer 2008; Rouhier et al. 2008). Much of the work contributing to our understanding of the synthesis and function of glutathione centers on the *Brassicaseae*, in particular *Arabidopsis* and *B. juncea*; however, additional studies completed in legumes, such as soybean, expand the role of this peptide to species-specific analogs.

Glutathione synthesis requires the activities of two dedicated ATP-dependent enzymes: glutamate-cysteine ligase (GCL) and glutathione synthetase (GS) (Fig. 1c). The first enzyme, GCL, utilizes L-glutamate and L-cysteine to generate γ-glutamylcysteine (Hell and Bergmann 1990; Jez et al. 2004). From this dipeptide and glycine, GS, the second enzyme in the pathway, then synthesizes glutathione (Jez and Cahoon 2004; Herrera et al. 2007), as follows:

$$\text{GCL} : \text{glutamate} + \text{cysteine} + \text{ATP} \leftrightarrow \gamma\text{-glutamylcysteine} + \text{ADP} + \text{P}_\text{i}$$

$$\text{GS} : \gamma\text{-glutamylcysteine} + \text{glycine} + \text{ATP} \leftrightarrow \text{glutathione} + \text{ADP} + \text{P}_\text{i}$$

In *Arabidopsis,* a reporter gene fused to the 5′-untranslated region (UTR) indicates that GCL localizes to the chloroplast, while both the chloroplast and the cytosol contain GS (Wachter et al. 2005). Earlier fractionation work in *Pisum savitum* also demonstrated the cytosolic localization of GS (Klapheck et al. 1987). Although transcript analysis and activity assays in bean (*Phaseolus vulgaris*) nodules are consistent with those from *Arabidopsis*, localization of GCL to both plastids and the cytoplasm occurs in cowpea (*Vigna unguiculata*) nodules (Moran et al. 2000).

The differing expression profiles of GS and GCL in specific tissue types of various plants further complicate efforts to understand the enzymes' exact role. HPLC analysis of thiols in leaves, roots, and nodules indicates that γ-glutamylcysteine is absent from broad bean (*Vicia faba*) leaves, cowpea leaves, and all assayed legume roots, while glutathione is absent or scarce in bean leaves and roots, as well as mungbean (*Vigna radiata*) roots (Matamoros et al. 1999). Given the presence of glutathione in tissues lacking γ-glutamylcysteine, movement of metabolites between tissue types is likely. Systematic analysis of GCL and GS activities in different tissues of various legumes is necessary to settle localization uncertainties. Such studies in nodules from various legumes, with the exception of mungbean, indicate the presence of both GCL and GS activities. A detailed investigation of expression patterns in the infected zone and the cortex of bean nodules reveal comparable GS activity in both sectors and nearly twofold higher GCL activity in the infected zone as compared to the cortex (Matamoros et al. 1999). This may reflect either additional bacterial synthesis of γ-glutamylcysteine or increased demand for γ-glutamylcysteine resulting from homoglutathione synthesis.

Although the regulation of glutathione synthesis in legumes is unclear, in other plants a number of different control mechanisms target GCL. In Arabidopsis, transcription of GCL increases following exposure to either heavy metal stress or jasmonic acid, but not to other oxidative stresses that regulate the enzymatic activity of GCL (Xiang and Oliver 1998; May et al. 1998). Changes in the redox-state of GCL provide a post-translational mechanism for regulation of activity (Jez et al. 2004; Hothorn et al. 2006; Hicks et al. 2007; Gromes et al. 2008). Oxidation/reduction of disulfide bonds allows for the interconversion of GCL between active homodimeric and less active monomeric forms. The soybean genome contains two full-length copies of the GCL gene that are 91% identical. Localization tag analysis suggests that both of these transcripts are targeted multiple locations (Table 4); activity assays will likely be required to confirm this assessment in soybean and other as-yet-untested legumes.

