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ORIGINAL ARTICLE

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

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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

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

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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 (Tabe and Higgins 1998; Amir and Galili 2003; Amir and Tabe 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 β-substituted alanine synthase (BSAS) family of enzymes, which also contains the enzymes that produce β-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 (Blaszczyk 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_2 (Youssefian et al. 2001). Similarly, Arabidopsis plants overexpressing OASS exhibited tolerance to cadmium chloride presumably due to higher levels of GSH and phytochelatins (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′-CCAA**GGATCC**ATGCCGACGGGGTTACCGGC-3′ and 5′-GGTT**GCGGCCGC**GGGCTCAAAAGTCATGC TTT-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

recovered by digestion with BamHI and NotI and inserted into corresponding sites in the intermediate vector resulting in plasmid pHCS1 with a fused 6X-His tag at the 5′-end of the ORF. As a final step, the inserts from the intermediate plasmid of OASS were digested with BamHI and XbaI and then cloned into the corresponding sites of pZ35S1, resulting in pZCS1. The final plasmid consisted of cauliflower mosaic virus 35S promoter (CaMV 35S), the OASS coding region, together with the cassette containing the CaMV 35S promoter, the *bar*-coding region and the 3′-region of the nopaline synthase gene (nos). Transfer of pZCS1 into *Agrobacterium tumefaciens* (strain EHA105) was performed by triparental mating.

Production of transgenic soybean lines

Transformation of soybean (*Glycine max* L. cv. Maverick; seeds obtained from Dr. David Sleper, University of Missouri, Columbia, MO, USA) was performed by *Agrobacterium*-cotyledonary node transformation (Hinchee et al. 1988) utilizing glufosinate as a selective agent. Regenerated transgenic soybean plants were screened for tolerance to herbicide Liberty by a leaf-painting assay as described earlier (Zhang et al. 1999). The expression of the introduced OASS gene was confirmed by Western-blot analysis and by measuring OASS activity.

Plant growth

Soybean plants were grown in 2-gallon pots containing PRO-MIX (Premier Horticulture, Québec, Canada) medium and fertilized with Osmocote Plus (Scotts, Marysville, OH, USA) in greenhouse with supplemental lighting. Greenhouse settings were 16-h daylength and 30/18°C day/night temperatures.

Estimation of transgene copy number in transformed soybean plants

Transgene copy number determination in soybean plants was performed by quantitative real-time PCR (qRT-PCR) essentially as described earlier (Mason et al. 2002; Omar et al. 2008). The primers and TaqMan probes used in this study (Table 1) were designed with SciTools software (http://www.idtdna.com/scitools/Applications/RealTimePCR/) and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The $SyOASS_{trans}F$ primer was designed to anneal to the 3′end of OASS gene coding region, whereas $SyOASS_{trans}R$ primer was designed to anneal to potato proteinase inhibitor II terminator region in the soybean transformation vector. The 5′ end of the probes specific for endogenous homoserince dehydrogenase (HSD; Schroeder et al. 2010), a single-copy internal control gene, and OASS transgene were labeled with fluorescent reporter dye FAM (6-carboxy-fluoroscein, excitation wavelength = 494 nm, emission wavelength = 521 nm). To minimize background noise and background fluorescence the 3′ end of both probes were labeled with internal ZEN quencher and Iowa Black FQ quencher (Table 1).

The real-time-PCR reactions for absolute quantification were performed in a fluorometric thermal ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems-Perkin-Elmer, Foster City, CA, USA) in 25 µl reaction mixtures containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) with 25 ng DNA and 500 nM each gene-specific primers, 200 nM each probes. The amplification conditions and analysis of data were performed as described by Omar et al. (2008). To generate standard curve for the endogenous HSD and the OASS transgene, genomic DNA from one of the transgenic line was used as described by Mason et al. (2002). Standard curves were calibrated using four concentrations (25, 50, 75, and 100 ng per reaction) of genomic DNA of the chosen transgenic line. These standard curves were used for relative quantification of the endogenous gene and the transgene. Calculation of copy number and statistical analysis were performed as described by Mason et al. (2002).

