SULFUR METABOLISM IN *GLYCINE MAX* [L.] MERR: CHARACTERIZATION OF SERINE ACETYLTRANSFERASE AND *O*-ACETYLSERINE (THIOL) LYASE

A Dissertation presented to the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

---

by

DEMOSTHENIS CHRONIS

Dr. Hari B. Krishnan, Dissertation Supervisor

DECEMBER 2006

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

SULFUR METABOLISM IN *GLYCINE MAX* [L.] MERR: CHARACTERIZATION OF SERINE ACETYLTRANSFERASE AND *O*-ACETYLSERINE (THIOL) LYASE

presented by Demosthenis Chronis
a candidate for the degree of Doctor of Philosophy
and hereby certify that in their opinion it is worthy of acceptance

____
Dr. Hari B. Krishnan

____
Dr. Dale G. Blevins

____
Dr. Christopher G. Taylor

____
Dr. Joseph C. Polacco

____
Dr. Joseph M. Jez

...to my brother George for making my dream come true.

Many thanks to my parents Nikos and Georgia, and my wife Aggeliki for their endless support.

άντε ‘γεια μας (*gr.* cheers)

ACKNOWLEDGMENTS

The author would like to express his great gratitude to Dr. Hari B. Krishnan for his everyday support and guidance throughout this project. Many thanks to my committee members for finding the time to provide with feedback and advice. Special thanks to Drs. Leustek and Saito for providing the SAT antibody for *Arabidopsis* and the *E. coli* NK3 mutant respectively and Dr. Polacco for providing the soybean cDNA library. Last, but not least, a word of appreciation to current and past members of the Krishnan's lab for sharing their knowledge and expertise in this project.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....ii

TABLE OF CONTENTS.....iii

LIST OF ILLUSTRATIONS.....iv

LIST OF TABLES.....vii

ABSTRACT.....viii

CHAPTERS

1. INTRODUCTION: AN OVERVIEW OF SOYBEAN NUTRITIONAL VALUE AND ECONOMIC IMPORTANCE.....1

2. SULFUR METABOLISM IN PLANTS: THE PAST, THE PRESENT AND THE FUTURE.....14

3. MOLECULAR CLONING AND CHARACTERIZATION OF A CYTOSOLIC ISOFORM OF SERINE ACETYLTRANSFERASE (SAT).....27

4. MOLECULAR CLONING AND CHARACTERIZATION OF *O*-ACETYLSERINE (THIOL) LYASE (OAS-TL) FROM SOYBEAN.....59

5. TRANSGENIC STUDIES: OVEREXPRESSION OF *O*-ACETYLSERINE (THIOL) LYASE (OAS-TL) AND SERINE ACETYLTRANSFERASE (SAT) IN *ARABIDOPSIS* AND SOYBEAN.....86

VITA.....129

LIST OF ILLUSTRATIONS

Chapter 1

Figure Page

1. Soybean world production..8
2. U.S. supply of oilseeds..9
3. PDCAAS for selected food sources.. 10

Chapter 2

Figure

1. Overview of sulfur metabolism in plants.. 22
2. Regulation of sulfur assimilation in plants..23

Chapter 3

Figure

1. Multiple alignment of the amino acid sequence and phylogenetic
   tree of SAT from different plants...47
2. Southern blot analysis of soybean genomic DNA..48
3. Functional complementation of soybean SAT in *E. coli*..................................... 49

4. Semi-quantitative reverse transcriptase (RT)-PCR detection of SAT mRNA in developing soybean seeds....50

5. Expression and purification of the recombinant SAT....51

6. Western blot analysis of soybean SAT....52

7. SAT activity in soybean and *Arabidopsis thaliana*....53

8. Product inhibition of soybean recombinant SAT by cysteine....54

Chapter 4

Figure

1. Enzyme restriction map and nucleotide sequence of the isolated soybean OAS-TL cDNA....76

2. Amino acid comparison and phylogenetic tree of the soybean amino acid sequence of OAS-TL....77

3. Southern blot analysis of soybean genomic DNA....78

4. Functional complementation of Cys^- *E. coli* NK3....79

5. Reverse transcriptase (RT)-PCR detection of OAS-TL mRNA in developing soybean seeds....80

6. SDS-PAGE of total seed protein from 6 different developmental stages and western blot analysis of OAS-TL....82

7. OAS-TL enzyme activity on soybean developing seed....83

Chapter 5

Figure

1. Graphic representation of SAT structure.... 106

2. Overexpression of native and recombinant SATs in *E. coli* 107

3. Inhibition of native and recombinant SATs by L-cysteine................................ 108

4. Genetic maps of SAT and OAS-TL constructs used for plant transformation...109

5. Western blot from *Arabidopsis* plants transformed with pZSAT1......................110

6. Western blot from *Arabidopsis* plants transformed with pZSAT5......................111

7. Western blot from *Arabidopsis* plants transformed with pZSAT6......................112

8. Western blot from *Arabidopsis* plants transformed with pZCS1........................113

9. Western blot from soybean plants transformed with pZSAT1............................114

10. Western blot from soybean plants transformed with pZSAT5............................115

11. Western blot from soybean plants transformed with pZCS1..............................116

12. Serine acetyltransferase activity from *Arabidopsis* plants transformed with pZSAT1, pZSAT5 and pZSAT6...117

13. Serine acetyltransferase activity from soybean plants transformed with pZSAT1, and pZSAT5..118

14. *O*-acetylserine (thiol) lyase activity from transgenic *Arabidopsis* plants transformed with pZCS1...119

15. *O*-acetylserine (thiol) lyase activity from transgenic soybean plants transformed with pZCS1.. 120

16. Effect of heavy metal stress to transgenic *Arabidopsis*.....................................121

17. Effect of oxidative stress to transgenic *Arabidopsis*... 122

18. Effect of oxidative stress to transgenic soybean..123

LIST OF TABLES

Chapter 3

Table Page

I. Kinetic parameters and inhibition constants of SAT...55

Chapter 5

Table

I. Kinetic parameters and inhibition constants of native and recombinant SATs...124

SULFUR METABOLISM IN *GLYCINE MAX* [L.] MERR: CHARACTERIZATION OF SERINE ACETYLTRANSFERASE AND *O*-ACETYLSERINE (THIOL) LYASE

DEMOSTHENIS CHRONIS

Dr. Hari B. Krishnan, Dissertation Supervisor

ABSTRACT

Soybean (*Glycine max* [L.] Merr) is considered an excellent protein source for both humans and livestock. Presently the protein fraction of soybean accounts for 75% of the value of the crop. Further improvement of quantity and quality of soybean protein is vital for maintaining the utility of this versatile plant derived nutrient. Although high protein soybean lines are currently available, the cysteine and methionine content is still not adequate to meet the dietary needs of livestock and poultry, two major consumers of soybean meal. Currently, rations for these animals are supplemented with synthetic methionine, a procedure costing the animal industry millions of dollars annually. Efforts to enhance the sulfur amino acid content of soybean protein to meet FAO standards through genetic engineering and traditional breeding have met with limited success. Expression of genes for exogenous high methionine proteins in soybeans has not substantially increased the overall sulfur amino acid content. A possible explanation is that the availability of sulfur amino acids in developing seeds may be limiting. Effectively increasing the accumulation of sulfur amino acids in soybean will require metabolic engineering of the sulfur assimilatory pathway. In an attempt to improve the

nutritional quality of soybean seed proteins, molecular techniques are being employed to manipulate key enzymes involved in sulfur assimilation. As a first step, the molecular cloning and characterization of two key enzyme in sulfur assimilation are reported here: Serine acetyltransferase (SAT), catalyzes the formation of *O*-acetylserine (OAS) from serine and acetylCoA, and *O*-acetylserine (thiol) lyase (OAS-TL), catalyzes the final step in cysteine biosynthesis. Both genes were overexpressed in soybean in an attempt to increase the overall cysteine content. Western blot analysis and enzyme activity assays revealed that SAT is present in low levels in soybean, which could explain the low concentration of cysteine in soybean seeds. Plants overexpressing SAT showed elevated levels of SAT protein and enzyme activity, suggesting that overexpression of SAT could elevate cysteine concentration to adequate levels. Transgenic plants expressing OAS-TL exhibited resistance to oxidative stress and heavy metals. In conclusion, this study demonstrates the importance of SAT and OAS-TL in cysteine biosynthesis and show that production of cysteine and related sulfur-containing compounds can be enhanced by genetic manipulation of the enzymes involved is sulfur assimilatory pathway.

CHAPTER 1

INTRODUCTION: AN OVERVIEW OF SOYBEAN NUTRITIONAL VALUE AND ECONOMIC IMPORTANCE

Soybean (*Glycine max* [L.] Merr), a well known crop to ancient Chinese, was first recorded 5000 years ago in northeast China. The distant evolutionary ancestor of soybean is considered to be perennial vining plants that gave rise to the wild and weedly form of soybean, the *Glycine soja* (Hermann, 1962). Soybean spread from China to the rest of the Orient when traders took soybeans with them on sea voyages (Ho, 1969), but they remained unknown in the West until 1765 when it was first introduced to the United States (Hymowitz and Harlan, 1983). But it was not till the 1920s, that U.S. farmers first began growing soybeans in commercial quantities, mainly for animal feed. However, by the Second World War, when edible oils and traditional sources of protein were in short supply, soybean began to make its valuable contribution to the human diet, establishing soybean as one of the world’s major economic crop.

Today processed soybeans are the largest source of protein feed and vegetable oil in the world. The United States is the world's leading soybean producer (Fig. 1; Soybean: world supply and distribution, 2006) and exporter, with U.S. soybean exports reaching 1,103 million bushels for the crop year 2004, an amount corresponding to 35% of the total U.S. soybean production (Oil crops outlook report, 2006). Soybeans equal about

90% of U.S. total oilseed production, while other oilseeds such as cotton seed, sunflower seed, and cottonseed account for the remainder (Fig. 2; U.S. oilseeds supply and distribution, 2006). Planted soybean acreage in 2004 was estimated to be 75.2 million acres yielding a farm value for U.S. soybean production of $17.9 billion, the second-highest value among U.S. produced crops, trailing only corn. In Missouri, 27% of the cropland is planted by soybeans establishing soybean as the number one crop in the state. The year 2004 approximately $537.7 million was contributed to the economy of Missouri ranking the state number seven in the production of soybean among other states (State fact sheet:MO, 2006).

The economic importance of soybean is related to its nutritional value. Soybean is considered an excellent source of protein, with an average protein content around 40%. The nutritional value of soybean is mainly derived by two groups of seed proteins 7S and 11S, designated β-conglycinin and glycinin, respectively (Nielsen, 1996; Krishnan, 2000). Glycinin (11S) has a relatively high methionine content. In contrast, β-conglycinin is limited in this amino acid, thus lowering the overall content of sulfur-containing amino acids in soybean (Nielsen, 1985). Under the old protein scoring method, which used Protein Efficiency Ratio (PER), soybean protein was considered inferior to animal protein because methionine and cysteine were limiting and prevented optimal growth in rats (Young, 1991). Humans do not have as high a need for the sulfur-containing amino acids, methionine and cysteine, as rats; therefore, the PER score for soybean protein was considered an inaccurate score for humans. The new scoring method, the Protein

Digestibility Corrected Amino Acid Score (PDCAAS), has been recommended by the Food and Agricultural Organization (FAO) and World Health Organization (WHO) Expert Consultation on Protein Quality Evaluation (FAO/WHO, 1991). The PDCAAS method is based on a food protein's amino acid content, digestibility and ability to supply essential amino acids in the amounts adequate to meet human needs, thus providing a more accurate and concise evaluation for the quality of a protein. The amino acid content standard for the PDCAAS is based on the requirements of a two- to five-year old child. This represents the most demanding amino acid requirements of any age group except infants. Soybean protein isolates and concentrates receive a score of 1.00, which is the highest rating and comparable to milk or eggs (Fig. 3).

Recently there have been numerous reports doubting the validity of the PDCAAS. The main controversy is around the antinutritional agents and the bioavailability of amino acids, factors that PDCAAS doesn't take under consideration. Soybean meal protein contains protease inhibiting agents, including the Kunitz trypsin inhibitor and chymotrypsin inhibitor. These agents could cause pancreatic hypertrophy when raw soybeans are ingested (Booth et al., 1960). Food processing steps like heat and alkaline treatment are required to eliminate or lower the effect of the protease inhibitors. Though excessive treatment could downgrade the quality of soybean protein, since these steps induce the production of lysinoalanine in soybean protein isolate (Sarwar, 1997), minimize amino acid availability and, as a result, reduce animal weight gain (Lee and Garlich, 1992). Taking into consideration the bioavailability of individual amino acids

and amino acid analysis, it was shown in chicken that soybean meal protein is limiting in methionine and cysteine, whereas soybean protein concentrate and isolate were limiting in the sulfur containing amino acids and threonine (Emmert and Barker, 1995). Nevertheless, soybean is considered an excellent nutrient source for humans.

Although, the use of soybean in the food industry for humans has been grown tremendously the past few years with the production of soymilk, tofu, soy flour etc., soybeans are mainly used as a high-protein feed ingredient in livestock and poultry production. However, the limitation of sulfur-containing amino acids in soybean is a constraint because animals cannot produce these amino acids (Finkelstein et al., 1988) and as a result grain-soybean meal rations do not cover the dietary requirement for sulfur amino acids of young swine and poultry. An estimated $100 million is spent annually by the poultry and swine industry to supplement feeds with synthetic methionine in order to achieve optimal growth and development of animals consuming grain-soybean meal rations (Imsande, 2001). Since soybean is the principal seed meal used in feeds, developing soybean cultivars with high sulfur amino acid content could influence the economy and production of the livestock and poultry industry.

Conventional plant breeding methods have had limited success in the past to increase the overall sulfur protein content in soybean. Madison and Thompson (1988) identified soybeans cell culture lines that overproduce methionine. These lines accumulate methionine 8.7 fold higher than the parental lines. In 2001 Imsande produced, by a standard mutagenic procedure, high sulfur lines showing 31% higher methionine than

what is present in average soybean cultivar. The same lines had an increased cysteine content of appoximately 20%. It has been shown that the ratio between 11S and 7S globulins determines the content of sulfur amino acids (Peak et al., 1997; 2000). Since 7S globulins are deficient in sulfur-containing amino acids, attempts have been made to raise cultivars that down-regulate or do not express these proteins at all (Kitamura and Kaizuma, 1981; Ladin et al., 1984; Tsukada et al., 1986). Although, this was a promising approach of traditional plant breeding, the plants showed developmental abnormalities and were not able to reproduce (Kitagawa et al., 1991). Recently, a line from a Japanese wild soybean collection that completely lacks the 7S globulin protein was identified (Hajika et al., 1996). Soybean cultivars of this trait showed normal growth and development (Teraishietal., 2001). The entire absence of the β-conglycinin protein could serve as an approach to increase the overall content of sulfur in soybean seed. However further investigation is needed to establish the effect of such a modification on the nutritional value of soybean.

In recent years, molecular biology and genetic engineering have opened new horizons in plant research providing useful tools for optimization of soybean seed protein content by expressing heterologous genes for proteins high in sulfur. The sulfur-rich 2S albumin gene (BNA) from the Brazil nut (*Bertholletia excelsa*) has been successfully expressed in *Arabidopsis thaliana*, *Brassica napus* and *Nicotiana tabacum* (Clercq et al., 1990). Expression of the BNA gene in bean (*Phaseolus vulgaris*) resulted in 14 to 23% increase in methionine content when compared to untransformed plants (Aragao et al. 1999).

Similar results were obtained in transgenic soybean lines, but the increase in methionine content was accompanied by a reduction in protease inhibitors, a protein rich in cysteine (Townsend and Thomas, 1994; Streit et al., 2001). The expression of the Brazil nut 2S albumin provides a promising approach of increasing the sulfur content of grain-legumes. However, the BNA protein has been identified as a potential allergen (Nordlee et al., 1996) and preventing the commercial production of soybeans transformed with the BNA gene. Transgenic lupin (*Lupinus angustifolius* L.) seeds expressing a seed-specific gene for the sulfur-rich sunflower albumin (SSA), revealed 94% increase in methionine and 12% decrease in cysteine, resulting in a net 19% increase in total sulfur amino acid content (Molvig et al., 1997). Expression of the same gene in rice resulted to a minimal increase in total sulfur amino acid content. Interestingly enough, the demand for sulfur driven by the SSA expression caused a re-allocation of the limited sulfur reserves from the endogenous storage seed proteins to the new sulfur "sink" in the transgenic lines (Hagan et al., 2003), indicating that the available sulfur for incorporation to the seed could be in moderate or diminished amounts. In another study, based on the same strategy, amino acid analysis on transgenic soybeans expressing a 15 kDa maize sulfur-rich zein protein under the β-phaseolin promoter confirmed a 12 to 20% increase in methionine and 15 to 35% increase in cysteine content compared to control lines. However, this increase is not adequate to cover the dietary need (Tabe and Higgins, 1998). Recently, an 11 kDa methionine-rich delta-zein protein was expressed in soybean, but the overall methionine content was not enhanced in these transgenic plants (Kim and

Krishnan, 2004). Expression of exogenous high methionine proteins alone is not sufficient to enhance substantially the overall sulfur amino acid content in soybean. It is possible that the supply of methionine or cysteine is only moderate during seed development, and thus preventing the high accumulation of the heterologous expressed proteins. Metabolic engineering and genetic manipulation of the enzymes involved in sulfur assimilatory pathway could potentially increase the accumulation of sulfur amino acids. As a first step, one must elucidate the machinery and the various regulatory steps involved in the sulfur metabolism pathway.

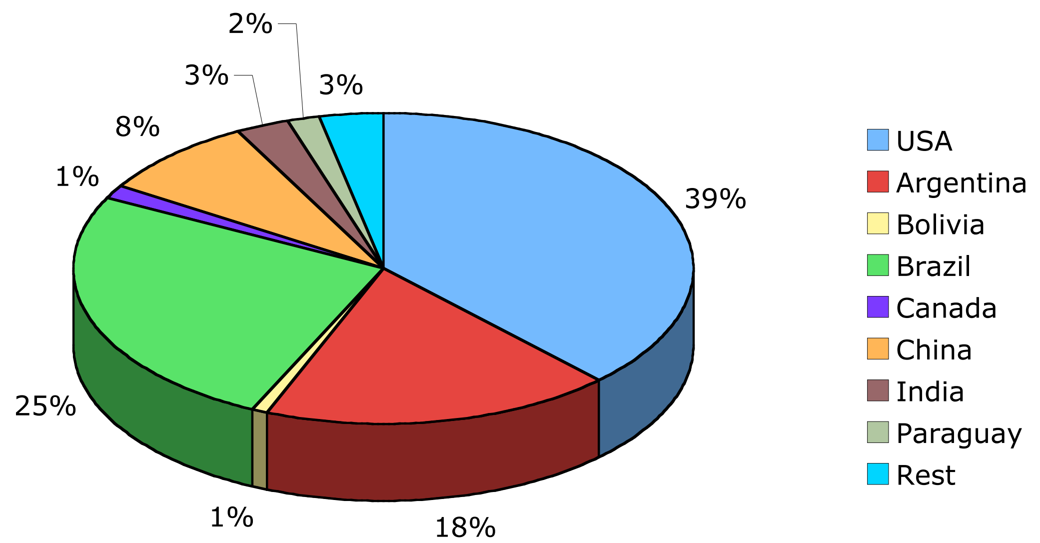


Figure 1: World soybean production for the crop year 2004. The United States, Brazil and Argentina are the top soybean production countries in the world (adopted from PDS/EAS 2006).

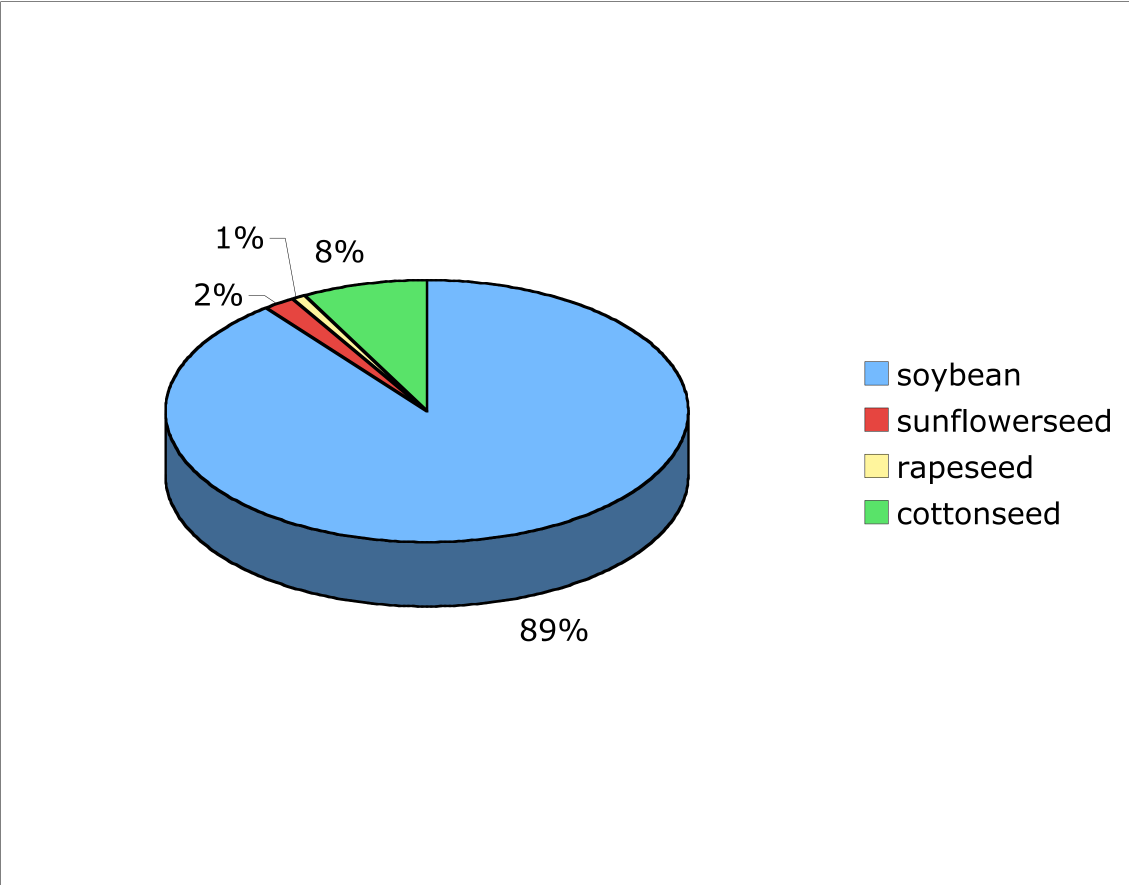


Figure 2: U.S. supply of oilseeds for the crop year 2004. Soybean is the main supply of oilseeds (adopted from PDS/EAS 2006).

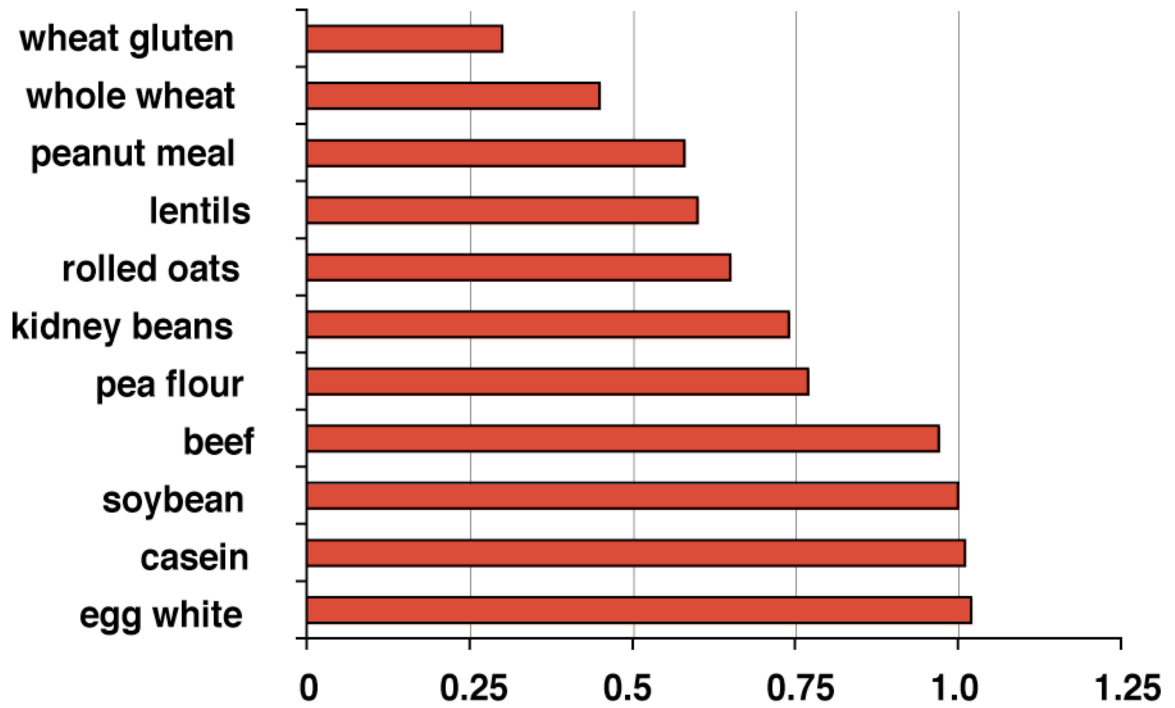


Figure 3: Protein Digestibility Corrected Amino Acid Score (PDCAAS) for soybean and other high protein foods.