Glutathione is the primary redox-regulatory molecule in eukaryotes, but it is not the only thiol-containing tripeptide in plants. Grasses and rice produce hydroxymethylglutathione, in which a serine replaces the terminal glycine, following exposure to heavy metals (Klapheck et al. 1994). Corn synthesizes an analog with a terminal glutamate and horseradish generates a tripeptide with a glutamine in place of the glycine following cadmium exposure (Meuwly et al. 1995; Kubota et al. 2000). Lastly, legumes substitute β-alanine for glycine to produce homoglutathione (Klapheck 1988; Skipsey et al. 2005).

As with glutathione, homoglutathione is synthesized in two ATP-dependent steps beginning with GCL; however, instead of GS, the second step requires homoglutathione synthetase (hGS) to catalyze the formation of homoglutathione from γ-glutamylcysteine and β-alanine (Moran et al. 2000). To date, homoglutathione is found in 14 different legumes (pea, alfalfa, soybean, bean, mungbean, lentil, chickpea, sweet pea, cowpea, Italian clover, red clover, blue fenugreek, sweet clover, and runner bean); however, two additional legumes (broad bean and lupine) appear to lack this glutathione analog in all assayed tissue types (Klapheck 1988; Matamoros et al. 1999). Additionally, two species—cowpea and pea—possess it in their roots and nodules, but not in their leaves.

Soybean produces more homoglutatione than glutathione (Klapheck, 1988; Matamoros et al. 1999). Leaves and seeds contain 50- to 200-fold and 135-fold more homoglutathione than glutathione, respectively. Nodules contain

Table 4 Summary of GCL, GS, and hGS isoforms in the soybean genome

Gene identification number	Isoform	Protein length/MW (kDa)	Predicted localization	cDNA expression/ enzyme activity
Glyma05g37850.1[a]	GmGCL1a	504 aa/57	–	Yes/ND
Glyma05g37850.2[a]	GmGCL1b	500 aa/57	–	Yes/ND
Glyma05g37850.3[a]	GmGCL1c	504 aa/57	–	Yes/ND
Glyma08g01750.1[a]	GmGCL2a	535 aa/60	–	Yes/ND
Glyma08g01750.2[a]	GmGCL2b	535 aa/60	–	Yes/ND
Glyma19g42610	GmGS1	527 aa/59	–	ND/ND
Glyma19g42620	GmGS2	478 aa/54	Cytosolic	ND/ND
Glyma03g40050	GmhGS1	547 aa/61	–	Yes/ND
Glyma19g42600.1[a]	GmhGS2a	547 aa/61	–	Yes/yes
Glyma19g42600.2[a]	GmhGS2b	436 aa/49	–	ND/ND
Glyma19g42600.3[a]	GmhGS2c	449 aa/50	–	ND/ND

Molecular weight was calculated based on the complete amino acid sequence using Protparam (http://www.expasy.ch/tools/protparam.html). Subcellular localization was analyzed using TargetP (http://www.cbs.dtu.dk/services/TargetP/). When a clear localization is not predicted, a "–" is shown. Confirmation of either cDNA expression or enzyme activity is noted, as follows: Yes or *ND* not determined

[a] Possible splice variants

almost fourfold more homoglutathione than glutathione, while the roots contain nearly 80-fold more homoglutathione. Physiologically, the exact role of homoglutathione is unclear. One possibility is that homoglutathione takes the place of glutathione as the dominant cellular redox-buffer; however, this leads to questions about why and how the transition from glutathione to homoglutathione took place. It is known that in nodules, the site of nitrogen fixation, homoglutathione is required for proper development (Frendo et al. 2005). Ultimately, there remains a great deal to discover about the interwoven roles of glutathione and homoglutathione in legumes.

The genomes of legumes, including soybean, show evidence for multiple rounds of genome duplication (Shoemaker et al. 2006; Van et al. 2008; Gill et al. 2009). Frendo et al. (2001) proposed that hGS arose from GS by divergent evolution following a duplication event. The distant phylogeny between legumes that produce homoglutathione makes it impossible to tell whether its synthesis originated as a trait at the base of the legume phylogeny and was subsequently lost in some species, or if the trait evolved independently in a subset of species. Nevertheless, examination of the soybean genome reveals the traces of genome duplication as it contains two copies each for GS and hGS (Table 4). Each pair of GS and hGS gene pairs shares 87 and 93% sequence identity, respectively.