Reverse transcriptase (RT)-PCR analysis

Total RNA from fully expanded soybean leaves at node 6 was extracted using Trizol reagent (Invitrogen) and treated with DNaseI (Invitrogen) to remove contaminating DNA. The extract was subjected to RT-PCR using the OneStep RT-PCR kit (Qiagen). Primers for the amplification of soybean cytosolic OASS were CSTF, 5′-dCCAAG GATCCATGGCTGTTGAAAGGTCCGG-3′ and 6HisR, 5′-dTCTAGAGTGGTGGTGGTGGTGGTGCTCGAG-3′. We took advantage of the six-histidine tag sequences to specifically amplify the introduced cytosolic OASS and not the endogenous OASS. A 700-bp fragment of soybean 18S rRNA was also reverse-transcribed under similar conditions and used as a loading control. Primer sequences were as follows: Forward: 5′-GCTTAACACATGCAAGTCG AACGTTG-3′, Reverse: 5′-ACCCCTACACACGAAATT CCACTC-3′.

Western-blot analysis

Soybean seeds collected at 1-week intervals were divided into eight developmental stages (DS1-8) based on seed size. Protein extracts from soybean leaf or seeds were fractionated by SDS-PAGE (Laemmli 1970) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA). The proteins were

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

Gene	Sequence	Specificity
HSD, Homoserine dehydrogenase (Phytozome, Glyma20g11950.1)		
$SyHSD_{endo}F$	5′-CACAGAAGCAGAAACAAGAACC-3′	Intron 6
$SyHSD_{endo}$probe	5′-/FAM/AAACCTCCC/ZEN/TTGCAGTCCACACTGAA/IABkFQ-3′	Intron 6
$SyHSD_{endo}R$	5′-GGAGAGGAATGGAGAAAAGGG-3′	Intron 6
OASS, *O*-acetylserine sulfhydrylase (GenBank Acc. No. AF452451)		
$SyOASS_{trans}F$	5′-TCCGTGCTATTTGAGTCAGTG-3′	pZCS1
$SyOASS_{trans}$Probe	5′-/FAM/TCTAGATCA/ZEN/GTGGTGGTGGTGGTGGT/IABkFQ-3′	pZCS1
$SyOASS_{trans}R$	5′-AAGTCTAGGGTCACATTGCAG-3′	Pin II terminator[a]

[a] 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-CVDITDFCYEPCKP 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 (Warrilow 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 $MgCl_2$, 1% Tween 80 and 10 mM dithiothreitol (DTT)]. The samples were transferred to microcentrifuge tubes and centrifuged at 4°C for 10 min at 12,000*g*. 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' 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 hexahistdine-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 trifoliate 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.

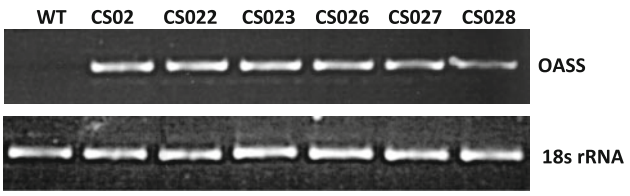

Fig. 1 RT-PCR analysis of OASS expression in transgenic soybean. Total RNA isolated from non-transformed plant (WT) and six transgenic soybean plants (CS02–CS028) were used in RT-PCR analysis. The *upper panel* shows RT-PCR product for soybean OASS (1,008 bp) and the *lower panel* shows the RT-PCR product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