LITERATURE CITED

Aragao F.J.L., Barros L.M.G., de Sousa M.V., Grossi de Sa M.F., Almeida E.R.P., Gander E.S., Rech E.L. (1999) Expression of a methionine-rich storage albumin from the Brazil nut (*Bertholletia excelsa* H.B.K., Lecythidaceae) in transgenic bean plants (*Phaseolus vulgaris* L., Fabaceae). Gen. Mol. Biol. **22 (3):** 445-449

Booth A.N., Robbins A.J., Ribelin W.E., DeEds F. (1960) Effect of raw soybean meal and amino acids on pancreatic hypertrophy in rats. Proc. Soc. Exp. Biol. Med. **104:** 681-683

Clercq A.D., Vandewiele M., Damme J.V., Guerche P., Montagu M.C., Vandekerckhove J., Krebbers E. (1990) Stable accumulation of modified 2S albumin seed storage proteins with higher methionine contents in transgenic plants. Plant Physiol. **94:** 970-979

Emmert J.L., Baker D.H. (1995) Protein quality assessment of soy products. Nutr. Res. **15:** 1647-1656

FAO/WHO (1991) Protein quality evaluation: Report of the joint Food and Agricultural Organization-World Health Organization expert consultation. FAO Food and Nutrition Paper. **51**, Rome, Italy

Finkelstein J.D., Martin J.J., Harris B.J. (1988) Methionine metabolism in mammals: the methionine-sparing effect of cysteine. J. Biol. Chem. **263:** 11750-11754

Hagan N.D., Upadhyaya N., Tabe L.M., Higgins T.J.V. (2003) The redistribution of protein sulfur in transgenic rice expressing a gene for a foreign, sulfur-rich protein. Plant J. **34:** 1-11

Hermann F.J. (1962) A ReVision of the genus Glycine and its immediate Allies. Technical Bulletin 1268, United States Department of Agriculture: Washington, DC

Ho P.-T. (1969) The loess and the origin of Chinese agriculture. Am. Hist. Rev. **75:** 1-36

Hymowitz T., Harlan J.R. (1983) The introduction of the soybean to North America by Samuel Bowen in 1765. Econ. Bot. **37:** 371-379

Imsande J. (2001) Selection of soybean mutants with increased concentrations of seed methionine and cysteine. Crop Sci. **41:** 510-515

Kim W-S., Krishnan H.B. (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 paranchyma cells. Plant Biotech Journal **2:** 199-210

Kitagawa S., Ishimoto M., Kikuchi F., Kitamura K. (1991) A characteristic lacking or remarkably decreasing 7S globulin subunits induced with gamma-ray irradiation in soybean seeds. Jpn. J. Breed. **41 (Supp. 2):** 460-461

Kitamura K., Kaizuma N. (1981) Mutant strains with low level of subunits of 7S globulin soybean (*Glycine max* Merr.) seed. Jpn. J. Breed. **31:** 353-359

Krishnan H.B. (2000) Biochemistry and molecular biology of soybean seed storage proteins. J. New Seeds **2:** 1-25

Ladin B.F., Doyle J.J., Beachy R.N. (1984) Molecular characterization of a deletion mutation affecting the α'-subunit of β-conglycinin of soybean. J. Mol. Appl. Genet. **2:** 372-380

Lee H., and Garlich J.D. (1992) Effect of overcooked soybean meal on chicken performance and amino acid availability. Poultry Sci. **71:** 499-508

Madison J.T., Thompson J.F. (1988) Characterization of soybean tissue culture cell lines resistant to methionine analogs. Plant Cell Rep. **7:** 473–476

Molvig L., Tabe L.M., Eggum B.O., Moore A.E., Craig S., Spencer D., Higgins T.J.V. (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. Proc. Natl. Acad. Sci. USA **94:** 8393-8398

Nielsen N.C. (1985) The structure and complexity of the 11S polypeptides in soybeans. J. Am. Oil Chem. Soc. **62:** 1680-1686

Nielsen N.C. (1996) Soybean seed composition. p. 127-163. *In* D.P.S. Verma and R.C. Shoemaker (ed.) Soybean: genetics, molecular biology and biotechnology. CAB International, Wallingford, UK

Nordlee J.A., Taylor S.L., Townsend J.A., Thomas L.A., Bush R.K. (1996) Identification of Brazil-nut allergen in transgenic soybeans. N. Engl. J. Med. **334:** 688-692

Oil crops outlook report (2006) Economic Research Service, United States Department of Agriculture, http://www.ers.usda.gov/News/soybeancoverage.htm

Paek N.C., Imsande J., Shoemaker R.C., Shibles R. (1997) Nutritional control of soybean seed storage protein. Crop Sci. **37:** 498–503

Paek N.C., Sexton P.J., Naeve S.L., Shibles R. (2000) Differential accumulation of soybean seed storage protein subunits in response to sulfur and nitrogen nutritional sources. Plant Prod. Sci. **3:** 268–274

Sarwar G. (1997) The protein digestibility - corrected amino acid score method overestimates quality of proteins containing antinutritional factors and of poorly digestible protein supplemented with limiting amino acids in rats. J. Nutr. **127:** 758-764

Soybean: world supply and distribution (2006) Production, Supply and Distribution (PSD) online database, Foreign Agricultural Service, United States Department of Agriculture, http://www.fas.usda.gov/psd/complete_tables/OIL-table2-24.htm

State fact sheet: MO (2006) Economic Research Service, United States Department of Agriculture, http://www.ers.usda.gov/statefacts/MO.htm

Streit L.G., Beach L.R., Register III J.C., Jung R., and Fehr W.R. (2001) Association of the Brazil nut protein gene and Kunitz trypsin inhibitor alleles with soybean protease inhibitor activity and agronomic traits. Crop Sci. **41:** 1757-176

Tabe L., Higgins T.J.V. (1998) Engineering plant protein composition for improved nutrition. Trends Plant Sci. **3:** 282-286

Townsend J.A., Thomas L.A. (1994) Factors which influence the Agrobacterium-mediated transformation of soybean. J. Cell. Biochem. Suppl. **18A:** 78

Tsukada Y., Kitamura K., Harada K., Kaizuma N. (1986) Genetic analysis of subunits of two major storage proteins (β-conglycinin) in soybean seeds. Jpn. J. Breed. **36:** 390-400

U.S. oilseeds supply and distribution (2006) Production, Supply and Distribution (PSD) online database, Foreign Agricultural Service, United States Department of Agriculture, http://www.fas.usda.gov/psd/complete_tables/OIL-table4-27.htm

Young V.R. (1991) Soy protein in relation to human protein and amino acid nutrition. J. Am. Diet. Assoc. **91:** 828-835

CHAPTER 2

SULFUR METABOLISM IN PLANTS: THE PAST, THE PRESENT AND THE FUTURE

Sulfur (S) is an essential macronutrient for plants. Only 0.1% of the plant dry matter corresponds to sulfur, fifteen times less than nitrogen, establishing sulfur as the least abundant macronutrient in plant tissue. However, sulfur is essential for protein structure and a fundamental element in a vast array of compounds with critical catalytic and electrochemical functions. Sulfur can be found in the amino acids cysteine (Cys) and methionine (Met) (Giovanelli et al., 1980; Saito, 1999). The thiol group of cysteine is important for the formation of disulfide bridges. The reversible formation of disulfide bonds between not adjacent cysteine residues, described as cysteinyl moiety, is essential for the tertiary structure of proteins and, therefore, catalytic activity of enzymes (Aslund and Beckwith, 1999). In addition, the thiol group is crucial for the function of antioxidant metabolites (glutathione, phytochelatins;), several cofactors (biotin, turgorin, phytosulfokine, thiamine pyrophosphate, lipoic acid, coenzyme A, Nod factors, thioredoxins), enzymes (nitrite reductase, ferredoxin:thioredoxin reductase) and structural components (sulfolipids) (Noctor et al., 1998; Rauser, 1995; Schlenk, 1965 Leustek et al., 2000).

Higher plants utilize sulfur mainly in the form of anionic sulfate (SO_4^{-2}). Other forms of sulfur can be employed, like the gaseous pollutant sulfur dioxide, but sulfate remains the primary source for plants, since it is relatively abundant in the environment. Sulfate is absorbed actively by the rhizosphere through the roots. Sulfur remains in the form of unmetabolized sulfate during translocation throughout the plant (Saito, 2004). Multiple transport steps are required for sulfate to reach the mesophyll cells of leaf tissue, where sulfur assimilation is believed to occur. This translocation is enabled by plasma membrane-localized sulfate transporters that exhibit 12 domains spanning through the plasma membrane and belonging to a large family of cotransporters (Hawkesford, 2003). Several genes have been identified that code for sulfate transporters, including 14 in *Arabidopsis* (Yoshimoto et al., 2002). The family of sulfate transporters is divided into five different groups according to their amino acid sequence and they are named SULTR 1 to 5. Each member shows distinct properties in translocation of sulfate, patterns of expression and tissue specificity. Evidence of how sulfate transporters function exists only for groups 1 and 2, that are classified as H^+/SO_4^{-2} transporters, and action is allowed by electrochemical gradient established by the plasma membrane proton ATPase pump (Buchner et al., 2004b). High affinity sulfate transporters of groups 1 and 2 are localized at the root epidermal cells and are involved in the uptake of sulfate into the plant (Takahashi et al., 2000). The long distance translocation from the root to the shoot is mediated by high affinity members of group 3 and movement of sulfate within the cell is mediated by the other subfamilies (Buchner et al., 2004b; Kataoka et al., 2004;

Yoshimoto et al., 2003). When sulfate reaches the epidermal cells, it can either be stored in the vacuole or enter the sulfur metabolic pathway.

The sulfur assimilatory pathway involves several regulatory steps that leads to the end product cysteine (Fig. 1). The pathway begins with the activation of sulfate by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS) and resumes with a two step reduction to sulfide by APS reductase and sulfite reductase. A relatively minor extension of this pathway is the phosphorylation of APS to 3'-phosphoadenosine-5'-phosphosulfate (PAPS), driven by APS kinase, and serves as a donor of activated sulfate for sulfation of jasmonates, flavonoids, glucosinolates and other compounds. The activation and reduction of sulfate occur exclusively in the plastids (Brunold and Sutter 1989; Lunn et al. 1990). However, a cytosolic isoform of ATP sulfurylase exists, which presumably is produced by structural genes for the plastidic isoforms, but it uses a different translational start codon (Hartzfeld et al., 2000). The function of the cytosolic ATP sulfurylase is yet unclear, since reduction of APS takes place entirely in plastids, but a possible action could be in generating APS for sulfation reactions (Rotte and Leustek, 2000). Incorporation of sulfide is the final step in sulfur assimilation leading to the formation of cysteine. Two enzymes are committed to this step, serine acetyltransferase (SAT) and *O*-acetylserine (thiol) lyase (OAS-TL). Serine and acetyl-CoA are the substrates of SAT which catalyzes the formation *O*-acetylserine (OAS). OAS is then coupled with sulfide to produce cysteine, reaction mediated by OAS-TL. In contrast to sulfate reduction

enzymes, isoforms of SAT and OAS-TL have been identified in three of the major compartments of plant cells, i.e. cytosol, chloroplasts and mitochondria (Saito, 2000).

The incorporation of sulfur into amino acids is regulated by a circuitous mechanism that involves OAS, sulfide, cysteine, glutathione and the unique enzyme complex between SAT and OAS-TL (Fig. 2). The cytosolic form of SAT is allosterically inhibited by cysteine, whereas the mitochondrial and chloroplastic isoforms are insensitive to this feedback inhibition. It is proposed that the cytosolic SAT is responsible for the production of OAS that serves as a regulatory factor in sulfur assimilatory gene expression (Noji et al., 1998; Innoue et al., 1999). Two residues at the allosteric site of SAT are responsible for this inhibition, glycine (Gly-277) and histidine (His-282). Both Gly-277 and His-282 exist in *Arabidopsis* cytoslic SAT, which the chloroplastic isoform does not contain any of these residues and mitochondrial isoform contains only the Gly-277 (Saito et al., 2000).

Specific protein-protein interactions between SAT and OAS-TL lead to the formation of an enzyme complex that plays an essential role in cysteine production (Bogdanova and Hell, 1997; Wirtz et al., 2001). The bound form of OAS-TL shows drastically lower catalytic activity than the free form. Kinetic studies revealed that the binding of one substrate to the free form of SAT does not affect the dissociation constant of the second substrate. However, in the complex, SAT shows higher affinity for its substrates. This positive cooperativity of SAT and the fact that OAS-TL is inactive in the complex have a

significant impact in the OAS rate formation and the production of cysteine (Droux et al., 1998).

In vivo, OAS-TL concentration is in considerable excess, approximately 300-fold, over to SAT, indicating that only a small fraction of OAS-TL binds to SAT. This distinctive difference in the molar concentration of the two enzymes controls the fate of sulfur assimilation. The levels of OAS and sulfide are critical regulatory factors of the activity of sulfur assimilatory enzymes. OAS triggers the dissociation of the complex, where sulfide compensates for this action, by promoting the binding of OAS-TL and SAT. It is believed that at low sulfide levels, OAS accumulates and hence slows its own synthesis by disrupting the enzyme complex. In contrast, when sulfide builds up, OAS-TL binds to SAT increasing the production of OAS for efficient cysteine synthesis (Droux et al., 1998).

A number of environmental factors determine the rate of sulfur metabolism. The levels of available sulfur and nitrogen are closely related to enzymes and metabolites of sulfur assimilation pathway. Under sulfur starvation, where the demand for sulfur metabolites is high, sulfur uptake and assimilation activity is induced (Wawrzynska et al., 2005). Studies in *Brassica* revealed that group 1, 2 and 4 sulfate transporters were up-regulated during sulfur deprivation, where group 3 was not affected at all (Buchner et al., 2004a). The limited availability of sulfur influences nitrogen metabolism causing increased levels of OAS (Nikiforova et al., 2005; Kim et al. 1999), which in turn promotes expression of APS reductase and sulfate transporters (Smith et al., 1997;

Koprivova et al., 2000). Supply of external OAS in potato mimics the effect of sulfur starvation leading to increased APS reductase and sulfate transporter gene expression (Hopkins et al., 2005). In the same study, transgenic lines of potato expressing the *cysE* gene from *Escherichia coli*, which encodes for SAT, showed increased cysteine and glutathione concentrations but a marginal increase in OAS pools, indicating that sulfur assimilation may be driven not only by OAS, but by depletion of sulfate, as well. In addition, sulfate transporter activity did not correlate with transcript and protein abundance, suggesting posttranslational regulatory mechanisms must exist. In contrast to OAS's positive effect, cysteine and glutathione down-regulate sulfur assimilation. In plants supplied with cysteine or glutathione, the elevated levels of thiols prevent sulfate uptake and decrease ATP sulfurylase activity, thus controlling sulfur efflux and cysteine production. (Lappartient and Touraine, 1996; Lappartient et al., 1999). The response of plants to sulfur starvation is reduced if at the same time the available nitrogen is also limited. Nitrogen limitation prohibits OAS accumulation and inhibits ATP sulfurylase and APS reductase activity, that are usually highly expressed under sulfur depleted conditions (Kim et al., 1999; Koprivova et al., 2000).

Abiotic stresses, such as heavy metal and oxidative stresses, influence sulfur assimilation. Once plants are exposed to heavy metals, they produce metal ion chelators, termed phytochelatins, that are derived from glutathione. These compounds may bind heavy metal cations through thiol groups and thus detoxify the metals (Rauser, 1995). As the synthesis of phytochelatins increases, the levels of glutathione and cysteine pools

decline. Therefore, the high demand for cysteine in response to heavy metal exposure drives the accumulation of ATP sulfurylase, APS reductase and OAS-TL (Heiss et al., 1999; Lee and Leustek, 1999), with plants overexpressing OAS-TL showing tolerance to heavy metals (Dominguez-Solis et al., 2001). Similar results have been described for salt stress. Plants supplemented with sodium chloride rapidly accumulate OAS-TL mRNA, cysteine and glutathione. Exposure to abscisic acid (ABA) mimicked the salt stress response, but mutants deficient or insensitive to ABA were not able to increase the OAS-TL levels as a consequence of salt addition (Barosso et al., 1999). Parallel to heavy metal and salt stress, oxidative stress induces sulfur metabolism. To combat oxidative stress caused by reactive oxygen species (ROS), plants have compiled a highly sophisticated antioxidant mechanism, where glutathione has a central role. Two glutathione molecules can be joined together through disulfide bridges between the cysteine residues. Disulfide bonds can be broken by reduction, freeing the thiol groups. Glutathione utilizes this property to function as an antioxidant, inactivating toxins, hormones, oxygen radicals and xenobiotic substances such as herbicides. The thiol group of cysteine links to the xenobiotic to form a conjugate, which is transferred into the vacuole. There the conjugate is hydrolyzed to a cysteine conjugate that is recycled (May et al., 1998). Transgenic lines of tobacco overexpressing OAS-TL showed elevated cysteine and glutathione contents, especially when exposed to sulfur oxide. The plants, accordingly, demonstrated dramatic reductions in the damage caused by the oxidative stress of sulfur oxide and the ROS generator methyl viologen, when compared to untransformed plants (Youssefian et al.,

2001). It is believed that oxidized glutathione derived in response to oxidative stress activates the APS reductase gene, *APR1*, which drives the production of cysteine. Under normal conditions the active site of *APR1*, APS reductase, is reduced and the enzyme is inactive. During oxidative stress reduced glutathione is consumed and the oxidized form accumulates, enabling activation of *APR1*. However, *APR2* and *APR3* genese are insensitive to redox regulation, indicating that these isoforms probably function in a non-oxidative stress environment (Bick et al., 2001).

During the past few years, remarkable progress has been made in understanding sulfur assimilation and the various regulatory steps involved in the synthesis of sulfur-containing metabolites. Most information comes from studies based on *Arabidopsis*, spinach and tobacco. Less is known about this pathway in soybean, a very important economic crop. This study was aimed increasing our information in sulfur metabolism in soybean with specific emphasis on SAT and OAS-TL, two key enzymes in sulfur assimilation. The information obtained will enhance our ability to increase the overall soybean sulfur content through genetic engineering.

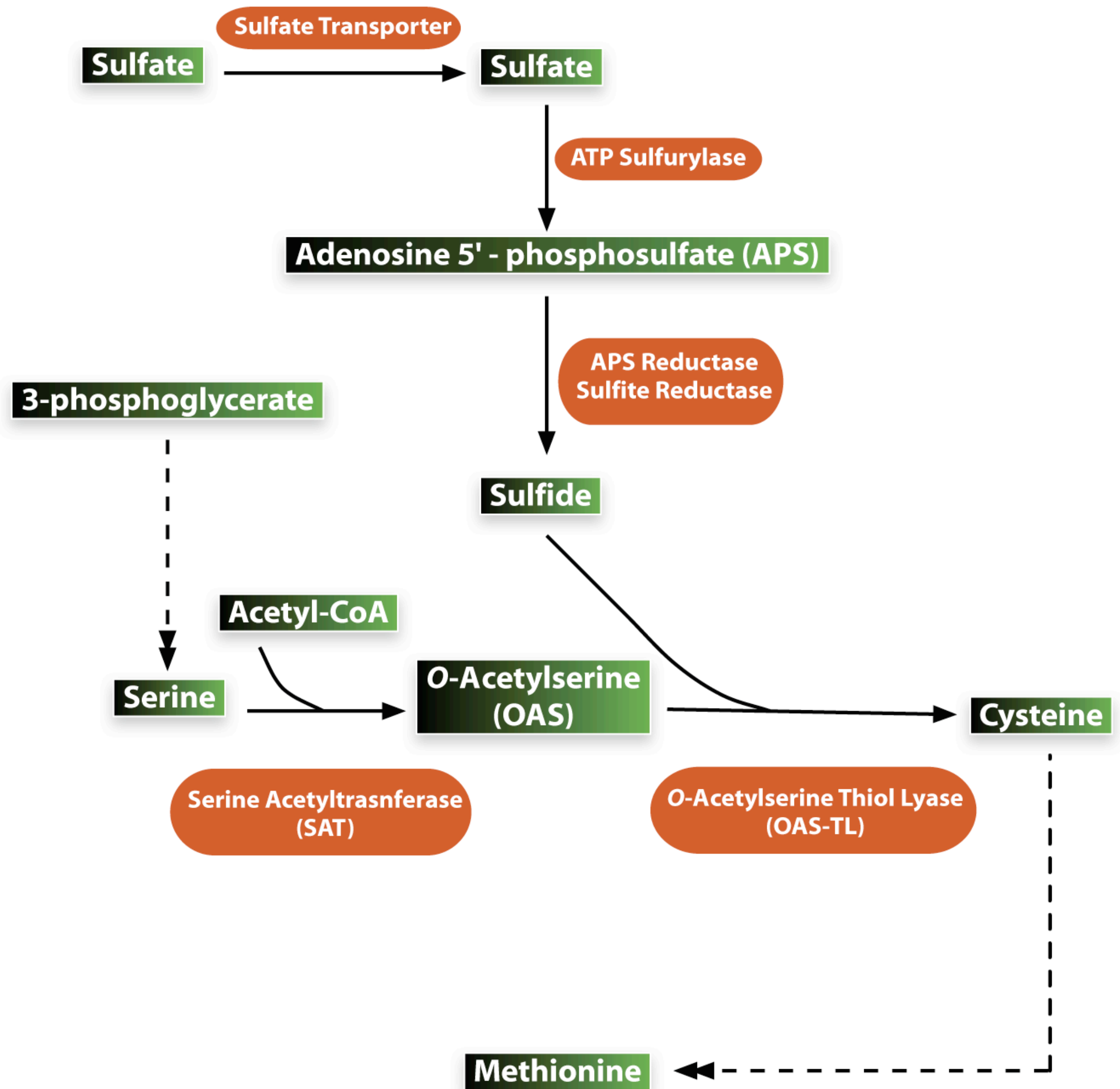


Figure 1: Overview of sulfur metabolism in plants. Sulfate is transported into the plant and is reduced to sulfide by the action of ATP sulfurylase, APS reductase and sulfite reductase. Serine acetyltransferase catalyzes the formation of *O*-acetylserine from serine and acetyl-CoA, where *O*-acetylserine (thiol) lyase combines *O*-acetylserine with sulfide to form cysteine. Several downstream steps lead to the production of methionine.

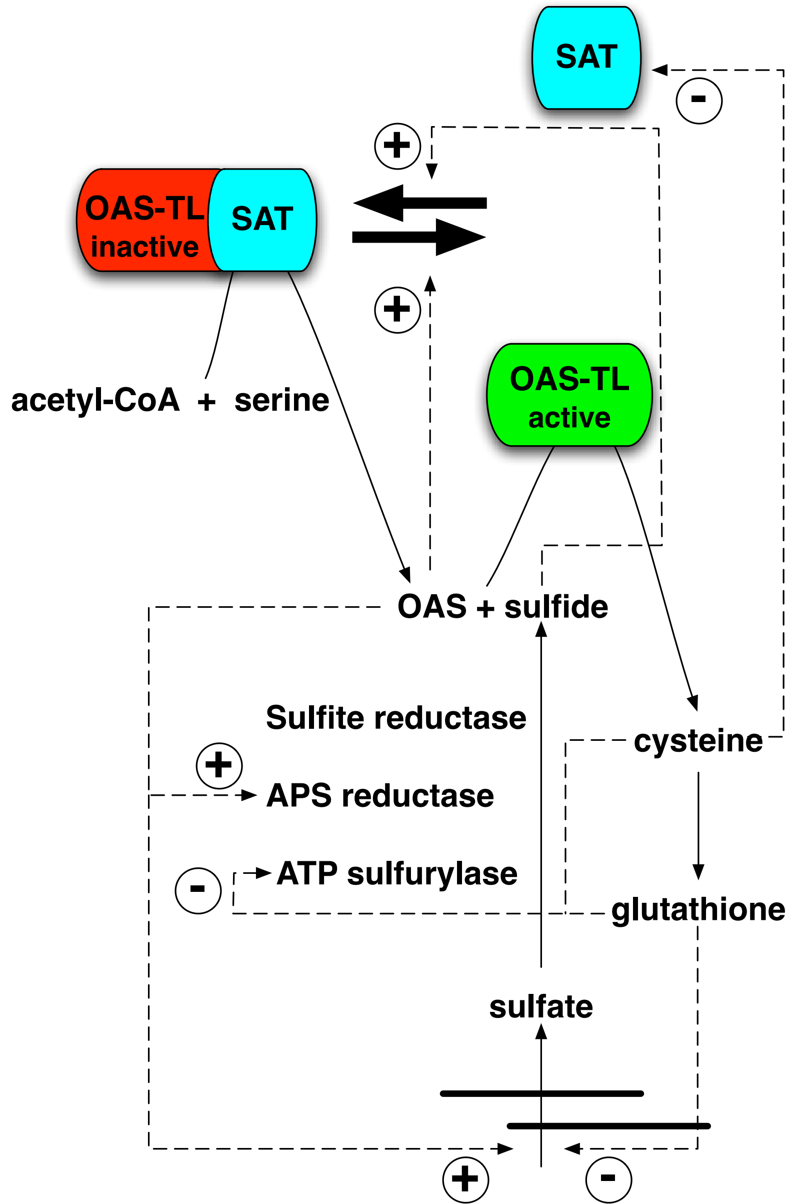


Figure 2: Regulation of sulfur assimilation in plants. Sulfide promotes the formation of the complex between serine acetyltransferase (SAT) and *O*-acetylserine (thiol) lyase (OAS-TL). In the complex SAT shows positive cooperativity, where OAS-TL is inactive. On the other hand *O*-acetylserine (OAS) promotes the dissociation of the complex, gene expression of APS reductase and sulfate transporter.. Cysteine down-regulates ATP sulfurylase and allosterically inhibits SAT. Finally glutathione down-regulates sulfate uptake and ATP sulfurylase. Catalytic steps are indicated with arrows. Regulatory steps are indicated with dashed-line arrows. Protein complex formation and dissociation are indicated with block arrows (Saito et al. 2000).

