

# Transgenic soybean plants overexpressing *O*-acetylserine sulfhydrylase accumulate enhanced levels of cysteine and Bowman–Birk protease inhibitor in seeds

Won-Seok Kim · Demosthenis Chronis ·  
Matthew Juergens · Amy C. Schroeder ·  
Seung Won Hyun · Joseph M. Jez · Hari B. Krishnan

Received: 11 May 2011 / Accepted: 7 July 2011 / Published online: 30 July 2011  
© Springer-Verlag (outside the USA) 2011

**Abstract** Soybeans provide an excellent source of protein in animal feed. Soybean protein quality can be enhanced by increasing the concentration of sulfur-containing amino acids. Previous attempts to increase the concentration of sulfur-containing amino acids through the expression of heterologous proteins have met with limited success. Here, we report a successful strategy to increase the cysteine content of soybean seed through the overexpression of a key sulfur assimilatory enzyme. We have generated several transgenic soybean plants that overexpress a cytosolic isoform of *O*-acetylserine sulfhydrylase (OASS). These transgenic soybean plants exhibit a four- to tenfold increase in OASS activity when compared with non-transformed wild-type. The OASS activity in the transgenic soybeans was significantly higher at all the stages of seed development. Unlike the non-transformed

soybean plants, there was no marked decrease in the OASS activity even at later stages of seed development. Overexpression of cytosolic OASS resulted in a 58–74% increase in protein-bound cysteine levels compared with non-transformed wild-type soybean seeds. A 22–32% increase in the free cysteine levels was also observed in transgenic soybeans overexpressing OASS. Furthermore, these transgenic soybean plants showed a marked increase in the accumulation of Bowman–Birk protease inhibitor, a cysteine-rich protein. The overall increase in soybean total cysteine content (both free and protein-bound) satisfies the recommended levels required for the optimal growth of monogastric animals.

**Keywords** Bowman–Birk protease inhibitor · Cysteine · Essential amino acid · Nutritional quality · *O*-acetylserine sulfhydrylase · Soybean

**Electronic supplementary material** The online version of this article (doi:10.1007/s00425-011-1487-8) contains supplementary material, which is available to authorized users.

W.-S. Kim · D. Chronis · H. B. Krishnan  
Division of Plant Sciences, University of Missouri,  
Columbia, MO 65211, USA

M. Juergens · A. C. Schroeder · J. M. Jez  
Department of Biology, Washington University,  
St. Louis, MO 63130, USA

S. W. Hyun  
Department of Statistics, North Dakota State University,  
Fargo, ND 58108, USA

H. B. Krishnan (✉)  
Plant Genetics Research Unit, United States Department of  
Agriculture/Agricultural Research Service, 108 Curtis Hall,  
University of Missouri, Columbia, MO 65211, USA  
e-mail: KrishnanH@missouri.edu

## Abbreviations

OASS *O*-acetylserine sulfhydrylase  
SAT Serine acetyltransferase  
HSD Homoserine dehydrogenase

## Introduction

Soybeans provide an excellent protein source for both humans and livestock. Commercial soybeans contain approximately 40% protein and 20% oil. Soybeans are widely used in animal feeds due to their high protein content. In the United States, the majority of soybean meal is used for animal diets, especially for poultry and swine. Regardless of the high protein content in soybeans, they are deficient in respect of monogastric diets and rations, in one

or more of the essential amino acids, especially the sulfur-containing amino acids—cysteine and methionine. Cysteine is considered as semi-essential amino acid because animals can convert methionine to cysteine. The concentration of methionine and cysteine in soybean is about 1.3 g per 100 g of protein, which falls short of the required amount of 3.5 g per 100 g protein for these two amino acids (Shewry 2000). Consequently, humans and animals that consume a grain-based diet require supplements with synthetic sulfur-containing amino acids for maintaining optimal growth and development.

A well-established approach used to improve the concentration of sulfur-containing amino acids in legumes involves the incorporation and expression of heterologous seed proteins rich in sulfur-containing amino acids (Jung 1997; Krishnan 2005, 2008; Müntz et al. 1998; Tabe and Higgins 1998; Ufaz and Galili 2008). Two examples of proteins rich in sulfur-containing amino acids are the 2S albumin protein isolated from Brazil nuts and sunflowers and the delta zeins protein isolated from corn. These sulfur-rich proteins have been successfully expressed in soybean seeds (Townsend and Thomas 1994; Dinkins et al. 2001; Kim and Krishnan 2004). Expression of these proteins resulted in a modest increase in the sulfur-amino acid content of soybean seeds. These studies effectively demonstrate the feasibility of increasing the methionine and cysteine content of soybean; however, the overall increase obtained by expressing heterologous seed proteins is insufficient to meet the amino acid requirements of monogastric animals. Furthermore, it has been reported that the accumulation of methionine-rich proteins in transgenic plants is often associated with a decline in the accumulation of endogenous methionine-rich proteins (Jung 1997; Streit et al. 2001; Tabe and Droux 2002; Hagan et al. 2003). This observation points to very low levels of free methionine in legume seeds (Tabé and Higgins 1998; Amir and Galili 2003; Amir and Tabé 2006).

The genetic manipulation of the enzymes involved in the sulfur assimilatory pathway has the potential to increase the cysteine and methionine content in plants. Most importantly, the final steps of cysteine biosynthesis are catalyzed by serine acetyltransferase (SAT) and *O*-acetylserine sulfhydrylase [OASS; also known as *O*-acetylserine (thiol) lyase]. OASS is part of the  $\beta$ -substituted alanine synthase (BSAS) family of enzymes, which also contains the enzymes that produce  $\beta$ -cyanoalanine from cysteine and cyanide. *O*-acetylserine (OAS) is synthesized by SAT from serine and acetyl-coenzyme A, while OASS catalyzes the reduction of inorganic sulfide with OAS resulting in the synthesis of cysteine (Leustek et al. 2000; Saito 2000). Protein–protein interaction between SAT and OASS allows for formation of a cysteine regulatory complex (Hell and Hillebrand 2001; Bonner et al. 2005; Francois et al. 2006;

Kumaran and Jez 2007; Kumaran et al. 2009; Yi et al. 2010a). Overexpression of SAT and/or OASS isoforms in transgenic plants has resulted in elevated levels of thiols and increased tolerance of plants to heavy metal toxicity (Błaszczuk et al. 1999; Harms et al. 2000; Noji et al. 2001; Sirko et al. 2004; Ning et al. 2010). Additionally, when a cytosolic and a chloroplastic isoform of OASS from spinach is overexpressed in tobacco, tolerance to toxic sulfur dioxide and sulfite and resistance to paraquat (methyl viologen), a herbicide that generates active oxygen species is displayed (Noji et al. 2001). Furthermore, the levels of cysteine and glutathione (GSH) were significantly elevated in these transgenic plants (Noji and Saito 2002). Similar results were obtained with tobacco plants overexpressing the wheat OASS gene (Youssefian et al. 2001). Transformed plants showed resistance to exposure of sulfur dioxide and displayed drastically reduced levels of chlorosis following methyl viologen treatment. Cysteine and GSH concentrations were also considerably higher in transgenic tobacco plants exposed to SO<sub>2</sub> (Youssefian et al. 2001). Similarly, Arabidopsis plants overexpressing OASS exhibited tolerance to cadmium chloride presumably due to higher levels of GSH and phytochelatin (Dominguez-Solis et al. 2001). These studies indicate that overproduction of OASS is a viable approach to increase the concentration of compounds containing reduced sulfur in soybean.