GS and hGS catalyze similar reactions and are related by ~70% sequence identity. GS uses glycine as the final substrate in its reaction, but the active site of hGS accepts β-alanine, a slightly longer molecule. Recent crystallographic studies of soybean hGS demonstrate the critical role of an active site loop in determining substrate specificity between these enzymes (Fig. 4) (Galant et al. 2009).

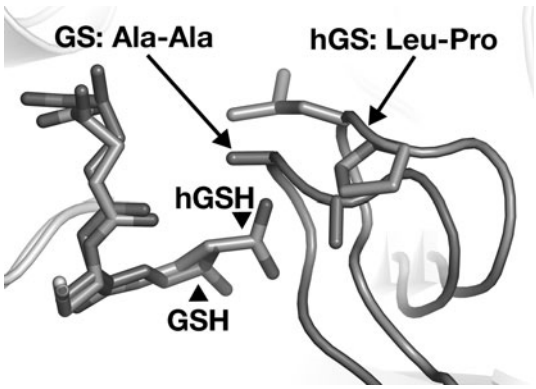

Fig. 4 Comparison of glutathione (*GSH*) and homoglutathione (*hGSH*) binding in the active sites of glutathione synthetase (*GS*) and homoglutathione synthetase (*hGS*). The active site loop differences determining substrate specificity of GS (*Ala-Ala*) and hGS (*Leu-Pro*) are indicated by the *arrows*. Modified from Galant et al. 2009

The apical residues of this loop contact the substrate carboxyl-terminal; the identity of these amino acids alters the size of the active site and determines specificity of the enzyme. In GS, the apical residues are two alanines, but in hGS, these residues are a leucine and a proline. Mutagenesis of the leucine-proline motif of soybean hGS to the alanine-alanine sequence reduces the catalytic efficiency with β-alanine by tenfold, while improving specificity for glycine by nearly 1,000-fold (Galant et al. 2009).

Conclusions

Thiol metabolism is critical for plant growth, development, and defense against a range of environmental stresses

(Rausch and Wachter 2005). Biochemical and physiological studies predominantly in Arabidopsis provide fundamental insights on sulfur assimilation, transport, and metabolism into a range of thiol-containing compounds. Yet, building the framework for optimizing crop quality and yield requires deeper understanding of sulfur demands, the basic pathways of thiol metabolism, and the regulation of these pathways in corresponding crops. Soybean is a major crop in many nations, but thiol metabolism in this crop is largely unexamined. Although the overall organization of sulfur metabolism in soybean, except the production of homoglutathione, does not differ from other plants, the whole genome duplication in soybean results in a more complicated set of isoforms for each thiol metabolic enzyme than observed in Arabidopsis. How this genetic expansion relates to the contribution of various isoforms to thiol metabolism in different organelles, tissues, and developmental stages remains unanswered. In this regard, analysis of the soybean genome will help define the protein components of these pathways and associated regulatory mechanisms. Do many of the biochemical regulatory systems found in plant thiol metabolism, including transport systems, formation of protein complexes, and redox regulation, also occur in soybean, or are they different (Yi et al. 2010)? Most of all, it is unclear if the possible matrix of interaction partners between SAT and OASS isoforms allows for additional fine control of thiol metabolism. Likewise, phosphorylation of SAT in soybean (Liu et al. 2006) suggests connections between metabolic systems and signal transduction pathways, but how these networks are linked needs to be unraveled for its functional importance. At the physiological level, soybean and other legumes also raise the question of how thiol metabolism evolves to diversify the production of sulfur-containing compounds like homoglutathione. Ultimately, studies of amino acid metabolism in soybean continue to provide new insights on these biochemical pathways (Schroeder et al. 2010).

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