LITERATURE CITED

Aslund F, Beckwith J. (1999) Bridge over troubled waters: sensing stress by disulfide bond formation. Cell **96:** 751-753

Barroso C., Romero L.C., Cejudo F.J., Vega J.M., and Gotor C. (1999) Salt-specific regulation of the cytosolic *O*-acetylserine(thiol)lyase gene from *Arabidopsis thaliana* is dependent on abscisic acid. Plant Mol Biol. **40:** 729-736

Bick J.A., Setterdahl A.T., Knaff D.B., Chen Y., Pitcher L.H., Zilinskas B.A., Leustek T. (2001) Regulation of the plant-type 5'-adenylylsulfate reductase by oxidative stress. Biochemistry **40:** 9040-9048

Bogdanova N., Hell R. (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. **11:** 251-262

Brunold C., Suter M. (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. Planta **179:** 228-234

Buchner P., Stuiver C.E.E., Westerman S., Wirtz M., Hell R., Hawkesford M.J., De Kok L.J. (2004a) Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulfate nutrition. Plant Physiol. **136:** 3396-3408

Buchner P., Takahashi H., Hawkesford M.J. (2004b) Plant sulphate transporters: co-ordination of uptake, intracellular and long distance transport. J. Exp. Bot. **55 (404):** 1765-1773

Dominguez-Solis J.R., Gutierrez-Alcala G., Romero L.C., 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., Ruffet M.L., Douce R., Job D. (1998) Interactions between serine acetyltransferase and *O*-acetylserine (thiol) lyase in higher plants: structural and kinetic properties of the free and bound enzymes. Eur. J. Biochem. **255:** 235-245

Giovanelli J., Mudd S.H., Datko A.H. (1980) Sulfur amino acids of plants. In: The Biochemistry of plants, Amino acids and derivatives, Miflin B.J. ed., Academic Press, New York, USA, pp. 453-505

Hatzfeld Y., Lee S., Lee M., Leustek T., Saito K. (2000) Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidopsis thaliana*. Gene **248:** 51-58

Hawkesford M.J. (2003) Transporter gene families in plants: the sulphate transporter gene family: redundancy or specialization? Plant Physiol. **117:** 155-163

Heiss S., Schafer H.J., Haag-Kerwer A., Rausch T. (1999) Cloning sulfur assimilation genes of *Brassica juncea* L.: cadmium differentially affects the expression of a putative low-affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. Plant Mol. Biol. **39:** 847-857

Hopkins L., Parmar S., Blaszczyk A., Hesse H., Hoefgen R., Hawkesford M.J. (2005) *O*-acetylserine and the regulation of expression of genes encoding components for sulfate uptake and assimilation in potato. Plant Physiol. **138:** 433-440

Inoue K., Noji M., Saito K. (1999) Determination of the sites required for the allosteric inhibition of serine acetyltransferase by L-cysteine in plants. Eur. J. Biochem. **266:** 220-227

Kataoka T., Watanabe-Takahashi A., Hayashi N., Ohnishi M., Mimura T., Buchner P., Hawkesford M.J., Yamaya T., Takahash H. (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. Plant Cell **16:** 2693-2704

Kim H., Hirai M.Y., Hayashi H., Chino M., Naito S., Fujiwara T. (1999) Role of *O*-acetyl-L-serine in the coordinated regulation of the expression of a soybean seed storage-protein gene by sulfur and nitrogen nutrition. Planta **209:** 282-289

Koprivova A., Suter M., Op den Camp R., Brunold C., Kopriva S. (2000) Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiol. **122:** 737-746

Lappartient A., Touraine B. (1996) Demand-driven control of root ATP sulfurylase activity and sulfate uptake in intact canola. Plant Physiol. **111:** 147-57

Lappartient A.G., Vidmar J.J., Leustek T., Glass A.D.M., Touraine B. (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compounds. Plant J. **18:** 89-95

Lee S., Leustek T. (1999) The affect of cadmium on sulfate assimilation enzymes in *Brassica juncea*. Plant Sci. **141:** 201-207

Leustek T., Martin M.N., Bick J-A., Davies J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 141-165

Leustek T., Saito K. (1999) Sulfate transport and assimilation in plants. Plant Physiol. **120:** 637-643

Lunn J.E., Droux M., Martin J., Douce R. (1990) Localization of ATP sulfurylase and *O*-acetylserine(thiol)lyase in spinach leaves. Plant Physiol. **94:** 1345-1352

May M.J., Vernoux T., Leaver C., Van Montagu M., Inzé D. (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J. Exp. Bot. **49:** 649-667.

Nikiforova V.J., Kopka J., Tostikov V., Fiehn O., Hopiking L., Hawkesford M.J., Hesse H., Hoefgen R. (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol. **138:** 304-318

Noctor G., Arisi A.C.M., Jouanin L., Kunert K.J., Rennenberg H., Foyer C.H. (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J. Exp. Bot. **49:** 623-647

Noji M., Inoue K., Kimura N., Gouda A., Saito K. (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine

acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J. Biol. Chem. **273:** 32739-32745

Rauser W.E. (1995) Phytochelatins and related peptides. Structure, biosynthesis, and function. Plant Physiol. **109:** 1141-1149

Rotte C, Leustek T. (2000) Differential subcellular localization and expression of ATP sulfurylase and 5'-adenylsulfate reductase during ontogenesis in Arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. Plant Physiol. **124:** 715-724

Saito K. (1999) Biosynthesis of cysteine. In: Plant Amino Acids: Biochemistry and Biotechnology, Singh B. ed., Dekker, New York, USA, pp 267-291

Saito K. (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr. Opin. Plant Biol. **3:** 188-195

Saito K. (2004) Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol. **136:** 2443-2450

Saito K., Takahashi H., Noji M., Inoue K., Hatzfeld Y. (2000) Molecular regulation of sulfur assimilation and cysteine synthesis. Sulfur nutrition and sulfur assimilation in higher plants, Bern, Switzerland, pp 59-72

Schlenk F. (1965) The chemistry of biological sulfonium compounds. Fortschr. Chemie Org. Naturst. **23:** 61-112

Smith F.W., Hawkesford M.J., Ealing P.M., Clarkson D.T., Vanden Berg P.J., Belcher A.R., Warrilow A.G. (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J. **12:** 875-884

Takahashi H., Watanabe-Takahashi A., Smith F.W., Blake-Kalff M., Hawkesford M.J., Saito K. (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. Plant J. **23:** 171-182

Wawrzynska A., Lewandowska M., Hawkesford M.J., Sirko A. (2005) Using a suppression subtractive library-based approach to identify tobacco genes regulated in response to short-term sulphur deficit. J. Exp. Bot. **56 (416):** 1575-1590

Wirtz M., Berkowitz O., Droux M., Hell R. (2001) The cysteine synthase complex from plants: mitochondrial serine acetyltransferase from *Arabidopsis thaliana* carries a bifunctional domain for catalysis and protein-protein interaction. Eur. J. Biochem. **268:** 686-693

Yoshimoto N., Inoue E., Saito S., Yamaya T., Takahashi H. (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in *Arabidopsis*. Plant Physiol. **131:** 1511-1517

Yoshimoto N., Takahashi H., Smith F.W., Yamaya T., Saito K. (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. Plant J. **29:** 465-473

Youssefian S., Nakamura M., Orudgev 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

CHAPTER 3

MOLECULAR CLONING AND CHARACTERIZATION OF A CYTOSOLIC ISOFORM OF SERINE ACETYLTRANSFERASE (SAT)

SYNOPSIS

A full-length cDNA clone encoding a cytosolic isoform of serine acetyltransferase (SAT) (EC 2.3.1.30) was isolated by screening a soybean seedling cDNA library with a ^{32}P-labeled expressed sequence tag. Nucleotide sequence analysis of the isolated cDNA revealed a single open-reading frame of 858 base pairs encoding a 30-kDa polypeptide. The deduced amino acid sequence of soybean SAT revealed significant homology with other plant SATs. Analysis of soybean genomic DNA by Southern blotting indicated that SAT is encoded by a small gene family. The authenticity of the isolated SAT cDNA was confirmed by the expression of the cDNA in an *Escherichia coli* cysteine auxotrophic mutant resulting in the growth of the mutant cells in minimal medium without cysteine. Expression of soybean SAT in *E. coli* resulted in the production of a 34-kDa protein that was subsequently purified by nickel-affinity column chromatography. The purified protein exhibited SAT activity, indicating that the *E. coli*-expressed protein is a functionally active SAT. The recombinant soybean SAT was inhibited by L-cysteine, the

end product of cysteine biosynthetic pathway. Antibodies raised against the recombinant soybean SAT cross-reacted with a 34-kDa protein from *Arabidopsis* leaves, but failed to detect any proteins from soybean leaves and seeds. Reverse transcriptase polymerase chain reaction analysis indicated that SAT mRNA was expressed at low levels during soybean seed development. In comparison to *Arabidopsis* leaves, the SAT activity was several-fold lower in soybean leaves and seeds, suggesting that SAT is a low-abundance enzyme.

INTRODUCTION

Soybeans are renowned for their high protein and high oil composition. Although high protein soybean lines are available, varieties expressing adequate levels of methionine and or cysteine have not been developed either through traditional breeding or with transgenic methods. Expression of a high methionine 2S albumin from Brazil nut (*Bertholletia excelsa*) in soybeans raised the methionine content approximately 40% (Townsend and Thomas 1994), but that is still is not sufficient to obviate supplementation with synthetic methionine in diets for many animals (Imsande 2001). Brazil nut protein elicits allergenic responses in certain individuals and thus, the feasibility of its use was compromised (Nordlee et al. 1996). A 15-kDa-zein protein, which is rich in methionine, was successfully introduced into soybean under a seed specific promoter and this raised the content of methionine between 12 and 20% (Dinkins et al. 2001). This modest increase, however, is not sufficient to meet the demands of monogastric anmal nutrition.

Plant nutrition studies have shown seed storage protein composition to be influenced by nutrient availability. A high nitrogen to sulfur ratio tends to increase the β subunit of β-conglycinin, which is essentially devoid of cysteine and methionine while a low ratio somewhat enhances accumulation of glycinin (Gayler and Sykes 1985; Paek et al. 1997; Sexton et al. 1998a, 1998b). Exogenously applied methionine in *in vitro* and whole-plant studies has shown that increased availability will enhance the accumulation of this amino acid (Holowach et al. 1984; Grabau et al. 1986). These results suggest that the supply of methionine/cysteine to the developing seed is a limiting factor in accumulation of glycinin. Other studies indicate that sulfur is derived from the soil during seed filling and not re-mobilized from maternal tissue, suggesting assimilation or transport as limiting the synthesis of sulfur rich-proteins (Anderson and Fitzgerald 2001).

Sulfur is stored in ionic form in vacuoles and in amino acids within protein in leaf tissue. Sulfate reduction and synthesis of the amino acids occurs in chloroplasts. Transport to the filial tissue from maternal tissue involves both symplastic and apoplastic pathways because there are no known vascular connections between plant and seed (Anderson and Fitzgerald 2001). Harvest index experiments suggest that partitioning of sulfur to seed is occurring at a maximum rate. Increasing protein quality would involve either more sulfur uptake or greater efficiency of fixation on a whole-plant basis. It has been calculated that a 50% increase in sulfur-containing amino acids would require between a 65 and 80% increase in the rate of sulfur uptake (Sexton et al. 1998a). In a high-sulfur-availability environment, there was an accumulation of sulfate in the stems. This available pool of sulfur was not mobilized during seed filling, indicating that rates of

downstream reactions were not alone sufficient to reduce and assimilate sulfur into amino acids, or there was an insufficient sink available to accommodate the increased supply of these sulfur-containing amino acids (Sunarpi and Anderson 1997).

Biochemical mechanisms involved in sulfur assimilation include uptake of the sulfate ion from growth medium, transport, reduction, amino acid synthesis and assimilation of the amino acids into storage proteins (Leustek and Saito 1999; Leustek et al. 2000; Saito et al. 2000). Any of the preceding steps have the potential to limit production of sulfur-rich seed storage proteins. In an attempt to improve the nutritional quality of soybean seed proteins, I utilized molecular techniques to identify and manipulate the enzymes involved in sulfur assimilation. Here, I report the molecular cloning and characterization of serine acetyl transferase, a key enzyme in cysteine synthesis.

MATERIALS AND METHODS

Plant material and growth conditions. Soybean cv. 'Williams 82' was grown at the Bradford Research and Extension Center near Columbia, Missouri, on a Mexico silt loam soil (Udollic Ochraqualf). Leaf and seed samples were collected from nodes 10 and 11 every five days for seven weeks beginning at growth stage R5 (Fehr and Caviness 1979). Seeds were sorted according to size, frozen in liquid nitrogen and stored at -80 ^{o}C. *Arabidopsis thaliana* (ecotype Columbia) plants were grown on soil (Sunshine no. 4 soil mix; Sun Gro Horticulture, Bellevue, WA., USA) in an environmental chamber that was programmed to provide an ambient temperature of 18.5^{o}C and a 16-hour photoperiod.

Leaves were collected four weeks after plant emergence and enzyme analysis performed on the day of harvest.

SAT cDNA isolation and sequence analysis. An examination of the soybean expressed sequence tag (EST) database revealed that one of the clones (GenBank accession no. AI495784) contained nucleotide sequences showing homology to SAT. Based on the sequence of this soybean EST clone, two primers were designed (Forward: 5'-ACGACCAGGGATGGTTGTGGA-3'; Reverse: 5'-GGAGAGGAGCGTGGATTAA-3') and used to amplify a 132-bp DNA fragment from soybean genomic DNA by polymerase chain reaction (PCR). The amplified PCR fragment was purified on a 0.8% agarose gel and radiolabeled with [α-^{32}P] dCTP (Perkin-Elmer Life Sciences Inc., Boston, MA, USA) using a random labeling kit (Takara Mirus Bio, Inc., Madison, WI, USA). A soybean seedling cDNA library constructed in lambda ZAP II (obtained from Dr. Joe Polacco, University of Missouri, Columbia, MO, USA) was screened with the radiolabeled probe following standard protocol (Sambrook et al. 1989). After three consecutive screening steps, four positive lambda clones were identified by colony hybridization. Plasmids from these clones were recovered using the Rapid Excision Kit (Stratagene, La Jolla, CA, USA) and the clone (pSSAT1) containing the largest cDNA insert was chosen for further analysis. DNA sequence was determined by the DNA Core Facility of the University of Missouri using a *Taq* Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The deduced amino acid sequence of soybean seed SAT was subjected to BLAST analysis (BLASTX,

National Center for Biotechnology Information - NCBI). Multiple sequence alignments were performed using CLUSTLAW software and BOXSHADE (University of California, San Diego, CA, USA; http://workbench.sdsc.edu) and phylogenic tree was constructed with the use of the same database.

Isolation of soybean genomic DNA and Southern blotting. Genomic DNA from soybean leaf (cv. Williams 82) was isolated by the hexadecyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). Ten μg aliquots of genomic DNA were digested overnight at 37°C with either *BamH*I, *EcoR*I, or *Hind*III. After electrophoretic fractionation on 0.8% agarose gel, the DNA was partially hydrolyzed (15 min depurination in 0.25 N HCl, 30 min denaturation in 0.4 M NaOH) and transferred to Hybond N^{+} membrane (Amersham Biosciences, Piscataway, NJ, USA). Prehybridization of the membrane proceeded overnight in a buffer containing 10% (w/v) bovine serum albumin (BSA), 500 mM Na_2HPO_4, 10 mM EDTA, 7% (w/v) SDS, 100 μg/ml salmon sperm DNA, in a total volume of 20 ml at 65°C. With the addition of the [α-^{32}P] dCTP labeled SAT probe, hybridization was initiated in the same solution and allowed to proceed at 65°C for 24 h. Following hybridization, the membrane was washed twice with 2x SSC, 1% (w/v) SDS, once with 1x SSC, 1% (w/v) SDS and then twice with 0.1x SSC, 1% (w/v) SDS. Each wash was carried out for 10 min at 65°C. Hybridizing bands were detected by autoradiography, using a DuPont (Wilmington, DE, USA) Cronex Lightening Plus intensifying screen for signal enhancement.

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA from developing soybean seeds was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen) to remove contaminating DNA. The extract was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) in a total volume of 50 µl. Primers specific for soybean SAT (Forward: 5'-CCAACATATGATGCCGACGGGGTTACCGGC -3'; Reverse: 5'-GGTTGCGGCCGCTCAAATGATATAATCTGACC -3'), soybean storage protein glycinin (Forward: 5'- TTCGCCCCTGAATTCTTGAAAGAAGCG3'; Reverse: 5'-CTCAAAGTTATCGCTCTGGGATTT -3') and soybean 18S rRNA (Forward: 5'-GCTTAACACATGCAAGTCGAACGTTG -3', Reverse: 5'-ACCCCTACACACGAAATTCCACTC -3') was employed in the reaction mixture. Glycinin and 18S rRNA served as internal and loading controls, respectively. The thermal cycler program was 50°C for 30 min, 95°C for 15 min, 26 cycles at 94°C (1 min), 58 °C (1 min), and 72°C (1 min), followed by a final cycle of 10 min at 72°C. Polymerase chain reaction products were separated on a 0.8% (w/v) agarose gel and quantified using the GeneWizzard bio-imaging system (LabRepco, Horsham, PA, USA).

Expression of soybean SAT in *E. coli* and antibody production. Serine acetyltransferase cDNA functioned as the template for amplifying the coding region of the soybean SAT by PCR. The N- and C-terminal specific primers were 5'-CCAACATATGATGCCGACGGGGTTACCGGC -3' and 5'-

GGTTGCGGCCGCTCAAATGATATAATCTGACC -3', which included a *Nde*I and a *Not*I restriction site, respectively, to facilitate cloning. The PCR product was purified from an agarose gel, digested with *Nde*I and *Not*I (Takara Mirus Bio, Inc.) and ligated into the *Nde*I / *Not*I site of *E. coli* expression vector pET 28(a)+ (Calbiochem-Novabiochem, San Diego, CA, USA) using the ExTaq ligase kit (Takara Mirus Bio, Inc.). The resultant plasmid, pSSAT10, was introduced into ER2566 *E. coli* strain (New England Biolabs, Beverly, MA, USA) by heat shock at 42°C for 1 min. Cultures of the ER2566 E. coli strain carrying the pSSAT10 plasmids were used to inoculate 100 ml Luria Broth medium in the presence of 100 μg/ml kanamycin, and grown at 37°C. When the culture reached an optical density of 0.9 (O.D.$_{600nm}$), isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth was allowed to continue overnight at 37°C. Recombinant SAT protein was purified under native conditions according to Hoffmann and Roeder (1990) at 4°C. Cells from the overnight-induced culture were harvested by centrifugation (4050g, 20 min, 4°C), resuspended in 5 ml of extraction buffer (10 mM Tris.HCl [pH 7.9], 10% glycerol, 0.5 M NaCl, 0.1% nonidet-P40, 5 mM DTT) and incubated on ice for 30 min. The suspension was centrifuged (11,300g; 10 min; 4°C) and imidazole was added to a final concentration of 1mM to the supernatant. After the supernatant was passed through a Ni-NTA agarose column (Qiagen), it was washed with two column volumes of BC100 (20% glycerol, 20 mM Tris.HCl [pH 7.9], 100 mM KCl, 5 mM dithiothreitol (DTT) and 0.5 mM PMSF) containing 20 mM imidazole and eluted with 5 ml of BC100 containing 80 mM

imidazole. Utilizing the DC Standard Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA), protein concentrations were spectrophotometrically determined using bovine serum albumin as a standard. Antibodies to the recombinant soybean SAT were raised in rabbits as described previously (Krishnan and Okita 1986).

Functional expression of soybean SAT in a cysteine-auxotrophic *E. coli* mutant. The *JM39/5 Cys^- E. coli* mutant [*F^+, cysE51, recA56*] (*E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA) was transformed with pSSAT10 or the cloning vector pET-28a. For the genetic complementation of the cysteine requirement, the transformed *E. coli* cells were cultured on M9 agar plates (Sambrook et al. 1989) supplemented with 100 µg/ml kanamycin and 1 mM IPTG) at 37°C for 2 days in the presence or absence of cysteine.

Immunoblot analysis. Total protein extracts from *Arabidopsis* leaf, soybean leaf and soybean seed were fractionated by SDS-PAGE (Laemmli 1970) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA). The proteins were 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) as described by Burnett (1981). The membranes were washed with TBS (80 mM Tris-HCl, 200 mM

NaCl, pH 7.5) for 5 min and incubated with TBS containing 5% (w/v) nonfat dried milk for 1 hr with gentle agitation at room temperature. Following this incubation, the membranes were incubated overnight with polyclonal antibodies raised against *Arabidopsis* SAT (obtained from Dr. Thomas Leustek, Rutgers University, NJ, USA) or antibodies raised against soybean recombinant SAT. Antibodies were diluted 1:2,000 in TBS containing 5% (w/v) nonfat dried milk. Following washes in TBST (TBS containing 1% (v/v) Tween 20) (3 x 10 min), the blots were incubated with HRP-conjugated goat anti-rabbit IgE (1:5,000 [v/v] dilution) in TBST containing 5% (w/v) nonfat dried milk for 1 hr with gentle agitation at room temperature. Final washes were carried out with TBST (3 × 10 min) and TBS (1 × 5 min). Immunoreactive polypeptides were visualized using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce Biotechnology, Rockford, IL, USA).

SAT assays. Recombinant soybean SAT activity was assayed according to Noji et al (1998). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, 1 mM L-serine, and a known amount of the purified recombinant soybean SAT in a final volume of 1 ml. The reaction was initiated by the addition of L-serine and the decrease in acetyl CoA was monitored spectrophotometrically. Serine acetyltransferase specific activity was calculated using the molar extinction coefficient for acetyl-CoA of ε=4500 at 232 nm. The kinetic parameters were determined by using the appropriate rate equations and the GraFit 5.0 software from Erithacus Software (Sigma-Aldrich Corp., St. Louis, MO, USA)

Serine acetyltransferase activity from the crude plant extracts was determined by the method of Kredich and Tompiks (1966). This enzyme assay is based on the disulfide exchange between CoA liberated by acetyl-CoA during the reaction and dithiobis 2-nitrobenzoic acid (DTNB). Production of thionitrobenzoic acid was followed spectrophotometrically at 412 nm. Freshly harvested soybean and *Arabidopsis* tissue samples (200 mg) were ground 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 DTT]. The samples were transferred to microcentrifuge tubes and spun (11,600*g*; 10 min; 4 ^oC). The clear supernatant obtained after centrifugation was used to measure SAT activity. The enzyme reaction mixture contained 0.1 mM acetyl-CoA, 50 mM Tris pH 7.6, 1 mM DTNB, 1 mM EDTA and 1 mM L-serine in 1 ml final volume. Subsequent to reaction initiation by addition of enzyme at room temperature, the initial velocity was estimated by monitoring the increase in absorbance at 412 nm. Rates were calculated using an extinction coefficient for thionitrobenzoic acid of ε=13,600 at 412 nm. Protein concentrations were determined spectrophotometrically using the DC Standard Protein Assay Kit (Bio-Rad Laboratories).

RESULTS

Isolation of a cDNA encoding SAT from soybean. Screening a soybean seedling cDNA library with a radiolabeled 132-bp DNA fragment of an EST clone (GenBank no. AI495784) resulted in the isolation of four possible SAT encoding sequences. Restriction

enzyme digestion of the DNA isolated from these four cDNA clones revealed the same restriction pattern, and one clone (pSSAT1) was chosen for further analysis. Nucleotide sequencing demonstrated that the cDNA insert was 1044 bp and contained a single, open-reading frame (ORF) of 858 bp, which could encode a 326 amino acid protein. The deduced molecular weight of this protein was 30.3 kDa with an isoelectric point of 7.82. The soybean SAT nucleotide sequence has been deposited in the GenBank and appears under accession number AF452452. Computer-assisted BLAST analysis showed that soybean SAT had significant homology with SATs from different sources (Fig. 1A). The boxed amino acid sequences in Fig. 1A were conserved between species and presumed involved in binding acetyl-CoA. The underlined region at the C-terminus of the protein is implicated in the allosteric inhibition by cysteine. Amino acids, Gly-277 and His-282, considered the residues principally involved in the interaction with cysteine (Inoue et al. 1999), are indicated by asterisks. Soybean SAT shows amino acid sequence similarities to the following enzymes: watermelon SAT, 82%; spinach SAT, 78%; onion SAT, 76%; *Arabidopsis* SAT-c, 76%; *Arabidopsis* SAT-p, 52%, and *Arabidopsis* SAT-m, 54%. The amino acid sequence between watermelon SAT and soybean SAT was 81% identical. A phylogenetic tree revealed that soybean SAT is closely related to the cytosolic SAT from several other plant species (Fig. 1B). This prediction is consistent with our observation that the soybean SAT exhibits neither chloroplastic nor mitochondrial amino terminal transit peptide.