Previous attempts to increase the sulfur amino acid content of soybeans through expression of heterologous methionine-rich proteins have been met with limited success (Kim and Krishnan 2004). Our research has taken an alternative approach to generate transgenic soybeans overexpressing a cytosolic isoform of soybean OASS. We display soybean plants overexpressing OASS contain elevated amounts of Bowman–Birk protease inhibitor when compared with non-transgenic plants. Additionally, amino acid analysis reveals our OASS overproducing transgenic soybean seeds contain a 58–74% increase in protein-bound cysteine content. These results demonstrate our ability to increase the sulfur amino acid content of soybeans through our approach of overexpressing OASS in soybeans.

## Materials and methods

### Plasmid construction

The coding region of soybean OASS was amplified from pSCS1 (Chronis and Krishnan 2003) with primers 5'-CCAAGGATCCATGCCGACGGGGTTACCGGC-3' and 5'-GGTTGCGGCCGCGGGCTCAAAGTCATGCTTT-3'. BamHI and NotI sites, indicated by bold letters, were created in these two primers for facilitation of cloning. The mature protein coding region of the soybean OASS was



**Table 1** Primers and probes for quantitative real-time PCR assays

Gene	Sequence	Specificity
HSD, Homoserine dehydrogenase (Phytozome, Glyma20g11950.1)		
SyHSD <sub>endo</sub> F	5'-CACAGAAGCAGAAACAAGAACC-3'	Intron 6
SyHSD <sub>endo</sub> probe	5'-/FAM/AAACCTCCC/ZEN/TTGCAGTCCACACTGAA/IABkFQ-3'	Intron 6
SyHSD <sub>endo</sub> R	5'-GGAGAGGAATGGAGAAAAGGG-3'	Intron 6
OASS, <i>O</i> -acetylserine sulfhydrylase (GenBank Acc. No. AF452451)		
SyOASS <sub>trans</sub> F	5'-TCCGTGCTATTTGAGTCAGTG-3'	pZCS1
SyOASS <sub>trans</sub> Probe	5'-/FAM/TCTAGATCA/ZEN/GTGGTGGTGGTGGTGGT/IABkFQ-3'	pZCS1
SyOASS <sub>trans</sub> R	5'-AAGTCTAGGGTCACATTGCAG-3'	Pin II terminator <sup>a</sup>

<sup>a</sup> Potato proteinase inhibitor II terminator sequences (GenBank Acc. No. X04118)

resolved on a slab gel (10 × 8 × 0.75 cm) consisting of a 13.5% (w/v) separation gel and a 4% (w/v) stacking gel. Electrophoresis was carried out at 20 mA constant current per gel at room temperature. After the completion of electrophoresis, the gels were equilibrated with electrode buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) for 15 min. Proteins from the gels were electroblotted onto pure nitrocellulose membrane (Midwest-Scientific, Valley Park, MO, USA). Immunoblot analysis was performed using antibodies raised against soybean OASS (Chronis and Krishnan 2003) or commercial His tag antibodies (SuperSignal West HisProbe Kit; Pierce Biotechnology, Rockford, IL, USA). Antibodies to soybean Bowman–Birk protease inhibitor were raised in rabbits with synthesized peptide (N-CVDITDFCYEPCPK SEDDK) which was coupled to a carrier protein prior to immunization.

#### Enzyme assay

Newly formed leaves at the tips of main stem (young leaf), fully expanded trifoliate leaves at node six (old leaf), the stem between nodes 3 and 6, inflorescence, and the roots from greenhouse-grown plants were harvested and stored at –80°C. Samples from four replications were pooled together and the assay was repeated three times. OASS activity from soybean leaf, root, stem, and inflorescence was measured according to the ninhydrin method (Warri-llow and Hawkesford 1998). Protein extracts were obtained by grinding samples (200 mg) in a chilled mortar and pestle with 2 ml of ice-cold extraction buffer [100 mM Tris–HCl pH 8.0, 100 mM KCl, 20 mM MgCl<sub>2</sub>, 1% Tween 80 and 10 mM dithiothreitol (DTT)]. The samples were transferred to microcentrifuge tubes and centrifuged at 4°C for 10 min at 12,000g. The clear supernatant was saved and used immediately for measuring the OASS activity. Protein concentration from plant extracts was determined spectrophotometrically with the help of Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

#### Amino acid analysis

Amino acid analysis was performed at the University of Missouri Agriculture Experiment Station Chemical Laboratories. Dry seeds of transgenic soybean lines (CS02, CS022, CS023) and untransformed soybean lines, which were grown under identical conditions in a greenhouse, were ground to a fine powder and subjected to hydrolysis for 16 h at 115°C with 6.0 mM HCl. For the quantification of methionine and cysteine, duplicate samples were oxidized with performic acid prior to acid hydrolysis. Amino acids were separated on a Beckman 6300 Amino Acid Analyzer (Beckman Instruments, Fullerton, CA, USA) equipped with a high-performance, cation exchange resin column.

Analysis of free amino acid content used the protocol of Hacham et al. (2002). Briefly, dry seeds of transgenic and untransformed soybean were frozen in liquid nitrogen and ground to a fine powder in the presence of water:chloroform:methanol (3:5:12, by vol.). Following a series of extractions, the free amino acids extracted are derivatized with *O*-phthalaldehyde and quantified using the manufacturer's instructions of the Waters AccQ•Tag Ultra kit on an Acquity UPLC system.