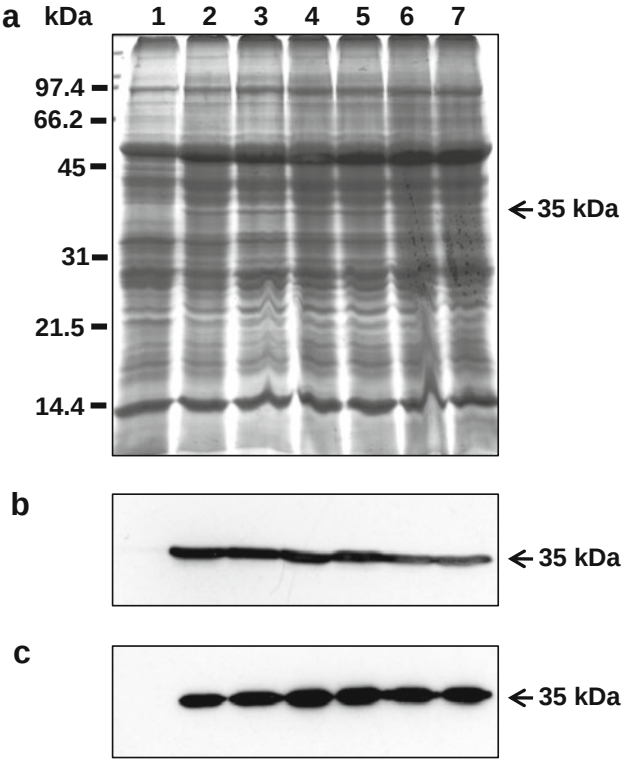

Fig. 2 Expression of OASS in the trifoliate leaves of transgenic soybean and non-transformed control plants. Total leaf proteins from non-transformed control (*lane 1*) and six independent transgenic lines (*lanes 2–7*) were fractionated by 13.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (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 $OASS_{transgene}$ 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

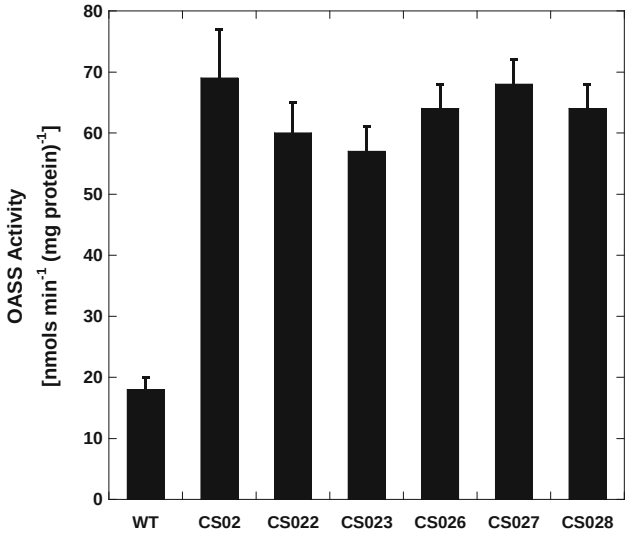

Fig. 3 OASS activity in leaf extracts of wild-type and six independent transgenic soybean lines. OASS activity was measured under standard assay conditions as described under "Materials and methods" using protein extracts from trifoliate leaves. OASS activity from non-transformed wild-type and transgenic soybeans are presented as the means ± 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$_{endo}$	SQ-OASS$_{trans}$	r_{line} (SQ-OASS$_{trans}$/SQ-HSD$_{endo}$)	(r_{line}/r_1)[a]	Copy number
Wild type	12.15 ± 1.85	1.21 ± 0.54	0.0995 ± 0.05	0.089 ± 0.04	0
CS02	19.09 ± 3.45	36.09 ± 4.26	1.8905 ± 0.41	1.703 ± 0.37	2
CS022	31.46 ± 10.88	41.73 ± 5.47	1.3264 ± 0.49	1.195 ± 0.44	1
CS023	17.08 ± 3.30	26.16 ± 5.62	1.5316 ± 0.44	1.380 ± 0.40	1
CS026	19.05 ± 4.24	36.19 ± 2.25	1.8559 ± 0.42	1.672 ± 0.38	2
CS027	13.72 ± 4.30	31.41 ± 14.81	2.2894 ± 1.30	2.062 ± 1.17	2
CS028	15.97 ± 2.82	27.63 ± 10.58	1.7301 ± 0.73	1.559 ± 0.66	2

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

[a] Calculated copy number for OASS transgene obtained as the ratio between r_{line} and virtual calibrator r_1

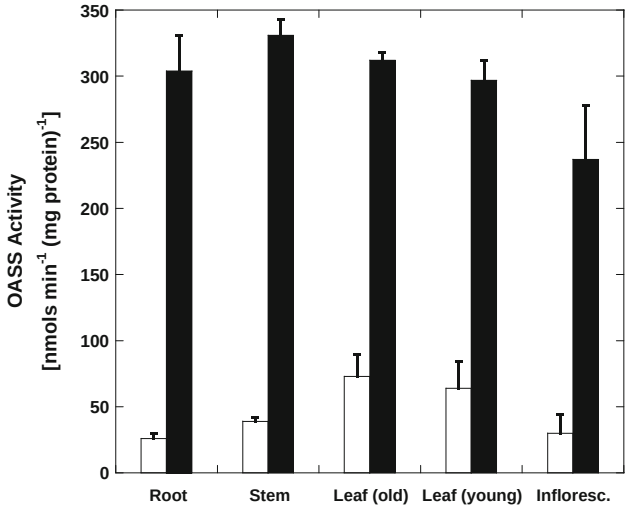

Fig. 4 OASS activity in different soybean tissues. OASS activity was measured under standard assay conditions as described under "Materials and methods" using protein extracts from roots, stems, young leaves, mature leaves, and inflorescences. OASS activity from non-transformed wild-type and transgenic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