Southern blot analysis was performed using soybean genomic DNA to determine the SAT copy number in the soybean genome. DNA was digested using *Bam*HI, *Eco*RI, or *Hind*III, transferred to a nylon membrane, and probed with ^{32}P-labeled SAT cDNA. Under stringent hybridization conditions (see materials and methods), I was able to detect one hybridizing band in both the *Bam*HI and *Hind*III digested genomic DNA (Fig. 2). In the case of *Eco*RI digestion, detection of two hybridizing bands is consistent with the fact that an internal *Eco*RI site exists within the coding region of SAT. The foregoing observations suggest that a single gene encodes the isolated cDNA SAT. However, a few weakly hybridizing bands were detected when the blot was hybridized under less stringent conditions. These bands possibly represent other SAT-related sequences in the genome of soybean.

Functional complementation of *JM39/5* cysteine *E. coli* auxotroph by soybean SAT. The authenticity of the isolated SAT was further verified by expressing the soybean cDNA in a cysteine auxotrophic mutant. *Escherichia coli JM39/5* lacks the gene for SAT and therefore is unable to grow in the absence of cysteine. To determine if soybean SAT can complement an *E. coli* cysteine auxotroph, the mutant *E. coli* was transformed with either the empty protein expression vector pET28a or the plasmid pSSAT10, which contains the coding region of the soybean SAT in the pET28a vector. Only *E. coli* JM39/5 cells transformed with pSSAT10 were able to grow in the absence of cysteine, indicating that the isolated cDNA codes for a functional SAT (Fig. 3).

Temporal expression of SAT mRNA during seed development. To monitor the SAT gene expression during seed development, I performed semi-quantitative RT-PCR analysis using total RNA isolated from seeds harvested at different developmental stages. The SAT mRNA was found to accumulate at low levels when compared to that of the glycinin, a major seed storage protein of soybean (Fig. 4). There was a small increase in the SAT expression levels during the seed developmental stages 3 and 4 followed by a decline in the expression at stage 5 (Fig. 4). The glycinin gene was expressed abundantly during the early stages of seed development with maximum expression detected at stage 2 followed by a gradual decline during the later stages of seed development (Fig. 4). In contrast to either the SAT or glycinin mRNA accumulation patterns, 18S RNA was expressed at similar levels at all stages of seed development (Fig. 4).

Expression of soybean SAT in *E. coli*. To characterize the soybean SAT, an initial attempt was made to purify SAT from developing soybean seeds by column chromatography. The quantity of purified protein recovered was not adequate for detailed biochemical analysis. Therefore, the soybean SAT was expressed in *E. coli* by cloning the coding region of the gene and placing it under the control of the T7 promoter. This process resulted in the introduction of six histidine residues to the N-terminus region. The expression of the 6X His-tagged recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside to the culture media. Total protein from the induced cultures, when resolved by SDS-PAGE, revealed the presence of an abundant 34-kDa protein (Fig. 5). The soybean SAT recombinant protein was purified by Ni-affinity column chromatography (Fig. 5). Since the recombinant protein has N-terminal His-tag,

the molecular mass of this protein is slightly larger than the one deduced from the DNA sequence analysis. Recombinant soybean SAT was also purified under native conditions to perform enzyme analyses.

Immunoblot analysis of SAT accumulation. Antibodies were generated against the purified recombinant soybean SAT and used to detect the protein in different organs. Repeated attempts to detect the SAT in both the leaves and seeds by immunoblot analyses were unsuccessful. This indicated that either the quality of soybean SAT antibody was poor or the concentration of SAT in soybean leaves and seeds was too low to be detected by Western blot analyses. Since the soybean SAT antibody reacted strongly against nanogram quantities of the purified recombinant SAT in Western blots, inability to detect SAT in soybeans could be related to their low abundance. To exclude the possibility that soybean SAT antibody was of poor quality, antibodies raised against *Arabidopsis* SAT (Dr. Thomas Leustek, Rutgers University, NJ, USA) were used to perform Western blot analyses. *Arabidopsis* SAT antibodies reacted strongly against a 34-kDa protein from *Arabidopsis* leaves, but did not recognize any proteins in soybean leaves or developing soybean seeds (Fig. 6). The soybean SAT antibodies also strongly reacted against the 34-kDa protein from *Arabidopsis* leaves (Fig. 6). These observations suggest that soybean leaves and seeds contain a very low concentration of endogenous SAT. Further investigation was conducted by measuring the activity of SAT in crude plant extracts. SAT activity was readily detected in *Arabidopsis* leaves, while significantly lower activities were detected from soybean leaves and seeds (Fig. 7).

Catalytic properties of the recombinant soybean SAT. Serine acetyltransferase catalyzes the formation of OAS from L-serine and acetyl-CoA. This enzyme is regulated by feedback inhibition by cysteine (Saito et al. 2000). Enzyme activity and cysteine inhibition of recombinant soybean SAT were measured. According to the Michaelis-Menten model, the calculated K_m value for L-serine was 2.27 (mM) with a V_{max} of 11.34. Acetyl-CoA showed a K_m of 0.31 (mM) with a V_{max} of 14.32. These values are similar to those obtained for the cytosolic form of SAT from *A. thaliana* (Noji et al. 1998) and spinach (Noji et al. 2001). It has been demonstrated that only the cytosolic form of SAT in *Arabidopsis* was inhibited by cysteine, while the chloroplastic and mitochondrial forms are insensitive to this inhibition (Inoue et al. 1999, Noji et al. 1998). The activity of soybean SAT diminished as the concentration of cysteine increased (Fig. 8), suggesting that the isolated cDNA codes for a cytosolic isoform of SAT. Soybean SAT showed competitive inhibition in response to acetyl-CoA, and noncompetitive inhibition in presence of L-serine, with K_i values of 7.6 μM and 11.2 μM respectively.

DISCUSSION

In this study, I isolated a full-length cDNA clone encoding a soybean SAT. Two lines of evidence indicate that the cloned cDNA codes for a functional SAT. Expression of the soybean SAT cDNA in the *E. coli* cysteine auxotroph rescued bacterial growth in cysteine deficient media and produced a 34-kDa protein, which showed SAT activity. Serine acetyltransferase has been purified from several plant species including *A. thaliana*

(Roberts and Wray 1996; Howarth et al. 1997; Murillo et al. 1997), *Spinacea oleracea* (Noji et al. 2001), *Citrullus vulgaris* (Saito et al. 1995) and *Allium tuberosum* (Urano et al. 2000). The molecular weight of SAT isolated from this diverse group of plants ranges from 30 to 42-kDa. Based on N-terminal signal sequences and subcellular localization studies, SAT can be classified as a cytosolic (SAT-c), plastidic (SAT-p), or mitochondrial (SAT-m) isoform. Direct evidence for the specific subcellular location of these isoforms was provided by transient expression studies utilizing a chimera of N-terminal sequences and green fluorescent protein (GFP) (Saito et al. 2000). Subcellular location of SAT-p-GFP in *Arabidopsis* leaves varied in a time-dependent manner. In 4-week-old leaves the SAT-p-GFP was localized in the chloroplasts, while in 6-week-old leaves, about 90% of the SAT-p-GFP was found in the cytosol suggesting that the subcellular location of SAT changes with plant developmental stages (Saito et al. 2000).

The subcellular compartmentation of SAT plays an important role in regulation of the enzyme. In *A. thaliana*, the cytosolic isoform of SAT is subject to cysteine inhibition, whereas the plastidic and the mitochondrial isoforms are insensitive to this feedback mechanism. (Inoue et al. 1999). The soybean recombinant SAT also shows feedback inhibition by cysteine. The amino acid sequence of soybean SAT reveals neither chloroplastic nor mitochondrial signal sequences (Fig. 1A). Deletion studies have established that the amino acid residues Gly-277 and His-282, lying within a short carboxyl-terminal domain, are primarily responsible for cysteine feedback inhibition (Inoue et al. 1999). These residues are conserved among the cytosolic isoforms of SAT

(Fig. 1A). The preceding data indicate that the isolated soybean SAT encodes a cytosolic isoform.

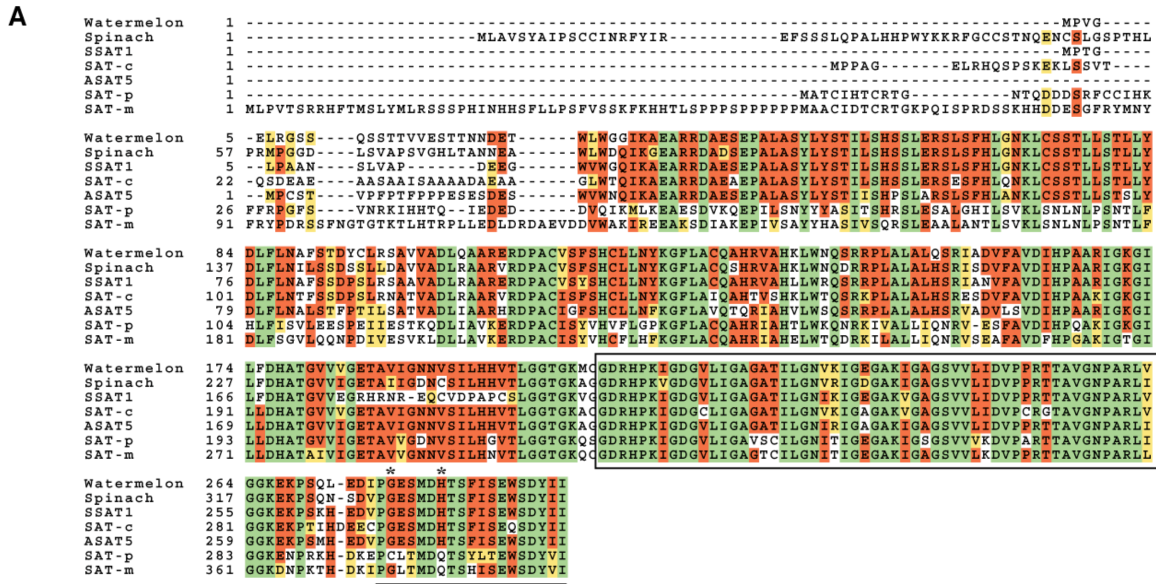
Serine acetyltransferase contains a catalytic domain, a protein-protein interaction domain, and an allosteric domain (Saito et al. 2000). The interaction domain facilitates complex formation with *O*-acetylserine (thiol)-lyase (OAS-TL). Wirtz et al. (2001), using computational modeling and site-directed mutagenesis, demonstrated that the protein-protein interaction domain lies at the carboxyl terminus of the enzyme. The amino acid sequences in this region are highly conserved in all of the SATs (Fig. 1A). The SAT-OAS-TL complex formation is regulated by OAS. Bimolecular interaction analysis using *A. thaliana* showed that accumulation of OAS promoted dissociation of the complex, resulting in an active OAS-TL, while low levels OAS had the opposite effect (Bogdanova and Hell 1997, Berkoqitz et al. 2001). Kinetic studies revealed that when in a complex with OAS-TL, the K_m of SAT decreases. *O*-acetylserine (thiol)-lyase is inactivated while complexed with the SAT (Droux et al. 1998). Based on conservation of amino acid sequences, it is apparent that soybean SAT contains the same distinct domains seen in the enzymes from other species. I have shown that soybean recombinant SAT is inhibited by L-cysteine. Since SAT forms a complex with OAS-TL, and the kinetic properties of the individual enzymes and enzyme-complex are distinct, it is important to determine if the enzyme-complex is also inhibited by cysteine. I have recently cloned OAS-TL from soybean and expressed this protein in *E. coli* (Chronis and Krishnan 2003). The availability of recombinant soybean OAS-TL and SAT will facilitate studies designed to

elucidate the mechanism and regulation of cysteine synthesis.

Southern blot analysis has shown that a multigene family encodes the isoforms of SAT in *A. thaliana* (Roberts and Wray 1996). Sequencing of the Arab*idopsis* genome has revealed the presence of five genes, which putatively encode SAT. Southern blot analysis indicates that soybean SAT is probably encoded by a small gene family. Isolation and characterization of the remaining members of this gene family and their products will enhance our understanding of sulfur assimilation in soybeans. Serine acetyltransferase activity in soybean leaves is significantly lower when compared to enzyme activity from *Arabidopsis* leaves (Fig. 7). I was unable to detect accumulation of SAT in soybean leaves and seeds by western blot analysis suggesting that the protein was present at extremely low levels. In contrast, western blot analysis indicated OAS-TL was present in substantial amounts in soybean seed (Chronis and Krishnan 2003). It has been reported that OAS-TL is present at a 300-fold molar excess over SAT (Leustek 2002, Ruffet et al. 1994). These findings suggest disparate amounts of SAT and OAS-TL may play an important role in the regulation of soybean sulfur assimilation. Recently, it has been proposed that calcium-induced phosphorylation is involved the control of cysteine synthesis (Yoo and Harmon 1997, Harmon et al. 2003). *In vitro* experiments have shown SAT to be a substrate of a calcium-dependent protein kinase. Phosphorylated SAT was insensitive to cysteine feedback inhibition (Yoo and Harmon 1997, Harmon et al. 2003). Thus, it appears that the SAT is also regulated by post-translational modification.

One of my goals is to increase the sulfur amino acid content of soybean seed proteins by genetic manipulation. Since my studies show that SAT is a low-abundance enzyme in soybean, over-expression of this enzyme may facilitate increased cysteine synthesis. It has been shown that expression of watermelon SAT in transgenic *Arabidopsis* resulted in over accumulation of OAS and cysteine (Noji and Saito 2002). However, this accumulation occurred only in plants transformed with the SAT that had a point mutation of Gly-277 to Cys, thus preventing feedback inhibition by cysteine. Similarly, transgenic potato plants expressing the *cysE* gene of *E. coli*, which encodes for SAT, exhibited significantly higher levels of cysteine compared to wild type (Hesse et al. 2000). These results suggest that a similar approach could be employed to enhance cysteine synthesis in soybeans.

A



B

Arabidopsis thaliana SAT-m

Arabidopsis thaliana SAT-p

Allium tuberosum ASAT5

Arabidopsis thaliana SAT-c

Citrulus vulgaris (watermelon)

Spinacia oleracea (spinach)

Glycine max SSAT1

Salmonella typhimurium

Escherichia coli

0.1

Figure 1: (A) Multiple alignment of the amino acid sequence of SAT from different plants. An underlying line at the C-terminus indicates the principal allosteric site for cysteine inhibition. Residues primarily responsible for this inhibition, Gly-277 and His-282, are indicated by asterisks. Boxed area corresponds to the binding region of acetyl-CoA. Dashes indicate gaps to facilitate best alignment. Green shading indicates conserved residues; red shading indicates residues showing more than 60% identity; yellow shading indicates those residues showing more than 60% similarity; **(B)** Phylogenetic tree of SAT. The phylogenetic tree was constructed using the University of California Data Base. *Escherichia coli* SAT (Accession No. NC_000913), *Salmonella typhimurium* SAT (Accession No. X59594), *Arabidopsis* SAT-m [identical to Sat-1 (Roberts and Wray 1996)], *Arabidopsis* SAT-p [identical to SAT1 (Murillo et al. 1997)], *Arabidopsis* SAT-c [identical to SAT52 (Howarth et al. 1997)], watermelon SAT2 (Saito et al. 1995), spinach SAT (Accession No. D88529), *Allium tuberosum* ASAT5 (Urano et al. 2000; Accession No. AB040502), *Glycine max* SAT1 (this study; Accession No. AF452452).

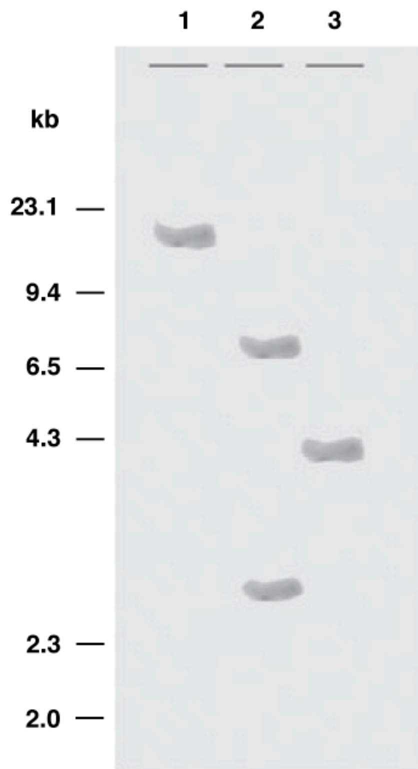
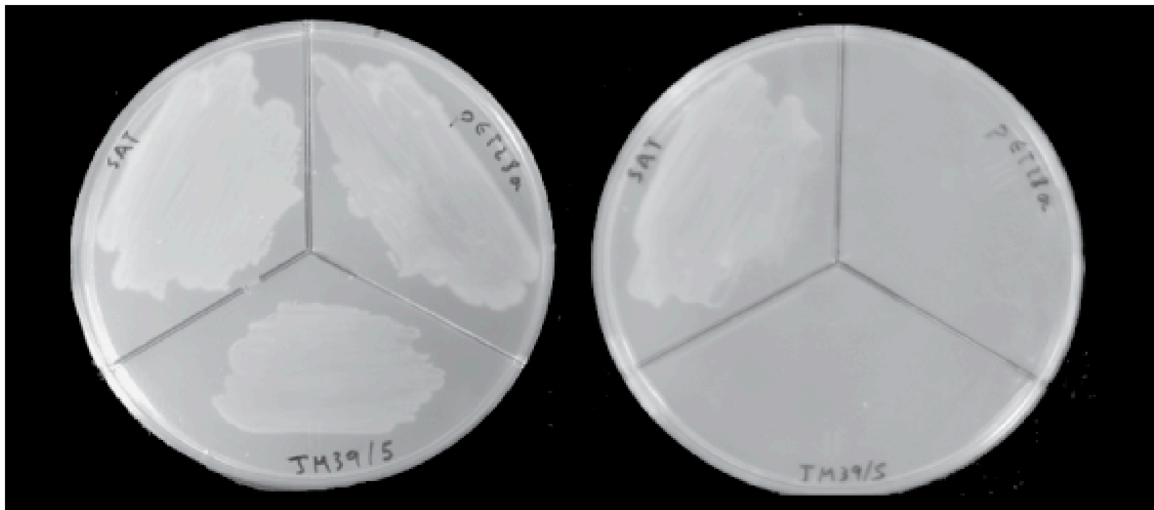


Figure 2: Southern blot analysis of soybean genomic DNA. Ten µg of soybean genomic DNA was restricted with *BamH*I (lane 1), *EcoR*I (lane 2), and *Hind*III (lane 3) and resolved on a 0.8% agarose gel. The gel was blotted to Hybond N^+ membrane followed by hybridization with ^{32}P-labeled soybean seed SAT cDNA. The positions of the Lambda *Hind*III molecular weight markers are shown.



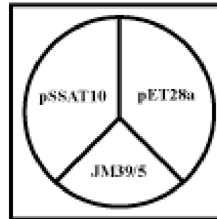


Figure 3: Functional complementation of Cys^- *Escherichia coli* JM39/5 by transformation with the expression vector carrying soybean SAT cDNA clone. The *E.coli* cysteine-auxotroph was transformed with pSSAT10 and streaked on M9 minimal agar plates in the presence (left plate) or absence of 0.5 mM cysteine (right plate). The empty vector pET28a was used as a negative control.

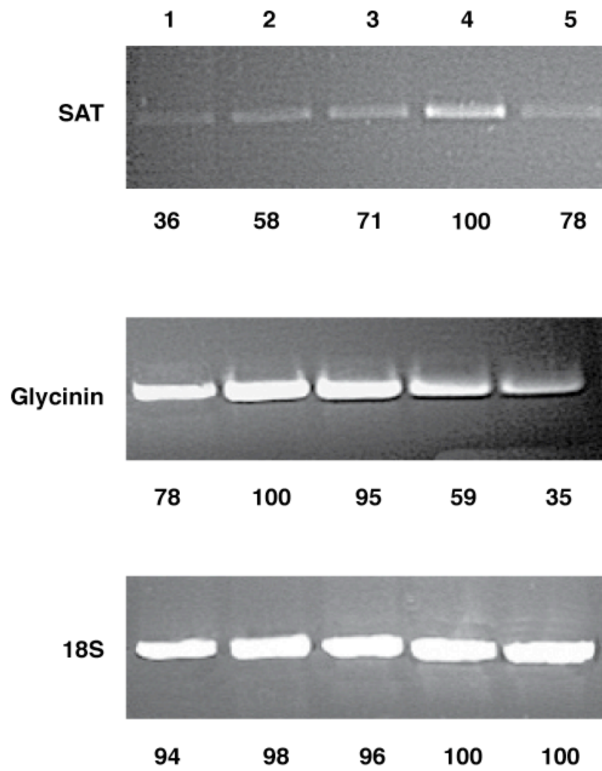


Figure 4: Semi-quantitative reverse transcriptase (RT)-PCR detection of SAT and glycinin mRNA in developing soybean seeds. Total RNA, isolated from soybean seeds harvested at 7-day intervals beginning at the R5 growth stage (lanes 1 to 5), was used as a template for RT-PCR. The 18S ribosomal mRNA was used as quantitative control. Band intensity was quantified using the GeneWizzard bio-imaging system and the values are indicated. Bands with the strongest signal were treated as 100%.

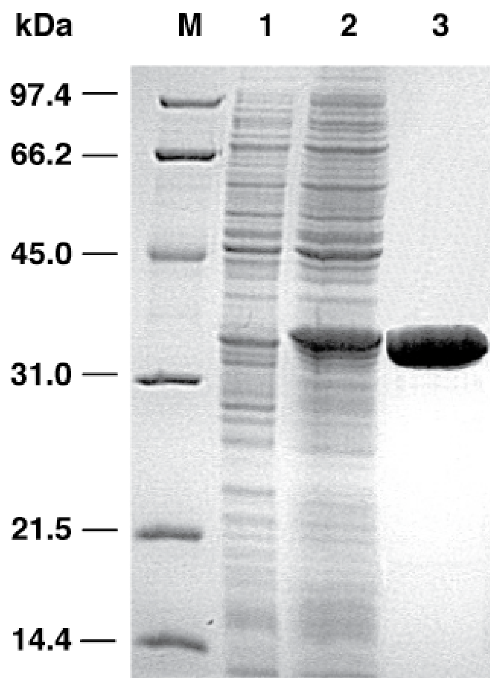


Figure 5: Expression and purification of the recombinant SAT. Cells of the *Escherichia coli* ER2566 strain were transformed with the plasmid pSSAT10. Protein expression was achieved by the addition of 1 mM IPTG to the transformed *E.coli* culture. The recombinant protein was purified on a Ni-NTA agarose chromatography column. Proteins were resolved on a 12.5% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. Lanes: M, Protein molecular weight markers (phosphorylase b, 97 400; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400); 1. total protein from uninduced cultures; 2. total protein from IPTG-induced cultures; 3. purified recombinant soybean SAT.

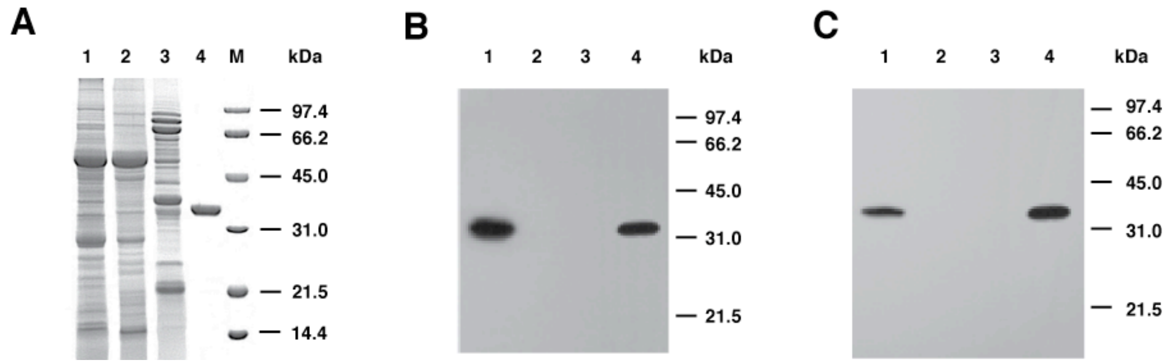


Figure 6: Western blot detection of SAT from *Arabidopsis thaliana* and soybean. Total proteins from *Arabidopsis* leaves (lane 1), soybean leaves (lane 2), and developing soybean seeds (lane 3) along with protein molecular weight markers (lane M) were resolved on a 12.5% SDS-PAGE gel. Fractionated proteins were visualized by staining with Coomassie Brilliant Blue (Panel **A**) Proteins from two identical gels were transferred to nitrocellulose and probed with the either *Arabidopsis* SAT antibodies (Panel **B**) or soybean recombinant SAT antibodies (Panel **C**). Note that both antibodies react with a 34-kDa protein from *Arabidopsis* leaves, but not with soybean proteins.