## Results

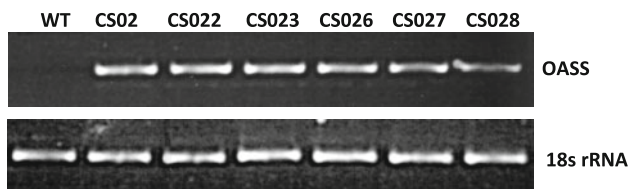
#### Generation of transgenic soybean overexpressing cytosolic isoform of OASS

To overexpress OASS in soybean, a plant transformation vector was constructed in which the coding sequences of the cytosolic OASS (Chronis and Krishnan 2003) were placed under the control of the CaMV 35S promoter. Codons encoding a hexahistidine-tag were fused in frame at the 5'-end of the OASS coding sequences to distinguish it from the native enzyme. This construct was ligated into the expression cassette containing the phosphinothricin acetyl

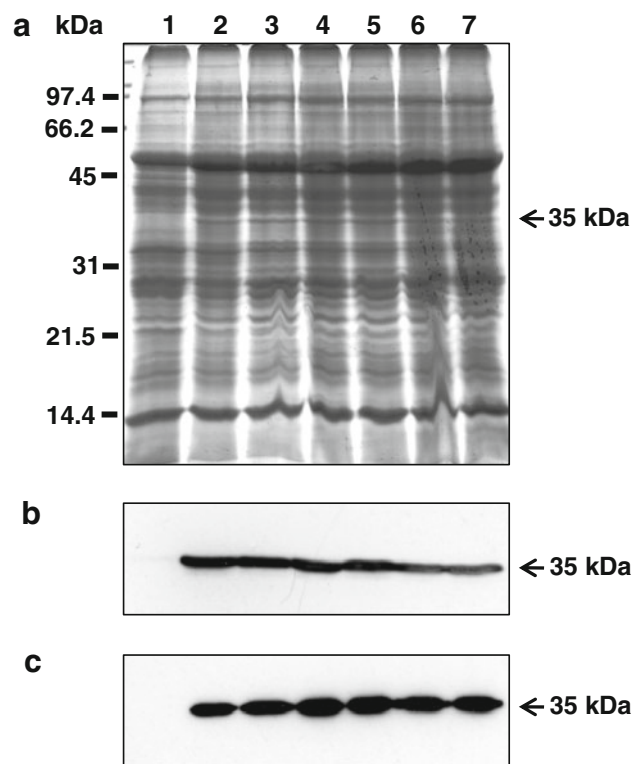


transferase gene (*bar*) under control of 35S promoter and nopaline synthase terminator. Six independent transgenic lines (CS02, CS022, CS023, CS026, CS026, and CS028) of soybean cultivar Maverick, expressing the cytosolic OASS, were obtained by *Agrobacterium tumefaciens*-mediated transformation (Hinchee et al. 1988; Zhang et al. 1999). To confirm expression of the introduced OASS gene in transgenic soybean, we isolated total RNA from soybean leaves and performed RT-PCR analysis (Fig. 1). Using primers that were designed to specifically amplify the introduced OASS, we amplified a 1,008-bp transcript from each of the six transgenic soybean lines (Fig. 1). Under identical conditions, an RT-PCR product was not generated using RNA from a non-transformed control soybean plant.

Expression of cytosolic OASS in these transgenic soybean lines was verified by Western-blot analysis (Fig. 2). Total leaf proteins from the six independent transgenic soybean lines and a non-transgenic soybean line were separated by SDS-PAGE (Fig. 2a) and subjected to immunoblot analysis using antibodies raised against soybean OASS (Fig. 2b; Chronis and Krishnan 2003). The antibody recognized a protein of about 35 kDa protein from the soybean leaf extracts, which corresponds to the molecular weight of OASS. The non-transgenic line showed a weak reaction when compared with the transgenic lines, indicating that these lines accumulated higher amounts of OASS (Fig. 2b). To confirm that the higher accumulation of OASS was due to expression of the introduced gene, which contains an N-terminal His-tag, we used anti-His-tag antibodies in immunoblot analysis. The antibodies clearly reacted against the 35-kDa protein from the transgenic soybean lines, but not from non-transformed soybean plant (Fig. 2c). We also examined the OASS activity from the first trifoliolate leaves of the transgenic soybean lines (Fig. 3). The six transgenic lines contain two- to threefold higher levels of OASS activity when compared with the non-transformed soybean plant. These transgenic soybean lines were advanced to generate R2 and R3 plants, and all the advanced transgenic lines exhibited significantly higher OASS activity.



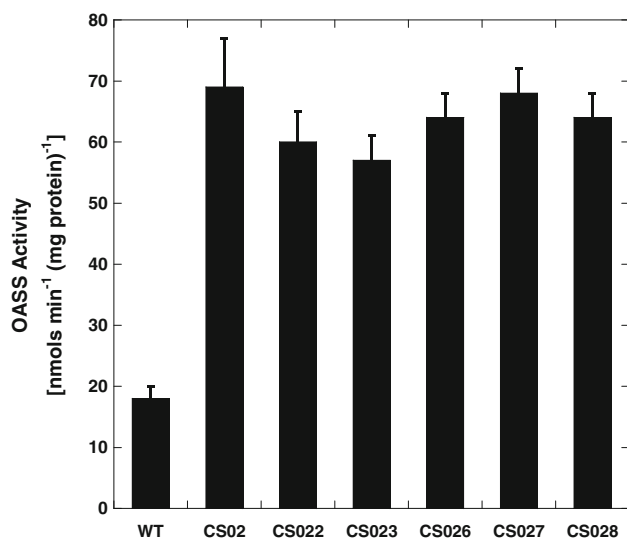
**Fig. 1** RT-PCR analysis of OASS expression in transgenic soybean. Total RNA isolated from non-transformed plant (WT) and six transgenic soybean plants (CS02–CS028) were used in RT-PCR analysis. The upper panel shows RT-PCR product for soybean OASS (1,008 bp) and the lower panel shows the RT-PCR product for the 18S ribosomal RNA (632 bp) control



**Fig. 2** Expression of OASS in the trifoliolate leaves of transgenic soybean and non-transformed control plants. Total leaf proteins from non-transformed control (lane 1) and six independent transgenic lines (lanes 2–7) were fractionated by 13.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue (a) or transferred to nitrocellulose membranes. The membrane was probed with antiserum against soybean OASS (b) or anti-His-tag antibody (c). Immunoreactive proteins were identified using anti rabbit IgG-horseradish peroxidase conjugate followed by chemiluminescent detection. Note the presence of 35 kDa protein only in the transgenic plants (a, arrow)

### Estimating the copy number of OASS transgene in soybean plants

To detect the endogenous homoserine dehydrogenase (HSD) and the OASS transgene in soybean plants, a pair of oligonucleotide primers and internal hybridization fluorogenic TaqMan probes were designed (Table 1) and used in quantitative real-time PCR. The amplification of the OASS transgene was compared with that of endogenous HSD gene. Quantitative RT-PCR analysis was performed with serial standard DNA dilutions of the OASS transgene and HSD genes, and standard curves were generated. Utilizing the standard curves the copy number of OASS<sub>transgene</sub> was determined by comparing the absolutely quantified OASS transgene with those of the endogenous HSD gene. The ratio between transgene and endogenous gene ( $r_{line}$ ) was calculated (Table 2). Using the  $r_{line}$  values, the virtual calibrator ( $r_1$ ) was calculated, which corresponds to one copy of the transgene. In our case, the value of  $r_1$  is 1.11



**Fig. 3** OASS activity in leaf extracts of wild-type and six independent transgenic soybean lines. OASS activity was measured under standard assay conditions as described under “Materials and methods” using protein extracts from trifoliolate leaves. OASS activity from non-transformed wild-type and transgenic soybeans are presented as the means  $\pm$  SE ( $n = 3$ )

for OASS transgene. Six independent transgenic soybean lines were examined, and the results indicated two transgenic lines (CS02, CS023) had one copy, and four transgenic lines (CS022, CS026, CS027, and CS028) had two copies of the transgene (Table 2).