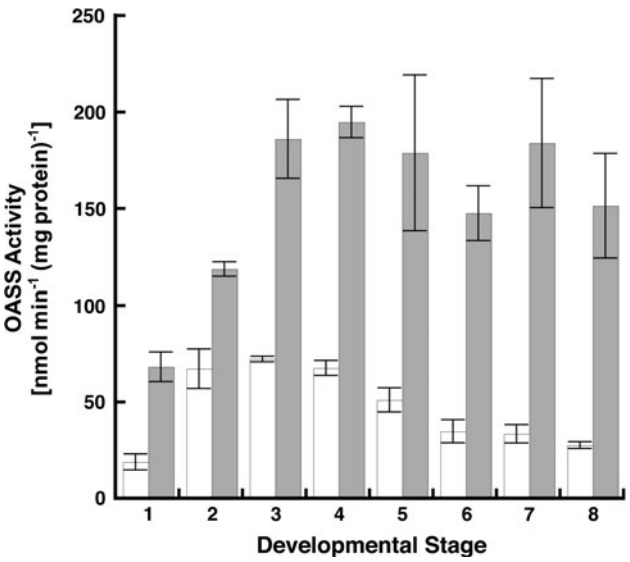

Fig. 5 OASS activity during soybean seed development. OASS activity was measured under standard assay conditions as described under "Materials and methods" using protein extracts from different seed developmental stages. OASS activity from non-transformed wild-type and transgenic soybeans are shown by *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 ± 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[a]	CS0022[a]	CS023[a]
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

[a] Transgenic T2 soybean lines overexpressing OASS. Dry seed powder from three independent plants were combined and subjected to amino acid analysis

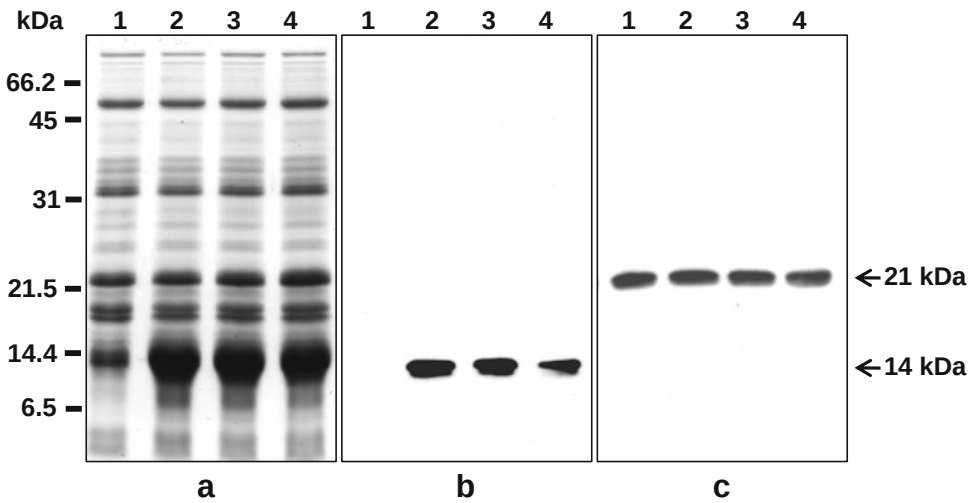

Fig. 6 Differential accumulation of Bowman–Birk protease inhibitor in the seeds of non-transformed wild-type and transgenic soybean plants. 50%-isopropanol extracted seed protein were separated by 13.5% SDS-PAGE and visualized with Coomassie Blue R-250 (**a**) or transferred to nitrocellulose membranes 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 *GmOASTL4* 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 γ-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 nutritionally 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 trails 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).

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