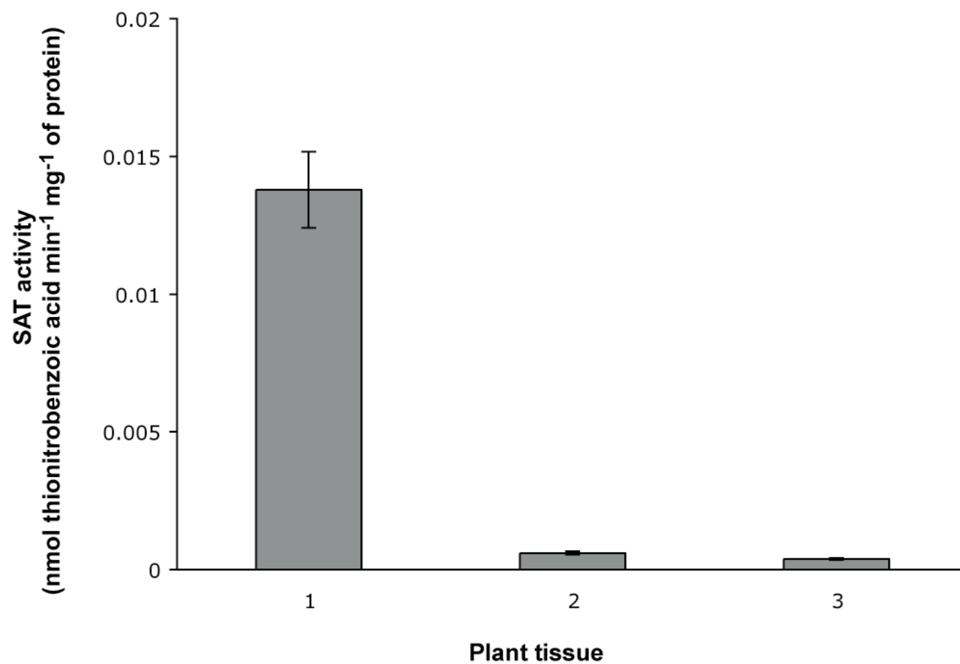


Figure 7: Serine acetyltransferase activity in soybean and *Arabidopsis thaliana*. Crude protein extracts from *Arabidopsis* leaves (sample 1), soybean leaves (sample 2), and developing soybean seeds (sample 3) were used to determine SAT activity. The production of thionitrobenzoic acid was followed spectrophotometrically at 412 nm. Bars represent the standard error of the mean.

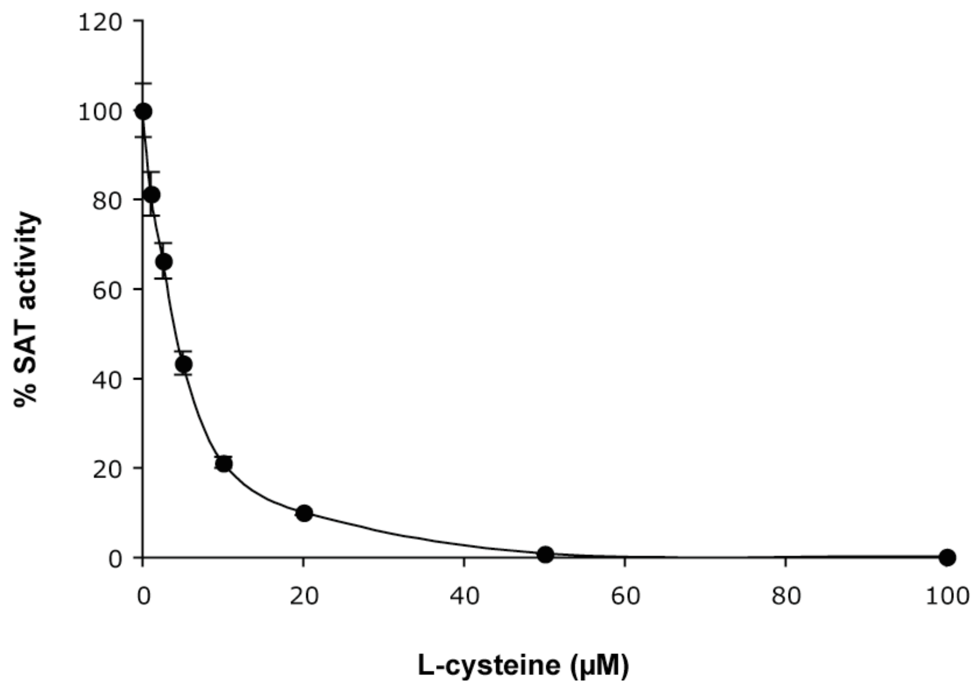


Figure 8: Soybean recombinant SAT undergoes feedback inhibition by cysteine. Bars represent the standard error of the mean.

Table I: Kinetic parameters for SAT

Variable substrate	K_m	V_{max}	K_i inhibition by L-cysteine (μM)
L-serine	2.27 ± 0.39	11.34 ± 0.57	11.2 ± 1.57 (noncompetitive)
Acetyl CoA	0.31 ± 0.03	14.32 ± 0.51	7.6 ± 0.83 (competitive)

LITERATURE CITED

Anderson J., Fitzgerald M.A. (2001) Physiological and metabolic origin of sulphur for the synthesis of seed storage proteins. Plant Physiol. **158:** 447-456

Beach L., Orman B., Townsend J., Thomas L. (1995) Reduction of endogenous seed protein levels in plants. U.S. Patent 9503784

Berkowitz O., Wirtz M., Wolf A., Kuhlmann J., Hell R. (2002) Use of biomolecular interaction analysis to eludiacte the regulatory mechanism of the cysteine synthase from *Arabidopsis thaliana*. J. Biol. Chem. **277:** 30629-30634

Bogdanova N., Hell R. (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. **11:** 251-262

Chronis D., Krishnan H.B. (2003) Sulfur assimilation in soybean: Molecular cloning and characterization of *O*-Acetylserine (thiol) lyase (cysteine synthase). Crop Sci. **43:** 1819-1827

Dinkins R.D., Reddy M.S.R., Meurer C.A., Yan B., Trick H., Thibaud-Nissen F., Finer J.A., Rarrott W.A., Collins G.B. (2001) Increased sulfur amino acids in soybean plants overexpressing the maize 15 kDa zein protein. In Vitro Cell Dev. Bio. Plant **37:** 742-747

Droux M., Ruffet M.L., Douce R., Job D. (1998) Interactions between serine acetyltransferase and *O*-acetylserine (thiol) lyase in higher plants: structural and kinetic properties of the free and bound enzymes. Eur. J. Biochem. **255:** 235-245

Fehr W.R., Caviness C.E. (1977) Stages of soybean development. Iowa Agricultural Experiment Station Special Report 80. Iowa Cooperative External Series**,** Iowa State University, Ames, Iowa

Gayler K.R., Sykes G.E. (1985) Effects of nutritional stress on the storage proteins of soybeans. Plant Physiol. **78:** 582-585

Grabau L.J., Blevins D.G., Minor H.C. (1986) Stem infusions enhanced methionine content of soybean storage protein. Plant Physiol. **82:** 1013-1018

Harmon A.C., Liu F., Yoo B.C., Strachan C., Stevens S., Sakiyama C., Denslow N., Hageman A., Harms A., Sussman M.R., Harper J.F. (2003) CDPK substrates in soybean and *Arabdiopsis*. Cur. Top Plant Biochem. Physiol. Mol. Biol. **21:** 64-65

Hesse H., Harms K., Ballmoos P.V., Brunold C., Willmitzer L., Hofgen R. (2000) Serine acetyltransferase: a bottleneck for cysteine and glutathione synthesis? Sulfur nutrition and sulfur assimilation in higher plants, Bern, Switzerland, pp 321-323

Holowach L.P., Thompson J.F., Madison J.T. (1984) Storage protein composition of soybean cotyledons grown *in vitro* in media of various sulfate concentrations in the presence and absence of exogenous L-methionine. Plant Physiol. **74:** 584-589

Hoffmann A., Roeder R.G. (1990) Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acid Res. **19:** 6337-6338

Howarth J.R., Roberts M.A., Wray J.L. (1997) Cysteine biosynthesis in higher plants: A new member of the *Arabidopsis thaliana* serine acetyltransferase small gene family obtained by functional complementation of an *Escherichia coli* cysteine auxotroph. Biochem. Biophys. Acta **1350:** 123-127

Imsande J. (2001) Selection of soybean mutants with increased concentrations of seed methionine and cysteine. Crop Sci. **41:** 510-515

Inoue K., Noji M., Saito K. (1999) Determination of the sites required for the allosteric inhibition of serine acetyltransferase by L-cysteine in plants. Eur. J. Biochem. **266:** 220-227

Kredich N.M., Tompikns G.M. (1966) The enzymatic synthesis of L-cysteine in *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. **21:** 4955-4965

Krishnan H.B., Okita T.W. (1986) Structural relationship among the rice glutelin polypeptides. Plant Physiol. 81:748-753

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685

Leustek T. (2002) Sulfate metabolism. In: Somerville CR, Meyerowitz EM (eds) The Arabidopsis book, American Society of Plant Biologists, Rockville, MD, pp 1-17

Leustek T., Saito K. (1999) Sulfate transport and assimilation in plants. Plant Physiol. **120:** 637-643

Leustek T., Martin M.N., Bick J.A., Davies J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 141-165

Murillo M., Foglia R., Diller A., Lee S., Leustek T. (1997) Serine acetyltransferase from *Arabidopsis thaliana* can functionally complement the cysteine requirement of a cysE mutant strain of *Escherichia coli*. Cell Mol. Biol. Res. **41:** 425-433

Neuenschwander U., Suter M., Brunold C. (1991) Regulation of sulfate assimilation by light and *O*-acetylserine in *Lemma minor* L. Plant Physiol. **97:** 253-258

Noji M., Inoue K., Kimura N., Gouda A., Saito K. (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J. Biol. Chem. **273:** 32739-32745

Noji M., Takagi Y., Kimura N., Inoue K., Saito M., Horikoshi M., Saito F., Takahashi H., Saito K. (2001) Serine acetyltransferase involved in cysteine biosynthesis from spinach: molecular cloning, characterization and expression analysis of cDNA encoding a plastidic isoform. Plant Cell Physiol. **42:** 627-634

Noji M., Saito K. (2002) Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolism engineering. Amino Acids **22:** 231-243

Nordlee J.A., Taylor S.L., Townsend J.A., Thomas L.A., Bush R.K. (1996) Identification of Brazil-nut allergen in transgenic soybeans. N. England J. Med. **334:** 688-692

Paek N.C., Imsande J., Shoemaker R.C., Shibles R. (1997) Nutritional control of soybean seed storage protein. Crop Sci. **37:** 498-503

Roberts M.A., Wray L. (1996) Cloning and characterization of an *Arabidopsis thaliana* cDNA clone encoding an organellar isoform of serine acetyltransferase. Plant Mol. Biol. **30:** 1041-1049

Ruffet M.L., Droux M., Douce R. (1994) Purification and kinetic properties of serine acetyltransfrease free of *O*-acetylserine (thiol) lyase from spinach chloroplasts. Plant Physiol. **104:** 597-604

Sexton P.J., Naeve S.L., Paek N.C., Shibles R. (1998a) Soybean sulfur and nitrogen balance under varying levels of available sulfur. Crop Sci. **38:** 975-982

Sexton P.J., Naeve S.L., Paek N.C., Shibles R. (1998b) Sulfur availability, cotyledon nitrogen:sulfur ratio, and relative abundance of seed storage proteins of soybean. Crop Sci. **38:** 983-986

Saghai-Maroof M.A., Soliman K.M., Jogensen R., Allard R.W. (1984) Ribosomal DNA spacer length in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA **81:** 8014-8018

Saito K., Yokoyama H., Noji M., Murakoshi I. (1995) Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. J. Biol. Chem. **270:** 16321-16326

Saito K., Takahashi H., Noji M., Inoue K., Hatzfeld Y. (2000) Molecular regulation of sulfur assimilation and cysteine synthesis. Sulfur nutrition and sulfur assimilation in higher plants, Bern, Switzerland, pp 59-72

Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular cloning: a laboratory manual, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 9.31-9.57

Sunarpi, Anderson J.W. (1997) Allocation of S in generative growth of soybean. Plant Physiol. **114:** 687-693

Townsend J.A., Thomas L.A. (1994) Factors which influence the *Agrobacterium*-mediated transformation of soybean. J. Cell Biochem. Suppl. **18A:** Abstract X1-014

Urano Y., Manabe T., Noji M., Saito K. (2000) Molecular cloning and functional characterization of cDNAs encoding cysteine synthase and serine acetyltransferase that may be responsible for high cellular cysteine content in *Allium tuberosum*. Gene **257:** 269-277

Wirtz M., Berkowitz O., Droux M., Hell R. (2001) The cysteine synthase complex from plants: mitochondrial serine acetyltransferase from *Arabidopsis thaliana* carries a bifunctional domain for catalysis and protein-protein interaction. Eur. J. Biochem. **268:** 686-693

Yoo B.C., Harmon A.C. (1997) Regulation of recombinant soybean serine acetyltransferase by CDPK. Plant Physiol. (suppl.) **114:** 267

CHAPTER 4

MOLECULAR CLONING AND CHARACTERIZATION OF *O*-ACETYLSERINE (THIOL) LYASE (OAS-TL) FROM SOYBEAN

SYNOPSIS

Soybean (*Glycine max* [L.] Merr.) is a good protein source for both humans and livestock. However, soybean seed proteins are deficient in the sulfur-containing amino acids, cysteine and methionine. This deficiency has stimulated efforts to improve the amino acid composition of soybean seed proteins. Our overall goal is to improve the sulfur amino acid content of soybean seed proteins by genetic manipulation. The objective of this study was to isolate and characterize *O*-acetylserine (thiol) lyase (OAS-TL), a key enzyme that catalyzes the last step in the production of cysteine. A full-length cDNA clone encoding a cytosolic isoform of OAS-TL was isolated by screening a soybean seed cDNA library with a ^{32}P-labeled expressed sequence tag (EST). Nucleotide sequence analysis of the cDNA revealed a single open-reading frame of 978 base pairs (bp) encoding a 34 kDa protein. The authenticity of the isolated cDNA was confirmed by the functional complementation of an *Escherichia coli* cysteine auxotrophic mutant. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that OAS-TL mRNA was abundant at early stages of seed development. Western blot analysis using

antibodies generated against the recombinant soybean OAS-TL demonstrated that the abundance of this protein gradually declined during later stages of seed development. The OAS-TL activity peaked in young developing seeds and declined steadily during the time period when the bulk of seed storage protein accumulation occurred. Thus, elevating the specific activity of OAS-TL during later stages of seed development could lead to an increase in cysteine synthesis in soybean seeds.

INTRODUCTION

Soybeans typically contain about 40% protein and 20% oil. Because of their high protein content, they are widely used as a protein source both for humans and animals. In the United States, soybeans are mainly used as animal feed. Soybean proteins are under-represented in sulfur-containing amino acids (methionine and cysteine). Unlike plants, animals have a dietary requirement for sulfur amino acids. As a consequence, animal diets are often supplemented with synthetic sulfur amino acids to achieve optimal growth. The use of supplemental amino acids costs the poultry and swine industry approximately 100 million dollars annually. Therefore, improving the sulfur-amino acid content of soybean proteins will greatly benefit the livestock industry and improve the overall profitability of soybean farmers.

Soybean seed proteins are classified into 7S and 11S proteins and these together represent about 70% of the total seed protein (Nielsen, 1996; Krishnan, 2000). The 11S proteins are named glycinin, while the 7S proteins are known as β-conglycinin. The 11S

glycinin contains more of the sulfur-containing amino acids than the 7S β-conglycinin. The β-conglycinin is made up of three subunits, namely α', α, and β. The β-subunit lacks methionine (Coates et al., 1985) and is considered to be of very poor nutritional quality. Elimination or reduction of the β-subunit of β-conglycinin, therefore, may lead to improvement of the nutritional quality of soybean seed proteins. In fact, soybean seed storage protein mutants have been obtained that have low levels of 7S globulins and such mutants have 15% more methionine than other cultivars (Kitamura and Kaizuma, 1981). Imsande in 2001 reported the isolation of soybean mutant lines with a methionine over-producing phenotype. It was reported such mutants had approximately 20% greater methionine and cysteine concentration in their seeds. The nutritional quality of soybean seed storage proteins has also been enhanced by expressing heterologous proteins that are rich in methionine. Introduction of methionine-rich 2S albumin from Brazil nut (*Bertholletia excelsa*) drastically improved the methionine content of soybean (Nordlee et al., 1996). However, the introduced Brazil nut protein was an allergen, and consequently, the transgenic soybeans were not commercialized. Interestingly, transgenic soybeans expressing Brazil nut 2S albumin showed lower accumulation of Kunitz trypsin inhibitor (KTI) and chymotrypsin inhibitor (CI). The protease inhibitors are rich in sulfur-containing amino acids and the heterologous expression of 2S Brazil nut albumin has presumably shunted the sulfur amino acids from the protease inhibitors (Streit et al., 2002). This study indicates that there is a limitation in the sulfur amino acid pool in developing soybean seeds.

I am interested in improving the protein quality of soybean seed storage proteins. One of the approaches that I have undertaken is genetic manipulation of enzymes that are involved in the sulfur biosynthetic pathway. In spite of the importance of sulfur amino acids in determining the protein quality of soybean, very little is known about sulfur metabolism in soybean. Therefore my objective was to identify and characterize enzymes involved in the cysteine biosynthetic pathway in soybeans. The cysteine biosynthetic pathway is responsible for the fixation of inorganic sulfur into L-cysteine (Leustek et al., 2000). Cysteine is the first reduced sulfur-containing compound and serves as the sulfur donor for methionine. The cysteine biosynthetic pathway involves several enzymatic steps (Leustek and Saito, 1999). *O*-acetylserine (thiol) lyase [OAS-TL; EC 4.2.99.8] catalyzes the formation of cysteine from *O*-acetylserine (OAS) and hydrogen sulfide with the release of acetic acid.

Cysteine is the principal starting material for the synthesis of sulfur-containing amino acids, coenzymes, and sulfolipids (Leustek et al., 2000). In spite of the importance of this enzyme in sulfur metabolism, no molecular characterization of this enzyme in soybean has been reported. Here, I report the molecular cloning and characterization of OAS-TL from soybean, an enzyme that catalyzes the final step of the cysteine biosynthetic pathway.

MATERIALS AND METHODS

Plant material. Soybeans cv. "Williams 82" were field-grown at the Bradford Research and Extension Center near Columbia, Missouri, on a Mexico silt loam soil (Udollic Ochraqualf). Samples were collected from nodes 10 and 11 over time starting from the R5 stage (Fehr and Caviness, 1979). Seeds from four replications were collected for each time point. The pod walls were split open and seeds were frozen immediately in liquid nitrogen and stored at –80°C until use.

cDNA Isolation and sequence analysis. Total RNA from developing soybean seeds was isolated by the LiCl precipitation procedure (Lizzardi, 1983). Poly $(A)^{+}$ RNA was isolated by oligo (dT)-cellulose chromatography. A cDNA library of soybean seed poly $(A)^{+}$ RNA was constructed in lambda ZAP II following the manufacturer's protocol (Stratagene, La Jolla, CA). To isolate the OAS-TL cDNA clone, we synthesized primers (Forward: 5' GGGTTACAAGCTCATAATTAC 3'; Reverse: 5' GCACCTGTCATTGTACCACGAG 3') based on published sequence of a EST clone (GenBank accession no. AW509442). These primers were utilized to amplify a 300 bp fragment from soybean genomic DNA. The polymerase chain reaction (PCR) fragment was purified from agarose gel and radiolabeled with [α-^{32}P] dCTP (PerkinElmer Life Sciences Inc., Boston, MA) using a random labeling kit (Takara Bio Inc., Shiga, Japan). The soybean seed cDNA library was screened with the radiolabeled probe according to standard protocol (Sambrook et al., 1989). Three positive lambda clones were identified

of the Rapid Excision Kit (Stratagene, La Jolla, CA). One of the positive clones (pSCS1) was chosen for further analysis. The cDNA insert was sequenced with a *Taq* Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at the DNA core facility of the University of Missouri using appropriate primers synthesized by Integrated DNA Technologies (Coralville, IA). The nucleotide sequence and the derived amino acid sequence of soybean seed OAS-TL were subjected to BLAST analysis (BLASTX, National Center for Biotechnology Information - NCBI). Restriction enzyme analysis was performed using NEBCutter 1.0 (New England Biolabs, Beverly, MA). Multiple sequence alignments were performed using CLUSTLAW software (European Bioinformatics Insitute, Germany; http://www.ebi.ac.uk/clustalw) and BOXSHADE (Pasteur Institute, France; http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). A phylogenic tree was constructed with the help of the University of California Data Base (University of California, San Diego, CA; http://workbench.sdsc.edu).

Isolation of soybean genomic DNA and southern blotting. Genomic DNA from soybean leaf (cv. Williams 82) was isolated by the standard hexadecyltrimethylammonium bromide (CTAB) method. Ten μg of genomic DNA were digested with *BamH*I, *EcoR*I and *Hind*III overnight at 37°C. The digested samples were fractionated on a 0.8% (w/v) agarose gel. After electrophoresis, the DNA was partially hydrolyzed (15 min depurination in 0.25 N HCl; 30 min denaturation in 0.4 M NaOH) before transfer to Hybond N^+ membrane (Amersham Biosciences, Piscataway, NJ). After transfer, the filter was prehybridized overnight in hybridization buffer at 65°C (1% [w/v]

BSA, 500 mM Na_2HPO_4, 10 mM EDTA, 7% [w/v] SDS, 100 μg/ml salmon sperm DNA, total volume of 20 ml). Hybridization was performed at 65°C for 24 h with [α-^{32}P] dCTP labeled OAS-TL probe. Following hybridization, the membrane was washed two times with 2x SSC, 1% (w/v) SDS , once with 1x SSC, 1% (w/v) SDS and finally two more times with 0.1x SSC, 1% (w/v) SDS. Each wash was carried out for 10 min at 65°C. Hybridizing bands were detected by autoradiography, using a DuPont (Wilmington, DE) Cronex Lightening Plus intensifying screen for signal enhancement.

Reverse transcriptase polymerase chain reaction (RT-PCR). analysis. Total RNA from developing soybean seeds was extracted using Trizol Reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Total RNA (0.1 μg) was used for the reverse transcriptase (RT) reaction. Prior to RT-PCR, the RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove any contaminating DNA. The RT reaction was carried out in a volume of 50 μl using the OneStep RT-PCR kit (Qiagen, Valencia, CA). Primers were designed from the 5' end and 3' end of the open-reading frame (ORF) of OAS-TL. The forward and reverse primers were 5'-CCAACATATGATGGCTGTTGAAAGGTCCGG-3' and 5'-GGTTGCGGCCGCTCAGGGCTCAAAAGTCATGC-3', respectively. The thermal cycler program was 50°C for 30 min, 95°C for 15 min, 30 cycles at 94°C (1 min), 58°C (1 min), and 72°C (1 min), followed by a final 10 min at 72°C. 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'-

G C T T A A C A C A T G C A A G T C G A A C G T T G - 3 ’, Reverse: 5 ’- ACCCCTACACACGAAATTCCACTC-3’. The PCR products were separated on a 7 g kg^{-1} agarose gel and photographed using an Eagle Eye II still video system (Stratagene, La Jolla, CA).

Western blot analysis. Seed proteins isolated from soybeans at different developmental stages were separated by SDS-PAGE (Laemmli, 1970) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA). The proteins were resolved on a slab gel (10 × 8 × 0.75 cm) consisting of a 13.5% separation gel and a 4% stacking gel. Electrophoresis was carried out at 20 mA constant current per gel at room temperature. After the completion of the electrophoresis, the gels were equilibrated with electrode buffer (25 nM^1 Tris, 192 mM glycine, and 20% [v/v] methanol, pH 8.3) for 15 min. Proteins from the gels were electroblotted onto pure nitrocellulose membrane (Midwest-Scientific, Valley Park, MO) essentially as described by Burnett (1981). The membranes were washed with TBS (80 mM Tris-HCl, 200 mM NaCl, pH 7.5) for 5 min and incubated with TBS containing 5% (w/v) nonfat dried milk for 1 hr at room temperature. Following this, the membrane was incubated overnight with polyclonal antibodies raised against soybean recombinant OAS-TL (Chronis and Krishnan, unpublished) that was diluted 1:5,000 in TBS containing 5% (w/v) nonfat dried milk. Following three washes in TBS containing 0.8 g L^{-1} of Tween 20 (TBST) for 10 min each, the blot was incubated with HRP-conjugated goat anti-rabbit IgE (1:5,000 [v/v] dilution) in TBST containing 5% (w/v) nonfat dried milk for 1 hr with gentle agitation at

room temperature. Final washes were carried out with TBST (3 × 10 min) and TBS (1 × 5 min). Immunoreactive polypeptides were visualized using horseradish peroxidase color development procedure recommended by the manufacturer (Bio-Rad Laboratories, Richmond, CA).

Complementation of *NK3 Cys⁻ E. coli* auxotroph. We amplified the coding region of soybean OAS-TL with gene-specific primers (Forward 5'-CCAACATATGATGGCTGTTGAAAGGTCCGG-3' and Reverse 5'-GGTTGCGGCCGCTCAGGGCTCAAAAGTCATGC-3') to which *Not*I and *Nde*I restriction sites were introduced at the 5'and 3' ends, respectively. The PCR product was cloned into the *Nde*I and *Not*I sites of the expression vector pET 28(a)+ (Calbiochem-Novabiochem, San Diego, CA) resulting in pSCS10. The *NK3 Cys⁻ E. coli* mutant [Δ*trpE5 leu-6 thi hsdR hsdM⁺ cysK cysM*], (obtained from Dr. Kazuki Saito, Chiba University, Chiba, Japan) was transformed with pSCS10 and the cloning vector pET-28a served as a negative control. For the genetic complementation of the cysteine requirement, the transformed *E. coli* cells were plated on M9 agar plates (Sambrook et al., 1989) supplemented with 100 mg L^{-1} kanamycin, 1 mmol L^{-1} IPTG and 0.2 g kg^{-1} leucine and tryptophan (Saito et al., 1992).