OASS is abundantly expressed in different organs of transgenic soybean

We isolated protein extracts from root, stem, inflorescence, young leaves, and old leaves from transgenic and non-transgenic soybean plants and measured the OASS activity. In non-transformed soybean plants the leaves had a higher amount of OASS activity when compared with other plant organs. In comparison, the transgenic soybean plants revealed significantly elevated levels of OASS activity in

all organs examined (Fig. 4). In both young and old soybean leaves, a fourfold increase in OASS activity was observed compared with non-transformed soybean. Moreover, a greater fold increase in enzyme activity was found in roots, stems, and inflorescences of transgenic soybeans (Fig. 4).

Transgenic soybean exhibits elevated levels of OASS throughout seed development

Previously, we reported OASS activity peaked in young developing seeds and declined steadily during the time when bulk of seed storage protein accumulation occurred (Chronis and Krishnan 2003). To see if similar situation also occurs in transgenic soybean plants, we measured OASS activity from whole seeds at eight different developmental stages (Fig. 5). The OASS activity in the non-transformed soybean was highest at the young stages and declined later during seed maturation, which is consistent with our earlier report (Chronis and Krishnan 2003). The OASS activity in the transgenic soybeans was significantly higher across all the stages of seed development. Unlike the non-transformed soybean plants, there was no marked decrease in the OASS activity even at latter stages of seed development (Fig. 5). Additionally, we measured 5'-adenylylsulfate reductase and ATP sulfurylase activities in these seeds and found no significant difference between wild-type and transgenic plants across developmental stages (Supplementary Fig. S1). Serine acetyltransferase activity was barely above background in both wild-type and transgenic plants due to low protein abundance, as reported previously for soybean (Chronis and Krishnan 2004; Liu et al. 2006).

Overexpression of OASS increases seed cysteine content

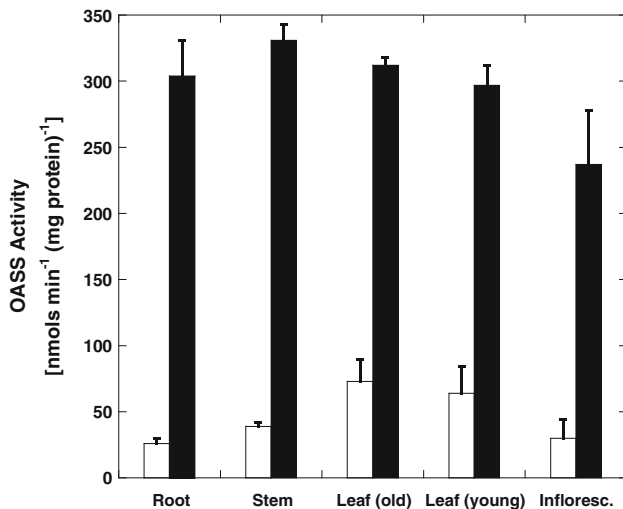
To test if the overexpression of OASS led to an increase in the accumulation of cysteine in soybean seeds, protein-

**Table 2** Estimated transgene copy number by quantitative real-time PCR

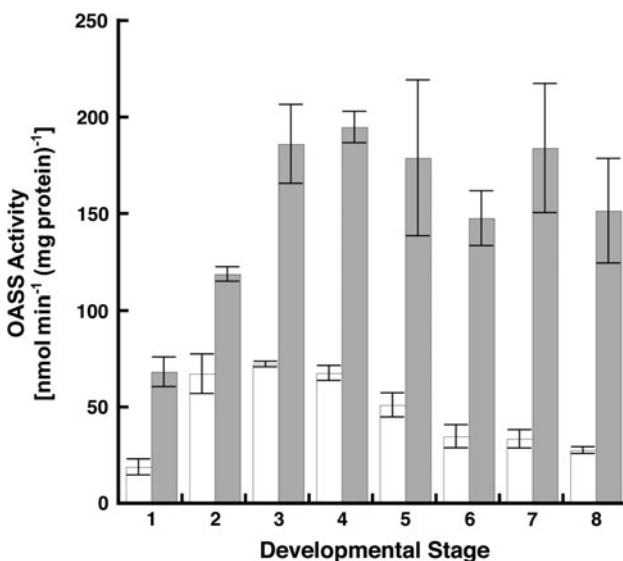
Line	SQ-HSD <sub>endo</sub>	SQ-OASS <sub>trans</sub>	$r_{line}$ (SQ-OASS <sub>trans</sub> /SQ-HSD <sub>endo</sub> )	$(r_{line}/r_1)^a$	Copy number
Wild type	12.15 $\pm$ 1.85	1.21 $\pm$ 0.54	0.0995 $\pm$ 0.05	0.089 $\pm$ 0.04	0
CS02	19.09 $\pm$ 3.45	36.09 $\pm$ 4.26	1.8905 $\pm$ 0.41	1.703 $\pm$ 0.37	2
CS022	31.46 $\pm$ 10.88	41.73 $\pm$ 5.47	1.3264 $\pm$ 0.49	1.195 $\pm$ 0.44	1
CS023	17.08 $\pm$ 3.30	26.16 $\pm$ 5.62	1.5316 $\pm$ 0.44	1.380 $\pm$ 0.40	1
CS026	19.05 $\pm$ 4.24	36.19 $\pm$ 2.25	1.8559 $\pm$ 0.42	1.672 $\pm$ 0.38	2
CS027	13.72 $\pm$ 4.30	31.41 $\pm$ 14.81	2.2894 $\pm$ 1.30	2.062 $\pm$ 1.17	2
CS028	15.97 $\pm$ 2.82	27.63 $\pm$ 10.58	1.7301 $\pm$ 0.73	1.559 $\pm$ 0.66	2

All calculated data in table are shown together with their 95% confidence interval

<sup>a</sup> Calculated copy number for OASS transgene obtained as the ratio between  $r_{line}$  and virtual calibrator  $r_1$



**Fig. 4** OASS activity in different soybean tissues. OASS activity was measured under standard assay conditions as described under “Materials and methods” using protein extracts from roots, stems, young leaves, mature leaves, and inflorescences. OASS activity from non-transformed wild-type and transgenic soybeans are shown by white and black bars, respectively. The data are presented as the means  $\pm$  SE ( $n = 3$ )