Determination of OAS-TL activity. The OAS-TL activity was determined according to the protocol of Warrilow and Hawkesford (1998). Soybean seed samples (200 mg) were ground in a chilled mortar and pestle with 2 ml of ice-cold extraction buffer [100 mM Tris-HCl pH 8.0, 100 mM[1] KCl, 20 mM $MgCl_2$, 1% Tween 80 and 10 mM

dithiothreitol (DTT)]. The samples were transferred to microcentrifuge tubes and centrifuged at 4ºC for 10 min at 12 000 g. The clear supernatant was saved and used immediately for measuring the OAS-TL activity. Protein concentrations from seed extracts were determined spectrophotometrically with the help of DC Standard Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). The enzyme reaction mixture contained 5 mM OAS, 3 mM sodium sulphide, 10 mM DTT and 0.1 M sodium phosphate pH 8 in total volume of 0.2 ml. The reaction was initiated by the addition of OAS and the mixture was incubated at 26ºC for 10 min. After the incubation period, 0.15 ml aliquots were removed and mixed with 0.35 ml of acidic ninhydrin reagent (1.3% ninhydrin in 1:4 HCl: HOAc) and heated at 100ºC for 10 min to allow color development. After cooling on ice, 0.7 ml of ethanol were added and absorbance measured at 550 nm. One unit of enzyme activity is defined as the conversion of 1 nmol of OAS into cysteine min^{-1} under the stated assay conditions. Assays were performed three times and each time was represented by two replications.

RESULTS

Isolation of a cDNA encoding OAS-TL from a soybean seed cDNA library. To isolate the OAS-TL cDNA clone from soybean seed cDNA library, I synthesized primers corresponding to 5' and 3' of an EST clone (AW509442) encoding OAS-TL. These primers were utilized to amplify a 300 bp fragment from soybean genomic DNA. This PCR product was labeled with ^{32}P and used as a hybridization probe to screen a soybean seed cDNA library constructed in lambda ZAP II vector resulting in the isolation of three

putative clones. Subsequent restriction enzyme digestion of the DNA isolated from the three positive cDNA clones showed the same restriction pattern for all of them, and one of these clones (PCS1) was chosen for further studies. The physical map of this cDNA clone is shown in Fig. 1A. To characterize the putative OAS-TL cDNA clone, the nucleotide sequence was determined at the DNA core facility of the University of Missouri. The nucleotide sequence revealed that the cDNA was 1267 bp long (Fig. 1A). Analysis of the DNA sequence using the ORF finder program identified a 978-bp-long ORF. The predicated ORF encodes a protein of 326 amino acids with a molecular mass of 34.2 kDa (Fig. 1B). The theoretical isoelectric point of the protein was estimated to be 5.83. *O*-acetylserine (thiol) lyase is a pyridoxal phosphate-dependent enzyme, and a lysine residue at the N-terminal region of this protein is involved in binding this cofactor (Saito et al. 1993). This lysine residue and the sequence around it are also conserved in soybean OAS-TL (Fig. 1B). The BLASTX program and pairwise amino acid comparison of the soybean seed OAS-TL showed significant homology to OAS-TL from plants and bacteria. Soybean OAS-TL had 81% identity with *Oryza sativa*, 80.5% identity with *Arabidopsis* and 53.3% identity with *E. coli* OAS-TL (Fig. 2). A phylogenetic tree revealed that the OAS-TL isoforms could be divided into three major groups (chloroplastic, mitochondrial and cytosolic) based on their cellular location. Soybean OAS-TL was closely related to the cytosolic isoforms of OAS-TL from several other plant species (Fig. 2B). This prediction is consistent with my observation that the soybean OAS-TL lacks an amino terminal chloroplastic or mitochondrial transit peptide.

To determine the gene copy number of OAS-TL in soybean genome, I performed Southern blot analysis using genomic DNA that was digested with different restriction enzymes. The restricted DNA was transferred to a nylon membrane and probed with ^{32}P-labeled OAS-TL cDNA. Under stringent hybridization conditions, I was able to detect more than one hybridizing band (Fig. 3) in the different restriction digestions. This observation suggests that the OAS-TL is probably encoded by a multigene family in soybean.

Functional complementation of *NK3* cysteine *E. coli* auxotroph by soybean OAS-TL. To verify if the isolated cDNA clone codes for a functional OAS-TL, I expressed the soybean cDNA in *E.coli NK3*, a cysteine auxotroph. This mutant lacks the gene for OAS-TL and therefore cannot grow in medium without supplemental cysteine. I cloned the coding region of the soybean OAS-TL in a protein expression vector (pET28a) resulting in a plasmid pSCS10. *Escherchia coli* NK3, transformed with pSCS10, was able to grow on M9 minimal medium without cysteine (Fig. 4). The cysteine auxotroph and the mutant carrying the cloning vector, however, were unable to support the growth in the absence of cysteine (Fig. 4). These results confirm that the cDNA isolated from soybean seed cDNA library codes for a functional OAS-TL.

Temporal expression of OAS-TL mRNA during seed development. For comparison of the OAS-TL gene transcription levels during seed development, we performed RT-PCR analysis using total RNA isolated from seeds at different developmental stages. Using primers designed to amplify the entire coding region of the

OAS-TL, I was able to obtain 1.0 kb RT-PCR product (Fig. 5). The RT-PCR product, which was abundant during the early stages of seed development, declined perceptibly at the late stages of seed development (Fig. 5). To exclude the possibility that the decline in the OAS-TL mRNA at later stages of seed development was due to differences in the amount of total RNA used as template in RT-PCR reactions, I performed control reactions by amplifying a 700 bp 18S ribosomal RNA. As expected, the abundance of the 18S ribosomal RNA RT-PCR products remained constant throughout the seed development (Fig. 5). The results from the RT-PCR analysis indicate that mRNA encoding the OAS-TL is abundant during the early stages and declines during the later stages of seed development.

Accumulation of OAS-TL polypeptide during seed development. Proteins extracted from developing soybean seeds when resolved by SDS-PAGE revealed the presence of prominent storage proteins (Fig. 6A). The 72 kDa and the 70 kDa and proteins are the α' and α subunits of β-conglycinin. The 52 kDa β-subunit of β-conglycinin, which accumulates at late stages of seed development, was present only at very low concentration. The 37 kDa and the 21 kDa abundant proteins represent the acidic and basic subunits of glycinin (Fig. 6A). To monitor the accumulation of the OAS-TL during seed development, Western blot analysis was performed using polyclonal antibodies raised against the purified soybean OAS-TL (Chronis and Krishnan, unpublished). The OAS-TL antibodies recognized a single 34 kDa protein from the total seed protein extract (Fig. 6B). The OAS-TL was detected throughout the seed

development, but was present at relatively higher concentration during the early stages of seed development (Fig. 6B). This protein accumulation followed a similar trend as the RNA accumulation pattern.

OAS-TL activity declines during seed development. The activity of OAS-TL was measured at different stages of seed development. The OAS-TL activity, which was measured spectrophotometrically, was readily detected in soybean seed extracts. The specific activity of the enzyme was highest during the earliest stage of seed development and declined eight fold during the last stage of seed development examined in this study (Fig. 8). The results from RT-PCR, Western blot analysis, and the enzyme activity assays all revealed similar temporal accumulation patterns.

DISCUSSION

Although the role of OAS-TL in cysteine biosynthesis has been extensively studied in several plants, to my knowledge this is the first report to identify a full-length cDNA clone of OAS-TL in soybean (GenBank accession no. AF452451). The amino acid sequence of the soybean OAS-TL cDNA shows significant homology to those of other plant species and bacteria. Soybean OAS-TL contains the conserved PXXSVKDR motif that is characteristic of cysteine synthase. The lysine residue in this conserved motif has been shown to bind the co-factor pyridoxal 5'-phosphate. The OAS-TL has been purified from several plant species including *Arabidopsis thaliana* (Hesse and Altmann, 1995), spinach (Saito et al., 1992; Warrilow and Hawkesford, 1998), the green algae

Chlamydomonas reinhardtii (Ravina et al., 1999), rice (Nakamura et al., 1999), *Allium tuberosum* (Ikegami et al., 1993; Urano et al., 2000), *Citrullus vulgaris* (Ikegami et al., 1988a), and *Brassica juncea* (Ikegami et al., 1988b). The enzyme consists of two identical monomers and a tightly bound co-factor pyridoxal 5'-phosphate (Rolland et al., 1996). Two to four isoforms of the enzyme have been isolated in higher plants by chromatographic separations and cDNA isolations (Ikegami et al., 1993; Kuske et al., 1996; Saito et al., 1992, 1993, 1994a, b; Warrilow and Hawkesford, 1998; Nakamura et al., 1999; Jost et al., 2000). The different isoforms of OAS-TL have been located in cytosol, plastids, and mitochondria. In *A. thaliana*, four genomic clones (*oasA*1, *oasA*2, *oasB*, and *oasC*) that encode OAS-TL have been identified and characterized. The *oasA*1, *oasB*, and *oasC* encode isoforms found in cytosol, the plastids, and the mitochondria, respectively. Based on the amino acid sequence homology, our soybean OAS-TL appears to be related to the cytosolic isoforms.

The OAS-TL plays an important role in linking sulfur and nitrogen assimilatory pathways and controlling the flux between these two pathways (Leustek and Saito, 1999; Leustek et al., 2000). Consequently, cysteine synthesis depends on the availability of sulfur, OAS, and the activity of OAS-TL. The accumulation of soybean seed storage proteins is regulated by sulfur and nitrogen availability. Under excess nitrogen supply, the accumulation of the β-subunit of β-conglycinin is enhanced, while that of glycinin is inhibited (Gayler and Sykes, 1985; Paek et al., 1997). Kim et al. (1999) have shown that the promoter of the β-subunit of β-conglycinin is up-regulated by sulfur deficiency and

down-regulated by nitrogen deficiency. Further, they have shown that OAS accumulates in soybean cotyledons that were cultured under sulfur deficiency. This study clearly establishes the pivotal role of OAS in regulating the accumulation of the soybean seed storage proteins. Since OAS-TL uses OAS as a substrate it should be interesting to examine if nitrogen and sulfur deficiency also influence its activity in soybean.

The activity of ATP sulfurylase, an enzyme that catalyzes the adenylation of sulfate, has been investigated in developing soybean seeds (Sexton and Shibles, 1999). It was reported that the ATP sulfurylase activity was highest in seeds harvested 15 days after the R5 stage (about 1600 nmol ATP g fresh wt^{-1} min^{-1}) and reached low levels (about 250 nmol ATP g fresh wt^{-1} min^{-1}) at the R7 stage. I have observed similar changes in the OAS-TL specific activity. The RT-PCR results indicated that OAS-TL mRNA was barely present in mature seeds and this led to the observed decline in OAS-TL activity during the later stages of seed development. It remains to be seen if a similar decline in ATP sulfurylase mRNA also occurs during seed maturation. The decline in the activity of two enzymes involved in the biosynthesis of cysteine may explain the low content of sulfur-rich amino acids in soybean seed proteins. Because the bulk of seed storage proteins are synthesized during the mid-stage of seed development, it would be desirable to have sufficient supply of cysteine during this period. However, the decline in the activity of OAS-TL and ATP sulfurylase during this period indicates that the supply of sulfur-amino acids may be limiting. The limitation on cysteine can be overcome by manipulating the expression levels of enzymes involved in cysteine biosynthetic pathway.

The cysteine biosynthetic pathway is tightly regulated at several levels (Leustek and Saito, 1999; Leustek et al., 20000; Noji and Saito, 2002). The end product of sulfur assimilation, cysteine, is an allosteric inhibitor of the cytosolic form of serine acetyltransferase (SAT; EC 2.3.1.30). Serine acetyltransferase catalyses the formation of OAS from acetyl-CoA and serine. The OAS-TL activity is also regulated by its interaction with SAT (Bogdanova and Hell, 1997; Droux et al., 1998). OAS-TL and SAT form an enzyme complex through specific protein-protein interactions. In the bound form, SAT shows positive cooperativity, meaning higher affinity for its substrates. On the other hand, OAS-TL is completely inactivated in the bound form. OAS triggers the dissociation of the complex, and sulfide counteracts the action of OAS (Bogdanova and Hell, 1997; Droux et al., 1998). A lag in sulfide production will result in accumulation of OAS, which will slow its own synthesis by promoting the dissociation of the complex. Alternatively, the accumulation of sulfide will act as positive regulator in the association of SAT and OAS-TL thereby speeding the formation of OAS. Because the level of OAS influences the composition of soybean seed storage proteins (Kim et al., 1999), it will be important to clone and characterize soybean SAT, the enzyme responsible for the generation of OAS.

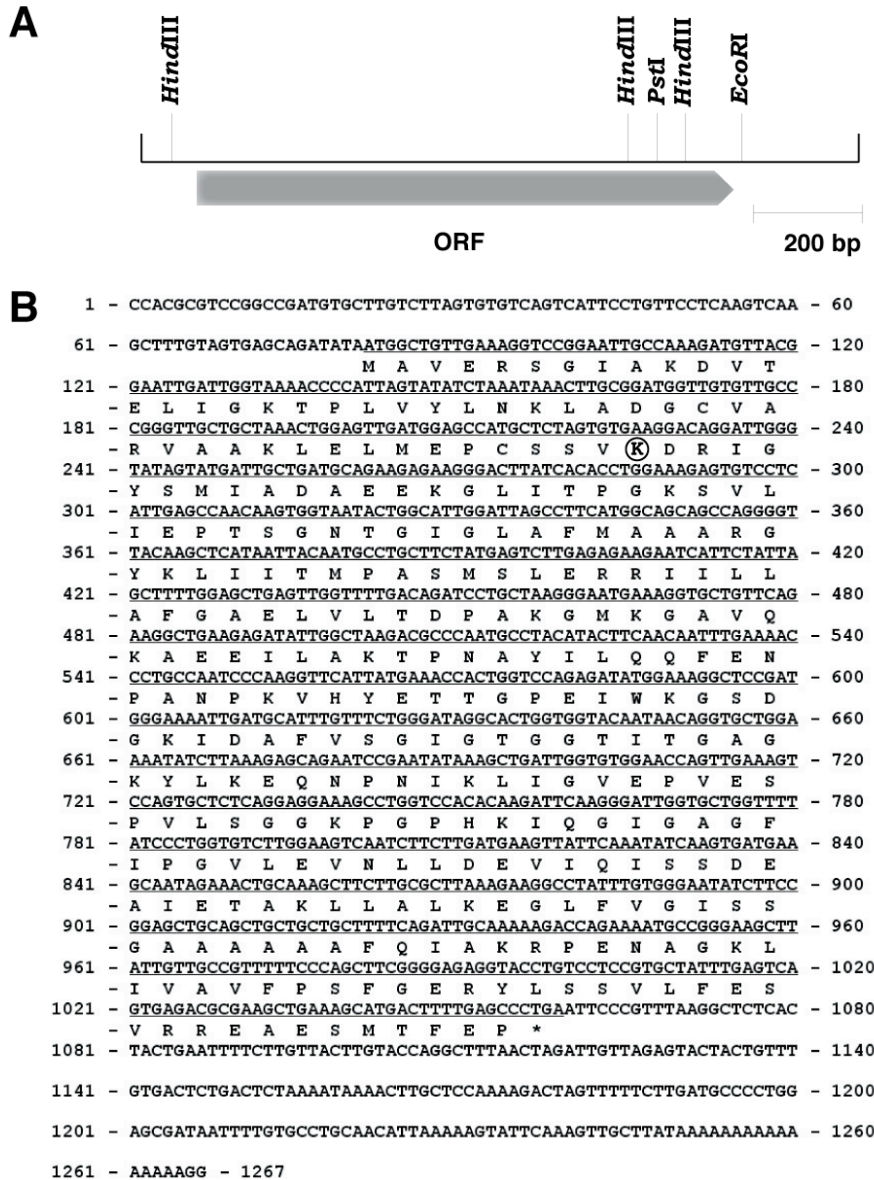
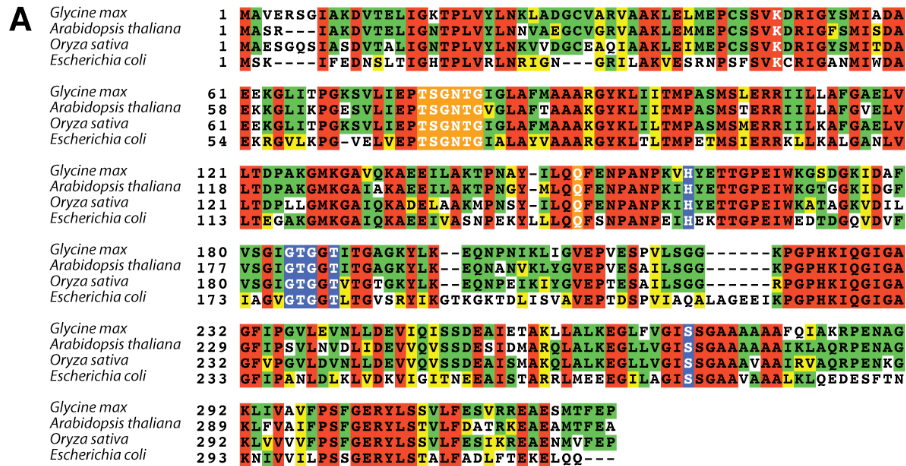


Figure 1: (A) Partial restriction map of soybean cDNA encoding the OAS-TL. The long arrow indicates the location of the open-reading frame (ORF); **(B)** Nucleotide sequence and deduced amino acid sequence of OAS-TL cDNA from soybean seed. The sequenced region covers 1267 nucleotides. The ORF for OAS-TL begins at position 82 and ends at position 1059 encoding a 34.2 kDa protein. The lysine residue that binds to pyridoxal 5'-phosphate is circled. The nucleotide sequence of OAS-TL cDNA from soybean appears in the GenBank database as accession No. AF452451.



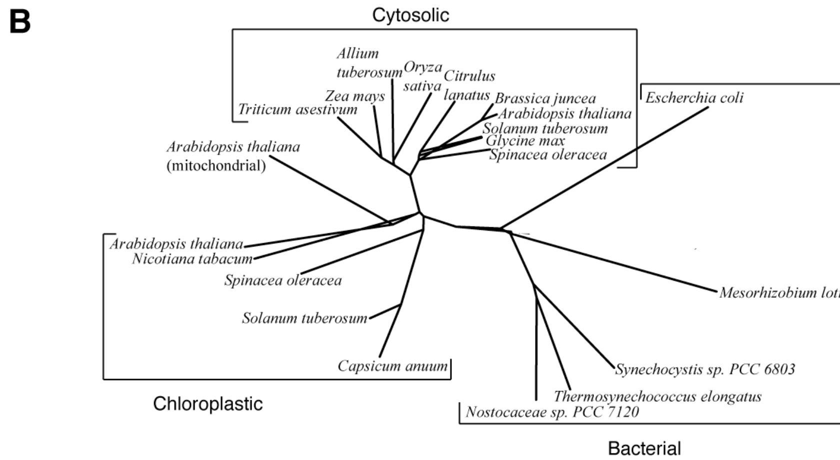


Figure 2: (A) Multiple alignment of the deduced amino acid sequence of OAS-TL. The sequences from rice (Accession No. Q9XEA8), *Arabidopsis* (Accession No. NP_193224), and *E. coli* (Accession No. P11096] are aligned with that of the soybean sequences from this study (Accession No. AF452451). Dashes indicate gaps to facilitate best alignment. Red shading indicates conserved residues; green shading indicates residues showing more than 60% identity; yellow shading indicates those residues showing more than 60% similarity. The active site lysine (white on red), the substrate loop (white on orange), and residues that interact with pyridoxal phosphate (white on blue) are indicated; **(B)** Phylogenic tree of OAS-Tl. The phylogenic tree was constructed using the University of California Data Base. Cytosolic isoforms: *Glycine max* (Accession No. AF452451), *Arabidopsis thaliana* (Accession No. NP_193224), *Solanum tuberosum* (Accession No. BAB20861), *Brassica juncea* (Accession No. O23733), *Spinacea oleracea* (Accession No. Q00834), *Citrulus lanatus* (Accession No. Q43317), *Oryza sativa* (Accession No. Q9XEA8), *Zea mays* (Accession No. P80608), *Allium tuberosum* (Accession No. BAA93051), and *Triticum aestivum* (Accession No. P38076). Chloroplastic isoforms: *Capsicum anuum* (Accession No. P31300), *Spinacea oleracea* (Accession No. D14722), *Nicotiana tabacum* (Accession No. AJ299249), *Solanum tuberosum* (Accession No. O81155), and *Arabidopsis thaliana* (Accession No. S48695). Mitochondrial isoform: *Arabidopsis thaliana* (Accession No. X81973). Bacterial OAS-TL: *Escherichia coli* (Accession No. P11096), *Nostocaceae* (Accession No. NC_003272), *Thermosynechococcus elongates* (Accession No. NC_004113), *Synechocystis* (Accession No. P73410), and *Mesorhizobium loti* (Accession No. NC_002678).

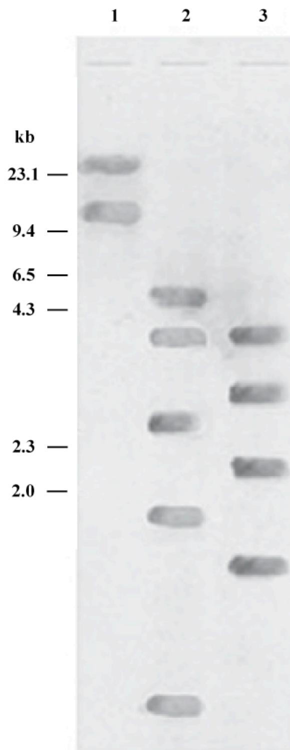
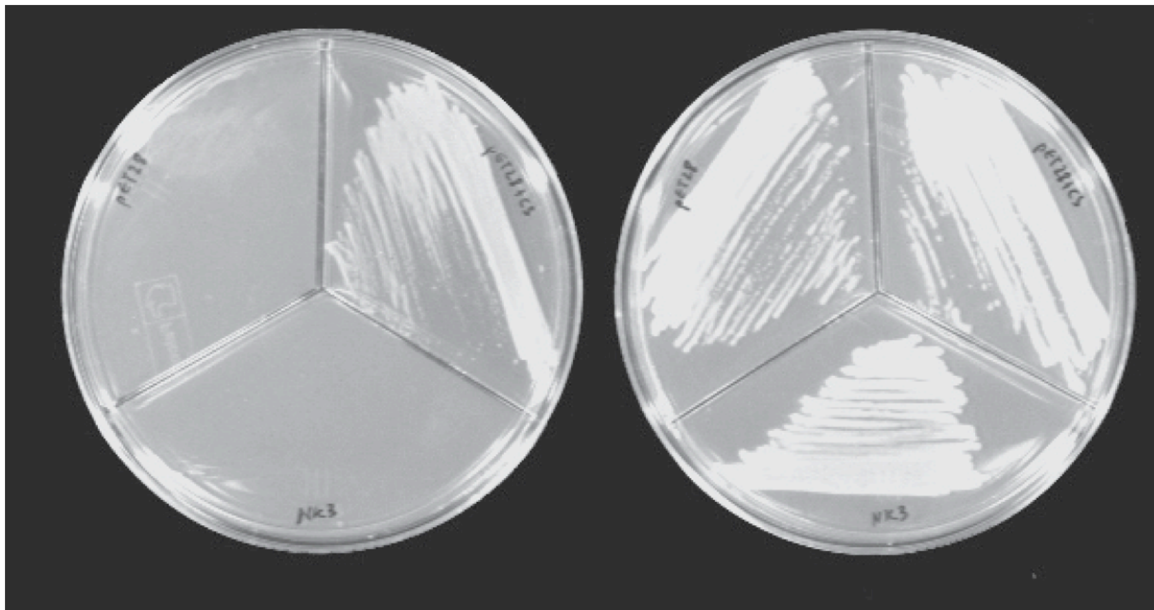


Figure 3: Southern blot analysis of soybean genomic DNA. Ten μg of soybean genomic DNA was restricted with *BamH*I (lane 1), *EcoR*I (lane 2), and *Hind*III (lane 3) and resolved on a 0.8% agarose gel. The gel was blotted to Hybond N^+ membrane followed by hybridization with ^{32}P-labeled soybean seed OAS-TL cDNA insert. The positions of the Lambda *Hind*III molecular weight markers are shown at the left side of the figure.



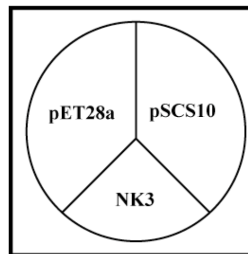


Figure 4: Functional complementation of Cys^{-} *E. coli* NK3 by transformation with the expression vector carrying soybean OAS-TL cDNA clone. The *E.coli* cysteine-auxotroph was transformed with pSCS10 and was streaked on M9 minimal agar plates with 0.5 mM cysteine (right plate) or without cysteine (left plate). The empty vector pET28a was used as a negative control.

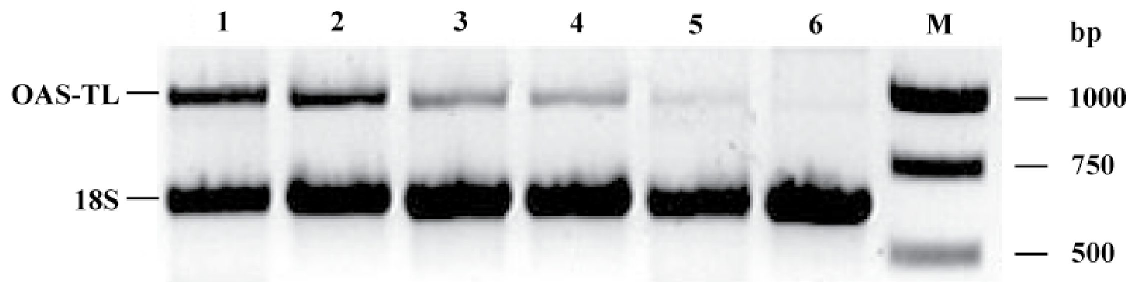


Figure 5: Reverse transcriptase (RT)-PCR detection of OAS-TL mRNA in developing soybean seeds. Seeds were harvested. Total RNA isolated from soybean seeds harvested at 7-day intervals from 5 days after R5 stage (lanes 1 to 6) was used as a template for RT-PCR. The 18S ribosomal mRNA was used as quantitative control. Sizes of the molecular weight markers are indicated on the right side of the figure.