**Fig. 5** OASS activity during soybean seed development. OASS activity was measured under standard assay conditions as described under “Materials and methods” using protein extracts from different seed developmental stages. OASS activity from non-transformed wild-type and transgenic soybeans are shown by white and black bars, respectively. Note the transgenic plants show two- to sixfold increase in OASS activity across all developmental stages. The data are presented as the means  $\pm$  SE ( $n = 3$ )

bound cysteine content of mature whole dry seeds from three independent transgenic events was quantified using a high-pressure liquid chromatography (HPLC; Table 3). Transgenic soybean seeds overexpressing OASS showed a significant increase in protein-bound cysteine levels

compared with the non-transgenic soybean seeds. The protein-bound cysteine content in control wild-type seeds was 1.59% compared with 2.77, 2.52, and 2.56% in three transgenic soybean events overexpressing OASS (Table 1). This represents a 74, 58, and 61% increase in the protein-bound cysteine content in these transgenic plants. Similarly, 17, 13, and 14% increases in protein-bound methionine content were also observed in the transgenic soybeans (Table 3). We also determined the free cysteine content in these seeds. The free cysteine content in the wild-type control and transgenic soybean seeds were 1.54 and 2.03, 1.88, and 1.92%, respectively. This represents a 32, 22, and 25% increase in the free cysteine content in these transgenic soybean seeds. This elevated level of cysteine was retained in the progeny of these transgenic soybean plants (Supplementary Table S1).

### Bowman–Birk protease inhibitor accumulation is enhanced in transgenic soybean seeds overexpressing OASS

Soybeans contain several low-molecular-weight proteins, including Bowman–Birk protease inhibitors that are rich in cysteine (Nielsen 1996). Since transgenic soybean seeds accumulate higher amounts of cysteine, we examined if the increase promotes the accumulation of cysteine-rich proteins such as Bowman–Birk protease inhibitor. We have previously shown that 50% isopropanol can be used to isolate low-molecular-weight proteinase inhibitors from soybean seeds (Krishnan 2004). A comparison of 50% isopropanol-soluble proteins isolated from the mature dry seeds of non-transformed wild-type control and transgenic soybean overexpressing OASS plants demonstrates a marked increase in the accumulation of a 14-kDa protein (Fig. 6a). Antibodies generated against a conserved Bowman–Birk peptide reacted specifically with this protein (Fig. 6b). The accumulation of the Bowman–Birk protease inhibitor was significantly higher in all the three transgenic lines when compared with the control plants. A faintly reacting band was detected in the control plants after a longer exposure of the X-ray film (data not shown). In contrast to the Bowman–Birk protease inhibitor, the accumulation of the Kunitz trypsin inhibitor was not significantly affected in the transgenic plants (Fig. 6c). A comparison of the intensity of the reactive proteins shows that the transgenic soybeans plants accumulate similar amounts of Kunitz trypsin inhibitor as the wild-type seeds (Fig. 6c).

### Discussion

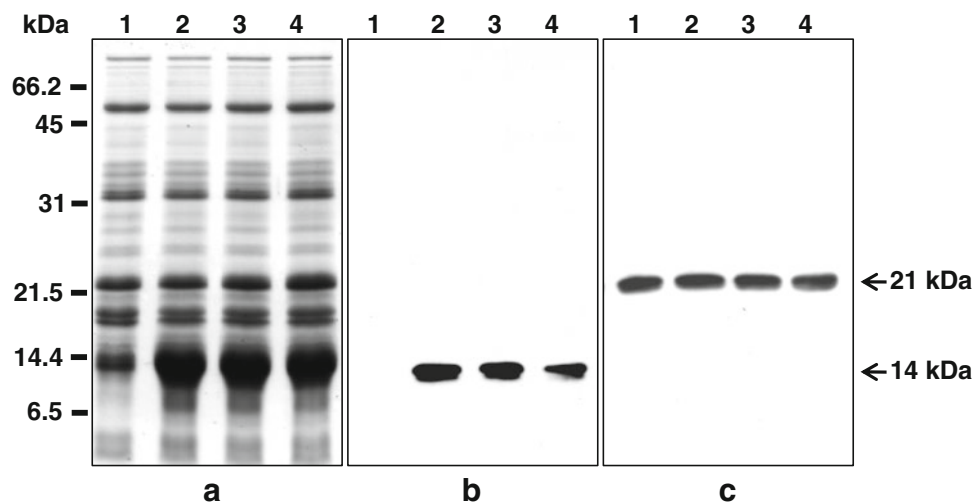
Relative to other feed crops, the concentration of sulfur-containing amino acids in soybean seeds is relatively

**Table 3** Amino acid composition of soybean seed (% per 100 g protein)

Amino acid	Control	CS02 <sup>a</sup>	CS0022 <sup>a</sup>	CS023 <sup>a</sup>
Aspartic acid	11.89 (0.17)	12.07 (0.11)	11.46 (0.12)	11.52 (0.07)
Threonine	4.20 (0.07)	4.28 (0.04)	4.12 (0.08)	4.02 (0.03)
Serine	4.69 (0.11)	5.28 (0.02)	5.02 (0.03)	4.92 (0.11)
Glutamic acid	17.14 (0.30)	17.14 (0.14)	17.70 (0.24)	17.86 (0.12)
Proline	5.09 (0.05)	5.05 (0.05)	5.10 (0.08)	5.09 (0.04)
Glycine	4.55 (0.07)	4.42 (0.03)	4.44 (0.08)	4.38 (0.03)
Alanine	4.45 (0.08)	4.50 (0.04)	4.40 (0.04)	4.46 (0.03)
Cysteine	1.59 (0.03)	2.77 (0.01)	2.52 (0.04)	2.56 (0.07)
Valine	5.09 (0.08)	4.67 (0.06)	4.86 (0.08)	4.62 (0.09)
Methionine	1.57 (0.03)	1.84 (0.02)	1.78 (0.02)	1.79 (0.01)
Isoleucine	4.82 (0.07)	4.55 (0.06)	4.66 (0.06)	4.65 (0.07)
Leucine	8.06 (0.12)	7.60 (0.08)	7.66 (0.07)	7.73 (0.03)
Tyrosine	3.63 (0.04)	3.15 (0.05)	3.49 (0.14)	3.50 (0.19)
Phenylalanine	5.07 (0.07)	4.67 (0.07)	4.72 (0.07)	4.94 (0.16)
Lysine	6.84 (0.11)	7.04 (0.06)	6.90 (0.10)	6.98 (0.02)
Histidine	2.92 (0.04)	2.92 (0.03)	2.94 (0.05)	2.92 (0.01)
Arginine	7.25 (0.09)	6.71 (0.07)	6.83 (0.06)	6.90 (0.06)
Tryptophan	1.14 (0.02)	1.35 (0.01)	1.40 (0.02)	1.36 (0.04)

Data are the average of three replicates from a single experiment and error for each value (reported in parenthesis) was calculated as the standard deviation of the mean

<sup>a</sup> Transgenic T2 soybean lines overexpressing OASS. Dry seed powder from three independent plants were combined and subjected to amino acid analysis



**Fig. 6** Differential accumulation of Bowman–Birk protease inhibitor in the seeds of non-transformed wild-type and transgenic soybean plants. 50%-isopropanol extracted seed protein were separated by 13.5% SDS-PAGE and visualized with Coomassie Blue R-250 (a) or

transferred to nitrocellulose membranes and subjected to immunoblot analysis using antiserum raised against Bowman–Birk protease inhibitor (b) or Kunitz trypsin inhibitor (c). Lane 1 non-transformed control, lane 2 CS02, lane 3 CS022, lane 4 CS023

lower. It has been speculated that this deficiency may be due to the paucity of sulfur amino acids in developing soybean seeds (Jez and Krishnan 2009). In this study, we demonstrate that it is possible to increase the availability of cysteine in the developing seed by manipulating the expression levels of OASS.

Previously, we demonstrated that OASS activity declines during the most active period of seed storage protein synthesis (Chronis and Krishnan 2003). We overcame this deficiency by generating transgenic soybeans that overexpress OASS throughout seed development, thus increasing the cysteine content of soybean. Several studies



have achieved a significant increase in the methionine content in model plants such as *Arabidopsis* and tobacco through the overexpression of key sulfur assimilatory enzymes, but there has been no previous report from important food crop plants. For example, Ning et al. (2010) overexpressed a soybean *O*-acetylserine (thiol) lyase-encoding gene *GmOASTLA* in tobacco and observed a marked increase in cysteine levels. These transgenic tobacco plants also exhibited increased tolerance to cadmium presumably due to induction of antioxidant enzymes (Ning et al. 2010). Recently, Avraham et al. (2005) reported enhanced levels of methionine and cysteine in transgenic alfalfa, a forage crop, by overexpressing *Arabidopsis* cystathionine  $\gamma$ -synthase gene. Importantly, they observed 2.6- and 2.3-fold increases in the levels of free and protein-bound cysteine content, respectively, in these transgenic alfalfa plants. The fold increase in cysteine we observed in our study is not as drastic as observed in alfalfa, but it is still sufficient to meet livestock nutritional needs.

In this study, we have demonstrated that the transgenic soybean plants overexpressing OASS contain higher amounts of Bowman–Birk protease inhibitor when compared with that of non-transgenic control plants. The Bowman–Birk family of isoinhibitors contributes significantly to the overall sulfur content of soybean seeds because they contain 14 cysteine residues (Birk 1985; Clarke and Wiseman (2000). The elevated level of cysteine in the transgenic soybean overexpressing OASS presumably promotes the synthesis of Bowman–Birk protease inhibitor. It will be interesting to see if the increased availability of cysteine also affects the accumulation of other sulfur-rich proteins in these transgenic soybean plants.

It has been estimated that 1 kg of animal ration contains approximately 150 g of soybean protein, which provides about 2.5 g of methionine to the animal feed (Imsande 2001). This value is based on the assumption that an average commercial soybean cultivar contains approximately 1.4% methionine. Additionally, to meet the nutritional requirement of animals 1 g of synthetic methionine is added to 1 kg of animal feed. Thus, the methionine content of soybean needs to be raised to approximately 1.7% to avoid methionine supplementation (Imsande 2001). The transgenic soybean seeds generated in our study contain about 2.7 and 1.8% cysteine and methionine, respectively. Thus, the total sulfur-containing amino acid (cysteine + methionine) content of soybean has been elevated to 4.5% which should be sufficient to meet the sulfur amino acid requirement of the livestock. However, conclusive evidence for this claim awaits feeding trials to evaluate the nutritional quality of the transgenic soybeans.

An important feature of cysteine biosynthesis is the formation of the cysteine regulatory complex (CRC) composed of SAT and OASS (Yi et al. 2010a). Interaction

between SAT and OASS provides an effective regulatory mechanism that readily responds to cellular concentrations of sulfide and *O*-acetylserine (Bogdanova and Hell 1997; Hell and Hillebrand 2001; Francois et al. 2006; Kumaran and Jez 2007; Kumaran et al. 2009). In vivo OASS is present at levels  $\sim$ 300-fold higher than SAT, indicating that only a small fraction of OASS binds to SAT (Ruffet et al. 1995; Droux 2003). This distinctive difference in the molar concentration of the two enzymes controls the fate of sulfur assimilation. The soybean genome contains multiple genes encoding OASS (Zhang et al. 2008a, b; Yi et al. 2010b). In *Arabidopsis*, it has been shown that the relative contribution of specific OASS isoforms to thiol metabolism may depend on organ type and growth conditions (Heeg et al. 2008; Lopez-Martin et al. 2008; Watanabe et al. 2008). Currently, we know little about the amount of each OASS isoform expressed during soybean seed development and their in vivo function. In this study, by expressing a cytosolic OASS, we were able to alter the overall rate of cysteine synthesis in soybean seeds. This increase in the cysteine synthesis may be accomplished by altering the molar ratio of OASS and SAT in soybean seeds and modulating the formation of the cysteine regulatory complex (CRC). Further studies are required to understand the biochemical basis for the observed increase in the synthesis of cysteine in soybean seeds.

Overproduction of SAT in transgenic plants can also serve as another viable approach to increase the sulfur-containing amino acids in transgenic plants. In potato, the overproduction of bacterial SAT resulted in increased levels of cysteine and glutathione in leaves, but not in tubers (Harms et al. 2000). Expression of feedback-insensitive SAT appears to be much more efficacious in increasing the cysteine levels in transgenic plants (Noji and Saito 2002). The magnitude of changes in the thiol content in transgenic plants was dependent on the cellular targeting compartment. In transgenic tobacco plants the cysteine levels were elevated sixfold by plastidic targeting of the *Arabidopsis* SAT, but only threefold increase was observed when SAT was targeted to the cytosol (Wirtz and Hell 2003). A recent study reported a dramatic increase in the concentrations of free cysteine in developing narrow leaf lupin seeds by overexpressing cysteine feedback-insensitive SAT (Tabe et al. 2010). This elegant work demonstrates for the first time that modification of cysteine biosynthesis in legumes can be achieved by manipulation of sulfur assimilatory pathways. A similar approach could also be employed to generate transgenic soybeans with higher content of cysteine. To follow this approach, we are currently generating transgenic soybean plants overexpressing a feedback-insensitive soybean SAT. Previous studies have shown that levels of soluble (free) essential amino acids can be greatly enhanced in transgenic plants

by metabolic engineering. Substantial increases in free lysine levels in the seeds of canola and soybean have been achieved by seed-specific expression of lysine-insensitive bacterial dihydrodipicolinate synthase (Falco et al. 1995). A recent study showed that seed-specific expression of the feedback-resistant variants of aspartate kinases could result in up to 100-fold increase in free threonine in transgenic soybean seeds (Qi et al. 2011). Interestingly, these soybean plants also accumulated increased levels of methionine, lysine, and isoleucine resulting in an upto 3.5-fold increase in the total free amino acid content; however, this study did not examine if there was also any increase in the levels of protein-bound essential amino acids. Improvement of crop nutrient quality cannot be achieved solely by enhancing the rates of biosynthesis of free essential amino acids. Free amino acids are easily lost or degraded during crop processing and high amounts of certain essential amino acids are regarded as deleterious for seed germination and seedling growth. To avoid these problems, it will be desirable to increase the concentration of protein-bound amino acids rather than that of free amino acids. In this regard, it is worth noting that the transgenic soybeans overexpressing OASS exhibit normal seed morphology with no observable defect in seed germination (data not shown).