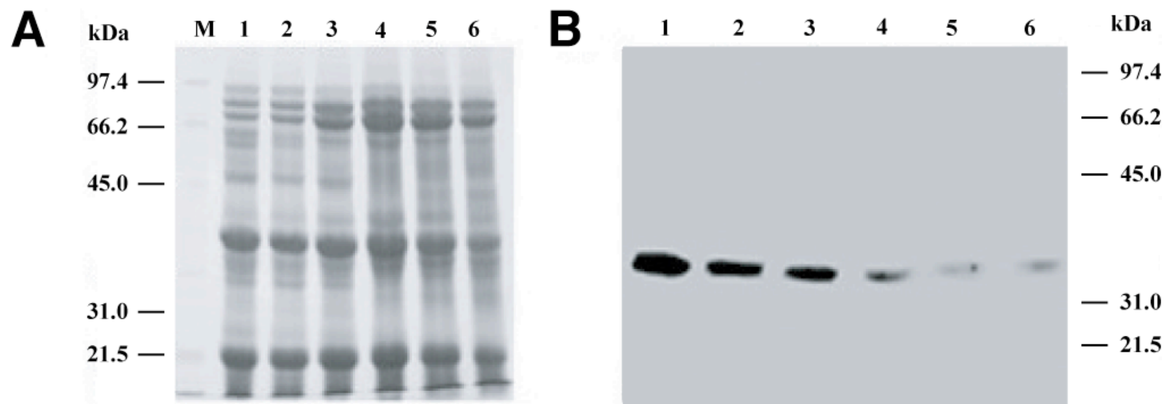


Figure 6: Accumulation of OAS-TL during soybean seed development. **(A)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein profiles of developing soybean seeds. Total seed proteins isolated from six different developmental stages (lanes 1 to 6) were resolved on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue; **(B)** Western blot analysis. Total protein from developing soybean seeds was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies raised against the soybean OAS-TL. Note that the antibody specifically recognizes a 34 kDa protein from the soybean seed extracts. The numbers in kDa shown at the sides of the figures represent Bio-Rad (Richmond, CA) protein molecular weight markers (phosphorylase b, 97 400; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400).

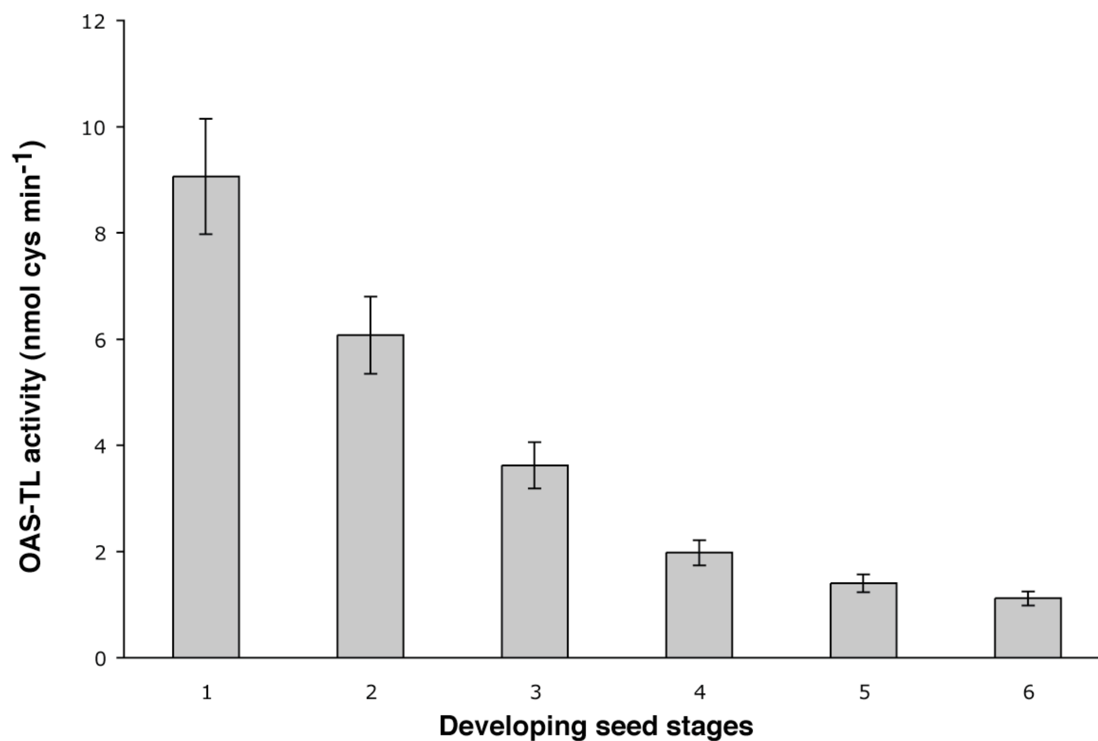


Figure 7: OAS-TL activity in soybean seeds. Seed samples from nodes 10 and 11 were collected at weekly intervals starting from R5 and cysteine synthase activity was measured using crude seed extracts. Formation of cysteine was determined with an OAS-ninhydrin assay. Bars represent the standard error of the mean.

LITERATURE CITED

Bogdanova N., Hell R. (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. **11:** 251-262

Burnett W.N. (1981) Western blotting: electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiodinated protein A. Anal. Biochem. **112:** 195-203

Coates J.B., Medeiros J.S., Thanh V.H., Nielsen N.C. (1985) Characterization of the subunits of β-conglycinin. Arch. Biochem. Biophys. **243:** 184-194

Droux M., Ruffet M.L., Douce R., Job D. (1998) Interactions between serine acetyltransferase and *O*-acetylserine (thiol) lyase in higher plants. Eur. J. Biochem. **255:** 235-245

Fehr W.R., Caviness C.E. (1979) Stages of soybean development. Iowa State Univ. Spec. Rpt. 80, Ames, IA

Gayler K.R., Sykes G.E. (1985) Effects of nutritional stress on the storage proteins of soybeans. Plant Physiol. **78:** 582-585

Hesse H., T. Altmann T. (1995) Molecular cloning of a cysteine synthase cDNA from *Arabidopsis thaliana*. Plant Physiol. **108:** 851-852

Ikegami F., Itagaki S., Kamiyama H., Murakoshi I. (1988a) Purification and characterization of two forms of cysteine synthases from *Citrullus vulgaris*. Photochemistry **27:** 697-701

Ikegami F., Itagaki S., Kamiyama H., Murakoshi I. (1988b) Purification and characterization of two forms of cysteine synthase from *Brassica juncea*. Photochemistry **27:** 3379-3383

Ikegami F., Itagaki S., Murakoshi I. (1993) Purification and characterization of two forms of cysteine synthase from *Allium tuberosum*. Photochemistry **32:** 31-34

Imsande J. (2001) Selection of soybean mutants with increased concentrations of seed methionine and cysteine. Crop Sci. **41:** 510-515

Jost R., Berkowitz O., Wirtz M., Hopkins L., Hawkesford M.J., Hell R. (2000) Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene **253:** 237-247

Kim H., Hirai M.Y., Hayashi H., Chino M., Naito S., Fujiwara T. (1999) Role of *O*-acetyl-L-serine in the coordinated regulation of the expression of a soybean seed-storage gene by sulfur and nitrogen nutrition. Planta **209:** 282-289

Kitamura K., Kaizuma N. (1981) Mutant strains with low level of subunits of 7S globulin in soybean (*Glycine max* Merr.) seed. Jpn. J. Breed. **31:** 353-359

Krishnan, H.B. (2000) Biochemistry and molecular biology of soybean seed storage proteins. J. New Seeds **2:** 1-25

Kuske C.R., Ticknor L.O., Guzman E., Gurley L.R., Valdez L.G., Thompson M.E., Jackson P.J. (1994) Purification and characterization of *O*-acetylserine sulfhydrylase isoenzymes from *Datura innoxia*. J. Biol. Chem. **269:** 6223-6232

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685

Leustek T., Martin M.N., Bick J-A., Davies J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 141-165

Leustek, T., Saito K. (1999) Sulfate transport and assimilation in plants. Plant Physiol. **120:** 637-643

Lizzardi P.M. (1983) Methods for the preparation of messenger RNA. Methods Enzymol. **96:** 24-38

Nakamura T., Yamaguchi Y., Sano H. (1999) Four rice genes encoding cysteine synthase: isolation and differential responses to sulfur, nitrogen and light. Gene **229:** 155-161

Nielsen N.C. (1996) Soybean seed composition. p. 127-163. *In* D.P.S. Verma and R.C. Shoemaker (ed.) Soybean: genetics, molecular biology and biotechnology. CAB International, Wallingford, UK

Noji M., Saito K. (2002) Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolism engineering. Amino Acids **3:** 231-243

Nordlee J.A., Taylor S.L., Townsend J.A., Thomas L.A., Bush R.K. (1996) Identification of Brazil-nut allergen in transgenic soybeans. N. Engl. J. Med. **334:** 688-692

Paek N.C., Imsande J., Shoemaker R.C., Shibles R. (1997) Nutritional control of soybean seed storage protein. Crop Sci. **37:** 498-503

Ravina C.G., Barroso C., Vega J.M., Gotor C. (1999) Cysteine biosynthesis in *Chlamydomonas reinhardtii* Molecular cloning and regulation of *O*-acetylserine (thiol) lyase. Eur. J. Biochem. **264:** 848-853

Rolland N., Ruffet M.L., Job D., Douce R., Droux M. (1996) Spinach chloroplast *O*-acetylserine (thiol) lyase exhibits two catalytically non-equivalent pyridoxal-5'-phosphate containing active sites. Eur. J. Biochem. **236:** 272-282

Saito K., Kurosawa M., Tatsuguchi K., Tagaki Y., Murakoshi I. (1994a) Modulation of cysteine biosynthesis in chloroplast of transgenic tobacco overexpressing cysteine synthase [*O*-acetylserine (thiol) lyase]. Plant Physiol. **106:** 887-895

Saito K., Kurosawa M., Tatsuguchi K., Tagaki Y., Murakoshi I. (1994b) Isolation and characterization of cDNA that encodes a putative mitochondrion-localized of cysteine synthase [*O*-acetylserine (thiol) lyase] from *Spinacea oleracea*. J. Biol. Chem. **269:** 28187-28192

Saito K., Miura H., Yamazaki M., Hirano H., Murakoshi I. (1992) Molecular cloning and bacterial expression of cDNA encoding plant cysteine synthase. Proc. Natl. Acad. Sci. USA **89:** 8078-8082

Saito K., Tatsuguchi K., Murakoshi I., Hirano H. (1993) cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacea oleracea*. FEBS Lett. **324:** 247-252

Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular cloning: a laboratory manual, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sexton P.J., Shibles R.M. (1999) Activity of ATP sulfurylase in reproductive soybean. Crop Sci. **39:** 131-135

Streit L.G., Beach L.R., Register III J.C., Jung R., Fehr W.R. (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

Urano Y., Manabe T., Noji M., Saito K. (2000) Molecular cloning and functional characterization of cDNAs encoding cysteine synthase and serine acetyltransferase that may be responsible for high cellular cysteine content in *Allium tuberosum*. Gene **257:** 269-277

Warrilow A.G.S., Hawkesford M.J. (1998) Separation, subecellular location and influence of sulphur nutrition on isoforms of cysteine synthase in spinach. J. Exp. Bot. **49:** 1625-1636

CHAPTER 5

TRANSGENIC STUDIES: OVEREXPRESSION OF *O*-ACETYLSERINE (THIOL) LYASE (OAS-TL) AND SERINE ACETYLTRANSFERASE (SAT) IN *ARABIDOPSIS* AND SOYBEAN

SYNOPSIS

Soybean (*Glycine max* [L.] Merr) is considered an excellent source of nutrient for humans and a main component of animal feed. Despite its nutritional value, the quality of soybean protein is lowered by the low content on sulfur amino acids, cysteine and methionine. In an effort to enhance the levels of cysteine and methionine in soybean, the coding sequence of serine acetyltransferase (SAT; EC 2.3.1.30), that produces *O*-acetylserine (OAS) from serine and acetylCoA, and *O*-acetylserine (thiol) lyase (OAS-TL; EC 4.2.99.8), the enzyme which converts OAS to cysteine, was introduced into the genome of soybean plants under the control of the cauliflower mosaic virus 35S promoter. *Arabidopsis* plants were also transformed with the same constructs. Since SAT is inhibited by cysteine (Cys), chimeric Cys insensitive forms of SAT were produced and used for plant transformation along with the sensitive wild type enzyme. Crude leaf extracts of the transgenic plants exhibited elevated levels of OAS-TL and SAT activity. Soybean SAT protein could not be detected by western blot analysis of wild type plants, indicating that the endogenous SAT levels are extremely low. Soybean transgenic plants

expressing either the Cys sensitive or insensitive form of SAT showed elevated SAT protein levels with enzyme activity reaching up to 20-fold higher than wild-type plants in some cases. Transgenic *Arabidopsis* plants expressing the OAS-TL gene displayed resistance to heavy metals and oxidative stress caused by methyl viologen. Similarly, transgenic soybean plants also exhibited tolerance to photooxidative stress. In conclusion, the results presented here demonstrate the importance of SAT and OAS-TL in cysteine biosynthesis and their protective role against heavy metals and oxidative stress.

INTRODUCTION

Soybean (*Glycine max* [L.] Merr) seeds with their 40% protein and 20% oil content are considered an excellent nutrient source. However, this nutritional value is compromised due to the low concentration of sulfur-containing amino acids, cysteine and methionine. Although traditional breeding methods have resulted in high protein soybean varieties, there has been only limited success in improving the cysteine and methionine content of soybean seed. Genetic engineering is a promising approach for improving soybean seed composition. Recently, several studies have reported the successful expression of heterologous proteins rich in sulfur in soybean. Transgenic soybean lines expressing the Brazil nut 2S albumin (BNA) protein showed an increase of the total methionine content upto 40% (Townsend and Thomas, 1994). Unfortunately, the BNA protein was identified as a potential allergen (Nordlee et al., 1996) and thus the commercial production of soybeans transformed with the BNA gene was abandoned. In

another study, soybeans expressing a 15 kDa maize sulfur-rich zein protein under the β-phaseolin promoter exhibited a 12 to 20% increase in methionine and 15 to 35% increase in cysteine content compared to untransformed lines. However, this increase is not adequate to cover the dietary need (Tabe and Higgins, 1998). Expression of the 11 kDa methionine-rich delta-zein protein in soybean failed to increase the overall content of sulfur amino acids (Kim and Krishnan, 2004).

It is clear that expression of exogenous sulfur rich proteins alone is not sufficient to raise the overall soybean sulfur amino acid content to significant levels. One possibility is that the available methionine or cysteine for incorporation is limiting during seed development, and thus preventing increased accumulation of the heterologous expressed proteins. Recent studies have shown that genetic manipulation of the enzymes involved in the sulfur assimilatory pathway could potentially increase the cysteine and methionine content in plants. *O*-acetylserine (thiol) lyase (OAS-TL; EC 4.2.99.8) or the synonymously termed cysteine synthase, catalyses the last committed step in cysteine biosynthesis, where *O*-acetylserine (OAS) combines with sulfide. OAS-TL cDNA clones have been isolated from *Arabidopsis* (Hell et al., 1994; Barroso et al., 1995; Hesse and Altmann, 1995), spinach (Saito et al., 1992, 1993, 1994; Hell et al., 1993; Rolland et al., 1993), watermelon (Noji et al., 1994), wheat (Youssefian et al., 1993), bell pepper (Romer et al., 1992), *Allium tuberosum* (Urano et al., 2000) and soybean (Chronis and Krishnan, 2003). It has been demonstrated that OAS-TL is induced under sulfur starvation and to a greater extent when both sulfur and nitrogen are depleted (Takahashi

and Saito, 1996). Transgenic tobacco overexpressing a cytosolic and chloroplastic isoform of OAS-TL from spinach displayed tolerance to toxic sulfur dioxide and sulfite (Noji et al., 2001). Furthermore, these transgenic plants showed resistance to paraquat (methyl viologen), a herbicide that generates active oxygen species. Levels of cysteine and glutathione (GSH), major end products of sulfur assimilation involved in plant defense under oxidative stress, were significantly elevated in resistant tobacco plants when compared to control plants (Noji and Saito, 2002). Similar results were obtained with tobacco plants overexpressing the wheat OAS-TL gene. Transformed plants were resistant to exposure to sulfur dioxide and showed drastically reduced levels of chlorosis following methyl viologen treatment. Cysteine and GSH concentration was considerably higher in transgenic tobacco plants. In addition Cu/Zn superoxide dismutase mRNA and activity were induced by cysteine and GSH (Youssefian et al., 2001). *Arabidopsis* plants overexpressing OAS-TL exhibited tolerance to cadmium chloride, compatible with the high cysteine biosynthesis requirements for the production of GSH and phytochelatins during exposure to heavy metals (Dominguez-Solis et al., 2001). Although overexpression of OAS-TL in transgenic plants resulted in an increase in cysteine content, OAS-TL activity can be controlled endogenously by serine acetyltransferase (SAT; EC 2.3.1.30), thus establishing an upper limit for the extent of increase. SAT catalyses the formation of OAS from acetylCoA and serine and has been isolated from several plants species, including watermelon (Saito et al., 1995), spinach (Noji et al., 2001), *Arabidopsis thaliana*, *Allium tuberosum* (Urano et al., 2000) and soybean (Chronis

and Krishnan, 2004). SAT is capable of binding OAS-TL to form a complex. The formation of the complex is promoted by sulfide and its dissociation by OAS. In this complex OAS-TL is completely inactive (Bogdanova and Hell, 1997; Droux et al., 1998). SAT is allosterically inhibited by cysteine, but only the cytosolic form and not the plastidic or the mitochondrial isoform of SAT (Noji et al., 1998; Noji et al., 2001; Urano et al., 2000). Experiments with chimeric SATs from watermelon and *Arabidopsis* revealed that SAT protein bears two distinct allosteric sites for inhibition by cysteine, one in the N-terminal and the other at the C-terminal. Two residues at the C-terminal allosteric site, Gly-277 and His-282, are primarily responsible for the sensitivity to cysteine (Innoue et al., 2000). To further understand the inhibition of SAT by cysteine and enhance the cysteine production in plants, Noji and Saito (2002) transformed *Arabidopsis* plants with a cysteine sensitive and insensitive SAT encoding gene. Although the SAT enzyme activity was significantly increased in cell-free extracts of all transformed plants, levels of OAS and cysteine were only higher than the wild type in the plants expressing the insensitive SAT, indicating that endogenously cysteine does inhibit SAT activity. Potato plants overexpressing the *cysE* gene from *Escherichia coli*, that codes for a cysteine insensitive form of SAT revealed remarkably higher SAT activity and increased levels of cysteine and GSH (Harms et al., 2000).

All previous evidence indicates that cysteine biosynthesis can be enhanced by manipulating the enzymes involved in sulfur assimilation. Here I report, generation of transgenic *Arabidopsis* and soybean expressing OAS-TL and SAT genes previously

isolated from soybean (Chronis and Krishnan, 2003; 2004). The results obtained from this study should aid our efforts to increase the overall cysteine and methionine content of soybean.

MATERIALS AND METHODS

Plasmid construction. Isolation of OAS-TL and SAT cDNA clones from soybean has been described previously (Chronis and Krishnan, 2003; Chronis and Krishnan, 2004). A unique *Spe*I site was created by PCR at position 819 of the SAT open reading frame (ORF) to facilitate single substitution of Gly-268 with Ala and His-273 with Arg, and double substitution of both residues. The region from position 819 and downstream to the stop codon of the SAT ORF was amplified using the primers: 5'-AACC**ACTAGT**TTTATCTCTGAGTGGTCAG-3' and 5'-GGTT**GCGGCCGC**TCAAATGATATAATCTGACC-3'; *Spe*I and *Not*I, annotated with bold letters, were created in these two primers. Amplification of the SAT coding region from position 819 and upstream to the start codon was carried out using the same forward primer (5'-CCAA**CATATG**ATGCCGACGGGGTTACCGGC-3') and different reverse primers to enable the point mutations at the SAT allosteric site (5'-GGTT**ACTAGT**ATGGTCCATAGACTCCGCAGG-3', Gly:Ala; 5'-GGTT**ACTAGT**ACGGTCCATAGACTCCCCAGG-3', His:Arg; 5'-GGTTA**CTAGT**ACGGTCCATAGACTCCGCAGG-3', double substitution), to which *Nde*I and *Spe*I restriction sites, indicated with bold letters, were introduced at the 5' and

3’ ends respectively. Each amplified fragment was individually inserted into pGEM-T easy vector (Promega, Madison, WI, USA). Utilizing unique restriction sites created by PCR, each flanking region from the start to 819 position was paired with the fragment starting from 819 position to stop codon and cloned to pGEM-T easy vector resulting in plasmids pGSAT3 (Gly substitution), pGSAT4 (His substitution) and pGSAT5 (double substitution). In similar approach, a plasmid with the SAT ORF lacking the allosteric site was created by amplifying the coding region with forward (5’-CCAA**CATATG**ATGCCGACGGGGTTACCGGC-3’; *Nde*I indicated with bold letters) and reverse (5’-GGTT**GCGGCCGC**TCACACATCCTCATGCTTAGAGGG C-3’; *Not*I indicated with bold letters). The amplified fragment was inserted into pGEM-T easy vector resulting in pGSAT6.

Constructs of modified SATs for overexpression in *Escherichia coli* were created as described previously (Chronis and Krishnan, 2004). Fragments from pGSAT3, pGSAT4, pGSAT5 and pGSAT6 were excised with *Nde*I / *Not*I double digestion and cloned into the *Nde*I and *Not*I sites of the expression vector pET 28(a)+ (Calbiochem-Novabiochem, San Diego, CA) resulting in pESAT3, pESAT4, pESAT5 and pESAT6 respectively, with a 6-His N-terminal fusion part. For overexpression of OAS-TL and SATs in plants five different plasmids were prepared. In the case of OAS-TL the coding region was amplified from pSCS1 (Chronis and Krishnan, 2003) with primers 5’-CCAA**GGATCC**ATGCCGACGGGGTTACCGGC-3’ and 5’-GGTT**GCGGCCGC**GGGCTCAAAAGTCATGCTTT-3’ (*BamH*I and *Not*I indicated

with bold letters). The PCR product was cloned into the intermediate vector pHK10 in the *BamH*I / *Not*I sites, resulting in plasmid pHCS1 with a fused 6X-His tag at end of the ORF. In the same manner, the coding region of native and modified SATs was amplified from pSSAT1 (Chronis and Krishnan, 2004), pGSAT5 and pGSAT6 with forward primer (5'-CCAA**GGATCC**ATGCCGACGGGGTTACCGGC-3', *BamH*I restriction site in bold letters) and reverse primer (5'-GGTT**GCGGCCGC**AATGATATAATCTGACC-3' for amplification from pSSAT1, pGSAT3 and pGSAT5; 5'-GGTT**GCGGCCGC**CACATCCTCATGCTTAGAGGGC-3' for amplification from pGSAT6; *Not*I restriction site in bold letters). The amplified fragments were introduced to the intermediate vector, resulting in plasmids pHSAT1, pHSAT3, pHSAT5 and pHSAT6 with the 6X-His tag at the end of each ORF. As a final step, the inserts from the intermediate plasmids of OAS-TL and SAT were digested with *BamH*I / *Xba*I and cloned into the corresponding sites of pZ35S1, resulting in pZCS1, pZSAT1, pZSAT3, pZSAT5 and pZSAT6 respectively. These final plasmids consisted of cauliflower mosaic virus 35S promoter (CaMV 35S), the different OAS-TL and SAT gene 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).

Overexpression of chimeric SATs in *Escherichia coli* (*E. coli*). Overexpression of the modified SATs was carried out using the ER2566 *E. coli* strain (New England Biolabs). A preparative culture (100 ml LB, 100 µg/ml kanamycin) of ER2566 strains carrying each SAT plasmid were grown at 37°C to an optimal density of 0.9 (550 nm) and

induced by addition of isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Induction was continued for 18 hr at 37°C. Recombinant SAT proteins were purified under native conditions according to Hoffmann and Roeder (1990) at 4°C. Cells from the overnight-induced cultures were harvested by centrifugation (4050g, 20 min, 4°C), resuspended in 5 ml of extraction buffer (10 mM Tris.HCl [pH 7.9], 10% glycerol, 0.5 M NaCl, 0.1% nonidet-P40, 5 mM DTT) and incubated on ice for 30 min. The suspensions were centrifuged (11,300g; 10 min; 4 °C) and imidazole was added to a final concentration of 1mM to the supernatants. The supernatants were passed through a Ni-NTA agarose column (Qiagen) and washed with two column volumes of BC100 (20% glycerol, 20 mM Tris.HCl [pH 7.9], 100 mM KCl, 5 mM dithiothreitol (DTT) and 0.5 mM PMSF) containing 20 mM imidazole. Elution was carried out with 5 ml of BC100 containing 80 mM imidazole. Utilizing the DC Standard Protein Assay Kit (Pierce, Rockford, IL, USA), protein concentrations were spectrophotometrically determined using bovine serum albumin as a standard.