**Acknowledgments** We thank the University of Missouri Plant Transformation Core Facility for the production of transgenic soybean lines. Work in the Jez lab was funded by a US Department of Agriculture grant (NRI-2005-02518) to J.M.J., M.J. and A.C.S. were also supported in part by American Society of Plant Biologists Summer Undergraduate Research Fellowships. Product names are necessary to report factually on available data; however, the University of Missouri and the USDA neither guarantee nor warrant the standard of product, and the use of the name by the University of Missouri and the USDA implies no approval of the product to the exclusion of others that may be suitable.

## References

- Amir R, Galili G (2003) Approaches to improve the nutritional values of transgenic plants by increasing their methionine content. In: Hemantaranjan A (ed) *Advances in plant physiology* 6. Scientific Publishers, Jodhpur, pp 61–77
- Amir R, Tabe L (2006) Molecular approaches to improving plant methionine content. In: Pawan KJ, Rana PS (eds) *Plant genetic engineering. Metabolic engineering and molecular farming II*, vol. 8. Studium Press LLC, pp 1–26
- Avraham T, Badani H, Galili S, Amir R (2005) Enhanced levels of methionine and cysteine in transgenic alfalfa (*Medicago sativa* L.) plants over-expressing the Arabidopsis cystathionine  $\gamma$ -synthase gene. *Plant Biotechnol J* 3:71–79
- Birk Y (1985) The Bowman–Birk inhibitor. *Int J Pept Protein Res* 25:113–131
- Blaszczyk A, Brodzik R, Sirko A (1999) Increased resistance to oxidative stress in transgenic tobacco plants overexpressing bacterial serine acetyltransferase. *Plant J* 20:237–243
- Bogdanova N, Hell R (1997) Cysteine synthesis in plants: protein–protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. *Plant J* 11:251–262
- Bonner ER, Cahoon RE, Knapke SM, Jez JM (2005) Molecular basis of plant cysteine biosynthesis: structural and functional analysis of *O*-acetylserine sulfhydrylase from *Arabidopsis thaliana*. *J Biol Chem* 280:38803–38813
- Chronis D, Krishnan HB (2003) Sulfur assimilation in soybean: molecular cloning and characterization of *O*-acetylserine(thiol)lyase (cysteine synthase). *Crop Sci* 43:1819–1827
- Chronis D, Krishnan HB (2004) Sulfur assimilation in soybean (*Glycine max* [L.] Merr.): molecular cloning and characterization of a cytosolic isoform of serine acetyltransferase. *Planta* 218: 417–426
- Clarke EJ, Wiseman J (2000) Developments in plant breeding for improved nutritional quality of soy beans II. Anti-nutritional factors. *J Ag Sci* 134:125–136
- Dinkins RD, Reddy MSS, Meurer CA, Yan B, Trick H, Thibaud-Nissen F, Finer JJ, Parrott WA, Collins GB (2001) Increased sulfur amino acids in soybean plants overexpressing the maize 15 kDa zein protein. In *Vitro Cell Dev Biol-Plant* 37:742–747
- Dominguez-Solis JR, Gutierrez-Alcala G, Vega JM, Romero LC, Gotor C (2001) The cytosolic *O*-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. *J Biol Chem* 276:9297–9302
- Droux M (2003) Plant serine acetyltransferase: new insights for regulation of sulphur metabolism in plant cells. *Plant Physiol Biochem* 41:619–627
- Falco SC, Guida T, Locke M, Mauvais J, Sandres C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. *Biotechnology* 13:577–582
- Francois JA, Kumaran S, Jez JM (2006) Structural basis for interaction of *O*-acetylserine sulfhydrylase and serine acetyltransferase in the Arabidopsis cysteine synthase complex. *Plant Cell* 18:3647–3655
- Hacham Y, Avraham T, Amir R (2002) The N-terminal region of Arabidopsis cystathionine gamma-synthase plays an important regulatory role in methionine metabolism. *Plant Physiol* 128:454–462
- Hagan ND, Upadhyaya N, Tabe LM, Higgins TJ (2003) The redistribution of protein sulfur in transgenic rice expressing a gene for a foreign, sulfur-rich protein. *Plant J* 34:1–11
- Harms K, von Ballmoos P, Brunold C, Hofgen R, Hesse H (2000) Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione. *Plant J* 22:335–343
- Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, Hell R (2008) Analysis of the Arabidopsis *O*-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. *Plant Cell* 20:168–185
- Hell R, Hillebrand H (2001) Plant concepts for mineral acquisition and allocation. *Curr Opin Biotech* 12:161–168
- Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Bio/Technology* 6:915–922
- Imsande J (2001) Selection of soybean mutants with increased concentrations of seed methionine and cysteine. *Crop Sci* 41:510–515
- Jez JM, Krishnan HB (2009) Sulfur assimilation and cysteine biosynthesis in soybean seeds: towards engineering sulfur amino acid content. In: Krishnan HB (ed) *Modification of seed composition to promote health and nutrition*. ASA-CSSA-SSSA Publishing, Madison, pp 249–261
- Jung R (1997) Expression of a 2S albumin from *Bertholletia excelsain* soybean. In: *The 39th NIBB Conference: Dynamic*

- aspects of seed maturation and germination. Okazaki: National Institute for Basic Biology. URL: <http://www.pubs.nrc-cnrc.gc.ca/ispmb/isqmb15/15393-4.pdf>
- Kim WS, Krishnan HB (2004) Expression of an 11 kDa methionine-rich delta-zein in transgenic soybean results in the formation of two types of novel protein bodies in transitional cells situated between the vascular tissue and storage parenchyma cells. *Plant Biotech J* 2:199–210
- Krishnan HB (2004) A simple and rapid method to isolate low molecular weight proteinase inhibitors from soybean. *Korean J Crop Sci* 49:342–348
- Krishnan HB (2005) Engineering soybean for enhanced sulfur amino acid content. *Crop Sci* 45:454–461
- Krishnan HB (2008) Improving the sulfur-containing amino acids of soybeans to enhance its nutritional value in animal feed. In: Jez JM (ed) *Sulfur: a missing link between soils crops and nutrition*. ASA-CSSA-SSSA Publishing, Madison, pp 235–249
- Kumaran S, Jez JM (2007) Thermodynamics of the interaction between *O*-acetylserine sulfhydrylase and the C-terminus of serine acetyltransferase. *Biochemistry* 46:5586–5594
- Kumaran S, Yi H, Krishnan HB, Jez JM (2009) Assembly of the cysteine synthase complex and the regulatory role of protein–protein interactions. *J Biol Chem* 284:10268–10275
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annu Rev Plant Physiol Plant Mol Biol* 51:141–165
- Liu F, Yoo BC, Lee JY, Pan W, Harmon AC (2006) Calcium-regulated phosphorylation of soybean serine acetyltransferase in response to oxidative stress. *J Biol Chem* 281:27405–27415
- Lopez-Martin MC, Becana M, Romero LC, Gotor C (2008) Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in *Arabidopsis*. *Plant Physiol* 147:562–572
- Mason G, Provero P, Vaira AM, Accotto GP (2002) Estimating the number of integrations in transformed plants by quantitative real-time PCR. *BMC Biotechnology* 2:20
- Müntz K, Christov V, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O, Wustenhagen T (1998) Genetic engineering for high methionine grain legumes. *Nahrung* 42:125–137
- Nielsen NC (1996) Soybean seed composition. In: Verma DPS, Shoemaker RC (eds) *Soybean: genetics molecular biology and biotechnology*. CAB, Wallingford, pp 127–163
- Ning H, Zhang C, Yao Y, Yu D (2010) Overexpression of a soybean *O*-acetylserine (thiol) lyase-encoding gene *GmOASTL4* in tobacco increases cysteine levels and enhances tolerance to cadmium stress. *Biotechnol Lett* 32:557–564
- Noji M, Saito K (2002) Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolism engineering. *Amino Acids* 22:231–243
- Noji M, Saito M, Nakamura M, Aono M, Saji H, Saito K (2001) Cysteine synthase overexpression in tobacco confers tolerance to sulfur-containing environmental pollutants. *Plant Physiol* 126:973–980
- Omar AA, Dekkers MG, Graham JH, Grosser JW (2008) Estimation of transgene copy number in transformed citrus plants by quantitative multiplex real-time PCR. *Biotechnol Prog* 24:1241–1248
- Qi Q, Huang J, Crowley J, Ruschke L, Goldman BS, Wen L, Rapp WD (2011) Metabolically engineered soybean seed with enhanced threonine levels: biochemical characterization and seed-specific expression of lysine-insensitive variants of aspartate kinases from the enteric bacterium *Xenorhabdus bovienii*. *Plant Biotechnol J* 9:193–204
- Ruffet ML, Lebrun M, Droux M, Douce R (1995) Subcellular distribution of serine acetyltransferase from *Pisum sativum* and characterization of an *Arabidopsis thaliana* putative cytosolic isoform. *Eur J Biochem* 227:500–509
- Saito K (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. *Curr Opin Plant Biol* 3:188–195
- Schroeder AC, Zhu C, Yanamadala SR, Cahoon RE, Arkus KA, Wachstock L, Bleeke J, Krishnan HB, Jez JM (2010) Threonine-insensitive homoserine dehydrogenase from soybean: genomic organization, kinetic mechanism, and in vivo activity. *J Biol Chem* 285:827–834
- Shewry PR (2000) Seed proteins. In: Black M, Bewley JD (eds) *Seed technology and its biological basis*. Sheffield Academic Press, Sheffield, pp 42–84
- Sirko A, Blaszczyk A, Liszewska F (2004) Overproduction of SAT and/or OASTL in transgenic plants: a survey of effects. *J Exp Bot* 55:1881–1888
- Streit LG, Beach LR, Register JC, Jung R, Fehr WR (2001) Association of the Brazil nut protein gene and Kunitz trypsin inhibitor alleles with soybean protease inhibitor activity and agronomic traits. *Crop Sci* 41:1757–1760
- Tabé LM, Droux M (2002) Limits to sulfur accumulation in transgenic lupin seeds expressing a foreign sulfur-rich protein. *Plant Physiol* 128:1137–1148
- Tabé L, Higgins TJ (1998) Engineering plant protein composition for improved nutrition. *Trends Plant Sci* 3:282–286
- Tabé L, Wirtz M, Molvig L, Droux M, Hell R (2010) Overexpression of serine acetyltransferase produced large increases in *O*-acetylserine and free cysteine in developing seeds of grain legume. *J Exp Bot* 61:721–733
- Townsend JA, Thomas LA (1994) Factors which influence the *Agrobacterium*-mediated transformation of soybean. *J Cell Biochem* 56, Suppl. 18A:78
- Ufaz S, Galili G (2008) Improving the content of essential amino acids in crop plants: goals and opportunities. *Plant Physiol* 147:954–961
- Warrilow AGS, Hawkesford MJ (1998) Separation, subcellular location and influence of sulphur nutrition on isoforms of cysteine synthase in spinach. *J Exp Bot* 49:1625–1636
- Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008) Physiological roles of the beta-substituted alanine synthase gene family in *Arabidopsis*. *Plant Physiol* 146:310–320
- Wirtz M, Hell R (2003) Production of cysteine for bacterial and plant biotechnology: application of cysteine feedback-insensitive isoforms of serine acetyltransferase. *Amino Acids* 24:195–203
- Yi H, Galant A, Ravilious GE, Preuss ML, Jez JM (2010a) Sensing sulfur conditions: simple to complex biochemical regulatory mechanisms in plant thiol metabolism. *Mol Plant* 3:269–279
- Yi H, Ravilious GE, Galant A, Krishnan HB, Jez JM (2010b) From sulfur to homogluthathione: thiol metabolism in soybean. *Amino Acids* 39:963–978
- Youssefian S, Nakamura M, Orudjev E, Kondo N (2001) Increased cysteine biosynthesis capacity of transgenic tobacco overexpressing an *O*-acetylserine(thiol) lyase modifies plant responses to oxidative stress. *Plant Physiol* 126:1001–1011
- Zhang Z, Xing A, Staswick P, Clemente TE (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell Tissue Organ Cult* 56:37–46
- Zhang C, Meng Q, Gai J, Yu D (2008a) Cloning and functional characterization of an *O*-acetylserine(thiol)lyase-encoding gene in wild soybean (*Glycine soja*). *Mol Biol Rep* 35:527–534
- Zhang C, Meng Q, Zhang M, Huang F, Gai J, Yu D (2008b) Characterization of *O*-acetylserine(thiol)lyase-encoding genes reveals their distinct but cooperative expression in cysteine synthesis of soybean [*Glycine max* (L.) Merr.]. *Plant Mol Biol Rep* 26:277–291