Plant material and transformation. Transformation of *Arabidopsis* was performed following the simplified floral dipping method of Clough and Bent (1998). *Arabidopsis thaliana* plants (ecotype Columbia) used for inoculations with *Agrobacterium tumefaciens* were grown in a moist potting soil (Premier Pro-mix potting soil, BareRoots Hydroponics ,Waterville, VT, USA) under 24 hr constant light at 22°C. When the primary bolts emerged, plants were clipped to increase growth and proliferation of many secondary bolts. Typically 3 to 4 days after clipping plants were inoculated with *A. tumefaciens* carrying pZCS1, pZSAT1, pZSAT5 and pZSAT6 plasmids. Overnight culture

of *A. tumefaciens* was resuspended in 5% (w/v) sucrose to a final OD_{600} of 0.8 containing 0.05% (v/v) of the surfactant Silwet L-77, and aerial parts of the plants were dipped for a few seconds with gentle agitation. After inoculation dipped plants were placed under a transparent plastic dome to increase humidity for 16 to 24 hrs. For selection of transgenic *Arabidopsis* plants, seeds were germinated in wetted soil. When cotyledons were fully opened plants were sprayed to saturation with 0.005% (w/v) glufosinate ammonium solution (Sigma-Aldrich Corp., St. Louis, MO, USA). Application of glufosinate ammonium was carried out for 3 consecutive days and repeated one more time 5 days after last application. Resistant plants were screened by western blot analysis for the presence of CS1, SAT1, SAT5 and SAT6 proteins and left to grow under constant illumination at 22°C till seeds were harvested.

Production of transgenic soybean lines (cv. Williams 82) was carried out by *Agrobacterium*-cotyledonary node transformation (Hinchee et al., 1988) utilizing glufosinate ammonium as a selective agent (Zhang et al., 1999). Plants were transformed with *Agrobacterium* carrying pZCS1, pZSAT1 and pZSAT5 plasmids. Regenerated transgenic soybean plants were screened for tolerance to glufosinate by a leaf-painting assay as described earlier (Zhang et al., 1999). The presence of the heterologous proteins in glufosinate-resistant plants was confirmed by western blot analysis.

Western blot analysis. Total protein extracts from *Arabidopsis* leaf and soybean leaf were fractionated by SDS-PAGE (Laemmli 1970) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA). The

proteins were 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) as described by Burnett (1981). Immunoblot analysis was performed following conventional western blot analysis with antibodies raised against soybean SAT and soybean OAS-TL as described previously (Chronis and Krishnan, 2003; 2004) and against the His tag of the recombinant proteins according to manufacturer's protocol (SuperSignal West HisProbe Kit; Pierce Biotechnology, Rockford, IL, USA).

Enzyme activity assays. SAT activity was assayed according to Noji et al (1998). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, 1 mM L-serine, and a known amount of the purified recombinant soybean SAT in a final volume of 1 ml. The reaction was initiated by the addition of L-serine and the decrease in acetyl CoA was monitored spectrophotometrically. SAT specific activity was calculated using the molar extinction coefficient for acetyl-CoA of ε=4500 at 232 nm. Cysteine inhibition effect on recombinant SAT activity was determined by following the deviation in SAT specific activity as concentration of [L]-cysteine in the enzyme reaction mixture increased. The kinetic parameters were determined by using the appropriate rate equations and the GraFit 5.0 software from Erithacus Software (Sigma-Aldrich Corp., St.

Louis, MO, USA). SAT activity from the crude plant extracts was determined by the method of Kredich and Tompiks (1966). Freshly harvested soybean and *Arabidopsis* tissue samples (200 mg) were ground 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 DTT]. The samples were transferred to microcentrifuge tubes and spun down (11,600*g*; 10 min; 4°C) and the clear supernatant was used to measure SAT activity. The enzyme reaction mixture contained 0.1 mM acetyl-CoA, 50 mM Tris pH 7.6, 1 mM DTNB, 1 mM EDTA and 1 mM L-serine in 1 ml final volume. Subsequent to reaction initiation by addition of enzyme at room temperature, the initial velocity was estimated by monitoring the increase in absorbance at 412 nm. Rates were calculated using an extinction coefficient for thionitrobenzoic acid of ε=13,600 at 412 nm. Protein concentrations were determined using the Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

OAS-TL activity from soybean and *Arabidopsis* leaf tissue 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 4ºC for 10 min at 12,000 g. The clear supernatant was saved and used immediately for measuring the OAS-TL activity. Protein

concentration from plant extracts was determined spectrophotometrically with the help of Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Heavy metal stress. Tolerance to heavy metal stress was determined according to Dominguez-Solis et al. (2001). Wild type *A. thaliana* (ecotype Columbia) and transgenic seeds were surfaced sterilized by treatment for 90 seconds in ethanol, then with 50% (v/v) bleach for 5 min, and rinsed three times with sterile water. Seeds were germinated on solid MS medium with and without 250 μM $CdCl_2$. The plants were grown in a growth chamber under constant illumination (25 μE m^{-2} s^{-1}) at 22°C.

Treatment of leaf discs with paraquat. The effect of oxidative stress and tolerance of transgenic soybean and *Arabidopsis* plants to reactive oxygen species (ROS) was established according to Noji et al. (2001). Leaf soybean discs (7 mm) and whole *Arabidopsis* leaves from wild type and transgenic plants of similar age were submerged in solution that contained 2 μM paraquat (methyl viologen; Sigma-Aldrich Corp., St. Louis, MO, USA) and 0.1% (w/v) Tween 20, followed by exposure to constant illumination (25 μE m^{-2} s^{-1}) at 22°C for 24 to 48 hr, and they were then examined visually for damage.

RESULTS

Construction of chimeric SATs and expression in *E. coli*. SAT protein harbors several domains with distinct functions (Fig. 1), including a catalytic domain, a protein-protein interaction domain with OAS-TL and two allosteric sites for cysteine binding.

Point mutation studies in *Arabidopsis* and watermelon revealed that between the two allosteric domains in SAT protein only the C-terminal domain containing amino acid residues glycine (Gly) at position 277 and histidine (His) at position 282 are responsible for SAT sensitivity to cysteine (Innoue et al., 2000). In this study I produced insensitive forms of the cytosolic soybean SAT, by performing point mutations to the previously isolated SAT cDNA from soybean (Chronis and Krishnan, 2004). A unique *Spe*I site was introduced to the cDNA sequence of SAT downstream of the Gly and His residues at the C-terminal to enable these substitutions. Four different SAT genes were generated resulting in four different plasmids for overexpression in *E. coli*: (1) pESAT3 (Gly residue was substituted with alanine (Ala)), (2) pESAT4 (His residue was substituted with arginine (Arg)), (3) pESAT5 (double substitution of Gly and His with Ala and Arg respectively) and (4) pESAT6 (complete elimination of the allosteric site). The chimeric SATs (SAT3, SAT4, SAT5 and SAT6) were overexpressed in *E. coli* and purified under native conditions (Fig. 2). As expected, SAT6 protein is smaller in size than the other recombinant proteins, since it is missing 19 amino acids at the C-terminal. In order to make sure that these mutations did not modify the enzyme properties of SAT, the K_m and V_{max} values of the recombinant SATs were measured. Kinetic analysis revealed comparable properties between mutated and native SAT, though the mutated SATs were not inhibited by cysteine (Table I). Even at high cysteine concentrations, the activity of the mutated SATs was not influenced, while native SAT enzyme activity was diminished as cysteine levels increased (Fig. 3).

Expression of SAT and OAS-TL in soybean and *Arabidopsis*. Native forms of SAT and those insensitive to cysteine inhibition and OAS-TL were expressed both in soybean and *Arabidopsis*. For expression of SAT in plants, three different constructs were designed: (1) pZSAT1 containing the wild-type soybean SAT, (2) pZSAT5 expressing the SAT5 gene with the double substitution of Gly and His residues, and (3) pZSAT6 where the allosteric site of SAT was completely eliminated (Fig. 4A). A construct for expression of OAS-TL was also created (pZCS1; Fig. 4B). All plasmids for plant transformation possess the same elements. SAT and OAS-TL genes were placed under the control of the CaMV 35S promoter, and a BAR cassette was incorporated in every plasmid to allow selection of transgenic plants resistant to glufosinate ammonium. Finally, a 6x His tag was introduced at the end of the ORF of each gene just before the STOP codon for confirmation of true transgenes by western blot analysis (see immunoblot analysis of transgenic plants). Soybeans were transformed with pZSAT1, pZSAT5 and pZCS1 plasmids, while *Arabidopsis* plants were transformed with all four constructs (pZSAT1, pZSAT5, PZSAT6 and pZCS1).

Immunoblot analysis of transgenic plants. In order to confirm the overexpression of SAT and OAS-TL in transgenic plants, total leaf protein was isolated from transgenic soybean and *Arabidopsis* plants showing resistance to glufosinate ammonium and resolved by SDS-PAGE. Proteins were subjected to western blot analysis with polyclonal antibodies raised against SAT (Chronis and Krishnan, 2004) and OAS-TL (Chronis and Krishnan, 2003). Western blots clearly showed the expression of SAT1, SAT5 and SAT6

proteins (Fig. 5A, 6A, 7A) and OAS-TL (Fig. 8A) protein in *Arabidopsis* plants. To further confirm the existence of introduced SAT and OAS-TL genes in transgenic *Arabidopsis* western blot analysis was performed with the same plant tissue samples but utilizing His tag antibodies. The antibody reacted with a single band corresponding to the expected size of SAT and OAS-TL His tagged proteins (Fig. 5B, 6B, 7B) or OAS-TL (Fig. 8B). In a previous study (Chronis and Krishnan, 2004) SAT protein could not be detected in wild type soybean plants by western blot analysis. Interestingly in transgenic soybean plants expressing SAT1 and SAT5 protein a single 34 kD protein was detected (Fig. 9A, 10A). The same pattern of expression was detected when the antibody against the His tag was used, indicating true overexpression of SAT in soybean (Fig. 9B, 10B). OAS-TL expression was also confirmed in soybean plants by western blot with antibodies against both OAS-TL and His tag (Fig. 11).

Enzyme activity assays in transgenic plants. Total leaf extracts from *Arabidopsis* and soybean that showed tolerance to glufosinate ammonium were used to determine the SAT and OAS-TL enzyme activity. SAT activity was enhanced in transgenic *Arabidopsis* when compared to wild type, with SAT5 and SAT6 plants displaying relatively higher activity than SAT1, which is inhibited by cysteine (Fig. 12). In soybean plants expressing SAT1 and SAT5 protein, the activity of SAT was significantly enhanced (~20-fold). Transgenic plants expressing the cysteine insensitive SAT5 showed higher activity than those expressing SAT1 (Fig, 13). Similarly, plants expressing OAS-TL exhibited elevated activity in both *Arabidopsis* (Fig. 14) and soybean (Fig. 15).

Effect of cadmium chloride in transgenic *Arabidopsis* expressing OAS-TL. The effect of the cadmium ion on the transformed plants was tested by growing wild type and transgenic *Arabidopsis* in MS medium containing 250 μM $CdCl_2$. The transgenic *Arabidopsis* plants overexpressing the OAS-TL gene were able to germinate and grow on this medium (Fig. 16D). Wild type seeds germinated poorly in the presence of the metal compared to control plants (Fig. 16A), and a few plants that germinated slowly did not produce leaves and died after 5–7 days (Fig. 16C).

Effect of photooxidative stress in transgenic *Arabidopsis* and soybean plants. The enhanced tolerance of transgenic *Arabidopsis* and soybean plants to active oxygen species was determined by treating leaf discs of soybean and whole detached leaves of *Arabidopsis* plants with paraquat. Paraquat is a reagent that generates active oxygen species in chloroplasts under constant illumination (Dodge, 1975). After a 24 hr incubation in 2 μM paraquat, wild type leaves of *Arabidopsis* developed chlorosis (Fig. 17C), an effect not seen in leaves from transgenic plants (Fig. 17D). Untransformed soybean leaf discs showed similar results with severe chlorosis after a 48 hr exposure to light (Fig. 18C), whereas transgenic leaf discs tolerated paraquat (Fig. 18D).

DISCUSSION

In this study, transgenic soybean and *Arabidopsis* plants were generated that overexpressed OAS-TL and cysteine feed back sensitive and insensitive forms of SAT. SAT is a crucial enzyme in sulfur metabolism, that catalyzes the formation of OAS. The

enzyme bears two distinct allosteric domain for cysteine inhibition. Two amino acid residues (Gly and His) located in the C-terminal of the protein are essential for the allosteric inhibition of SAT (Fig. 1). Only the cytosolic form of SAT, that contains both of these residues, is inhibited by cysteine, whereas the plastidic and mitochondrial isoforms are insensitive (Innoue et al., 2000). Four different insensitive forms of the soybean SAT were produced here with substitutions of either Gly or His, or by altering both residues and by complete elimination of the allosteric site. The kinetic parameters of the recombinant proteins were comparable to the wild type SAT with no significant differences, establishing that these modifications did not alter the activity of the enzyme (Table 1). These modifications rendered the SAT insensitive to cysteine inhibition (Fig. 3). Western blot analysis confirmed the overexpression of SAT and OAS-TL in transgenic *Arabidopsis* (Fig. 5 to 8) and soybean plants (Fig. 9 to 11). Interestingly enough, the SAT protein could not be detected in soybean wild type plants by western blot analysis, but the signal was evident in transgenic plants, indicating the low endogenous levels of SAT in soybean. Enzyme activity assays from the transformed plants reflected the results of the western blot with SAT activity in transgenic *Arabidopsis* plants being 4 times higher than the wild type (Fig. 12). In some transgenic plants the SAT activity was increased 20 fold (Fig. 13). Plants transformed with the insensitive forms of SAT showed higher activity compared to the ones transformed with the cytosolic form of SAT (Fig. 12 and 14). This difference can be explained due to inhibition of SAT from the endogenous cysteine. Plants overexpressing OAS-TL showed 3-fold higher activity than the wild type in both

Arabidopsis (Fig. 14) and soybean (Fig. 15). Previous studies have shown that overexpression of OAS-TL increased levels of cysteine and downstream products, like phytochelatins (Dominguez-Solis et al., 2001) and GSH (Noji et al/, 2001; Youssefian et al., 2001). Phytochelatins are derivatives of GSH and bind heavy metal cations through the thiol group and thus detoxify them. *Arabidopsis* plants that overexpress OAS-TL were resistant to cadmium chloride and germinated normally, while wild type seeds were either unable to germinate or produce leaves in the presence of cadmium (Fig. 16), indicating that overexpression of OAS-TL presumably leads to overproduction of phytochelatins and tolerance to heavy metals. Transgenic soybean and *Arabidopsis* plants were tolerant to methyl viologen, a ROS generator compound that triggers the production of GSH (Fig. 17 and 18). GSH functions as an antioxidant that inactivates toxins, hormones, oxygen radicals and xenobiotic substances such as herbicides. The transgenic *Arabidopsis* and soybean plants tolerates exposure to methyl viologen as indicated by the lack of chlorosis suggesting that overexpression of OAS-TL could lead to accumulation of GSH.

All previous studies involving transgenic plants have shown that overexpressing SAT and OAS-TL leads to elevated levels of OAS (Hopkins et al., 2005; Riemenschneider et al., 2005) and cysteine (Harms et al., 2000; Hopkins et al., 2005; Noji et al. 2001; Wirtz and Hell, 2003; Youssefian et al., 2001). However, improvement in crop nutrient quality cannot be achieved solely by enhancing the rates of biosynthesis of essential amino acids. Free amino acids are easily lost or degraded during crop processing and large quantities

of free cysteine or methionine are regarded as deleterious for plant metabolism. Considering that the concentration of seed proteins are much higher than that of free amino acids and proteins are less susceptible to degradation, it is desirable to express heterologous seed proteins that are rich in sulfur amino acids. To accumulate sufficient amounts of transgenic proteins genetic engineering must manage to achieve high expression rates, enhanced translation rates, increased protein stability, good nutritional accessibility and low allergenicity. The best candidate for genetic manipulation of the enzymes involved in sulfur metabolism seems to be SAT. It has been shown that overexpressing SAT in plants increases the levels of OAS and cysteine more than plants overexpressing OAS-TL (Youssefian et al., 2001). This study has established that SAT is present in extremely low levels in soybean (as shown from the western blot analysis and enzyme activity assays). It must be noted though that better results could be obtained if the insensitive form of SAT is expressed, so that cysteine inhibition is not a factor. Crosses between soybean plants overexpressing a form of SAT insensitive to cysteine with plants expressing heterologous sulfur rich proteins could enhance the nutritional value of soybeans to meet the dietary requirement for sulfur amino acids in animal feed.

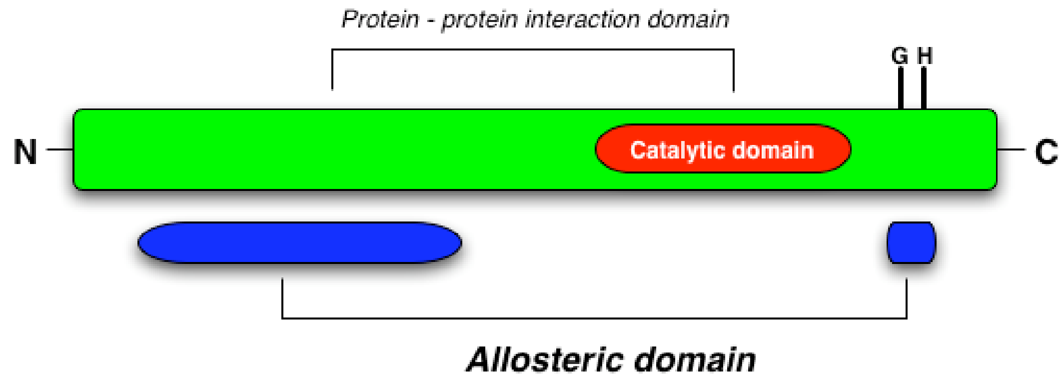


Figure 1: Graphic representation of SAT domain structure. The C and N terminal allosteric domains are with blue color and the catalytic domain with red. G and H indicate the Gly-277 and the His-282 responsible for the L-cysteine inhibition in the C-terminal domain, respectively (adopted from Saito et al., 2000).

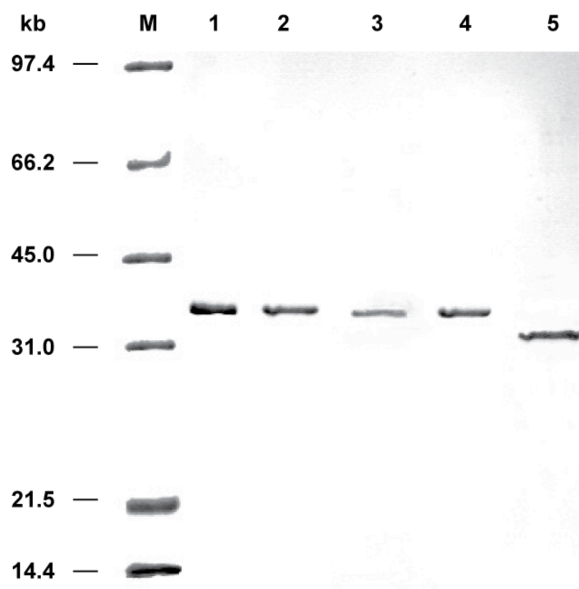


Figure 2: Expression and purification of the native and recombinant SATs. Lanes: M, Protein molecular weight marker; 1. purified native SAT1; 2. purified recombinant SAT3 (Gly:Ala); 3. purified recombinant SAT4 (His:Arg); purified recombinant SAT5 (Gly:Ala, His:Arg); purified recombinant SAT6 (complete elimination of allosteric site).

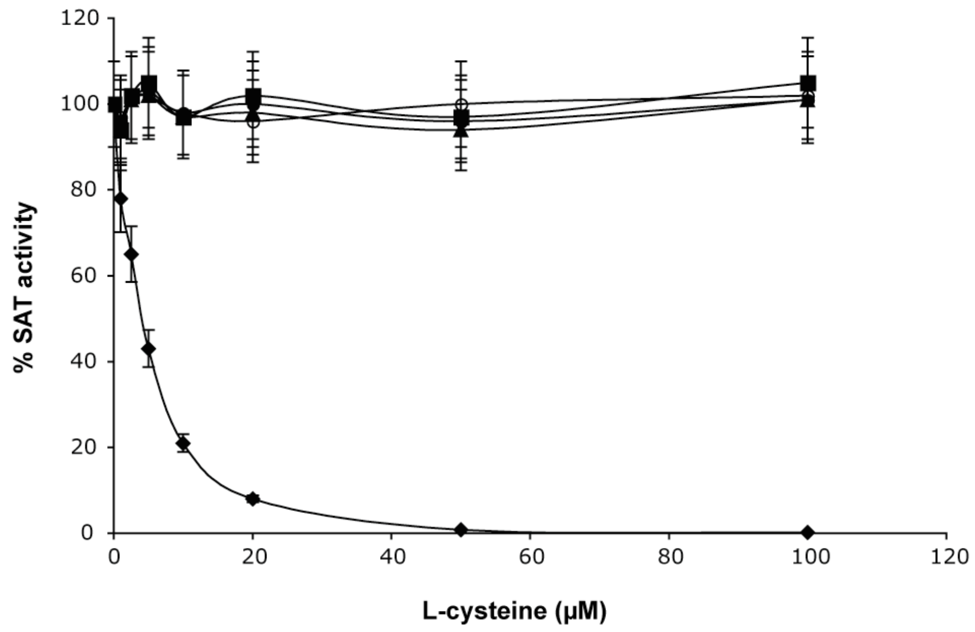


Figure 3: Native SAT1 undergoes feedback inhibition by L-Cys while the recombinant SATs are insensitive. Bars represent the standard error of the mean. (SAT1 ◆, SAT3 ▪, SAT4 ▲, SAT5 ●, SAT6 o).

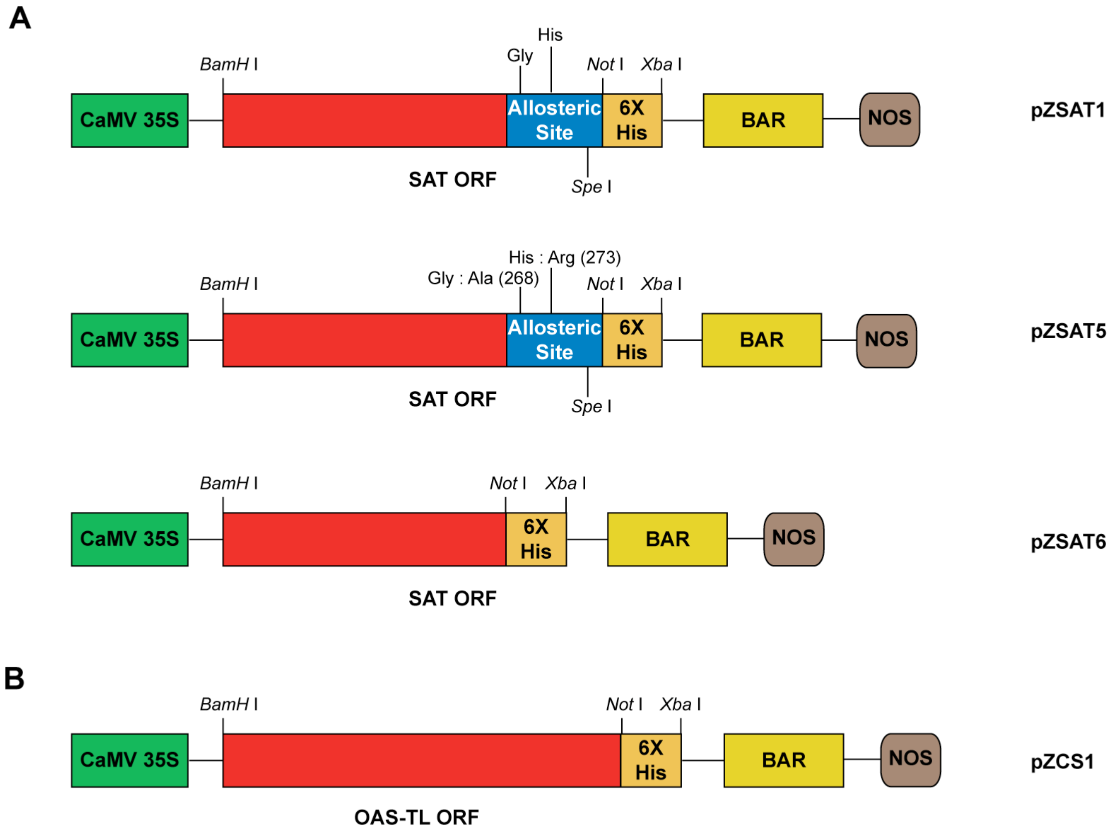


Figure 4: (A) Generic maps of SAT constructs used for soybean and *Arabidopsis* transformation. Each construct bears a 6x His tag at the end of the ORF, a CaMV 35S promoter and a BAR cassette for selection. Plasmid pZSAT1 bears the native cytosolic form of SAT. In pZSAT5 there is a double substitution of both Gly-268 and His-273 with Ala and Arg respectively, where pZSAT6 lacks completely the allosteric site of SAT; (B) Genetic map of the pZCS1 plasmid used for transformation of soybean and *Arabidopsis* plants. The CaMV 35S promoter, 6x His tag, BAR cassette, NOS terminator are indicated.

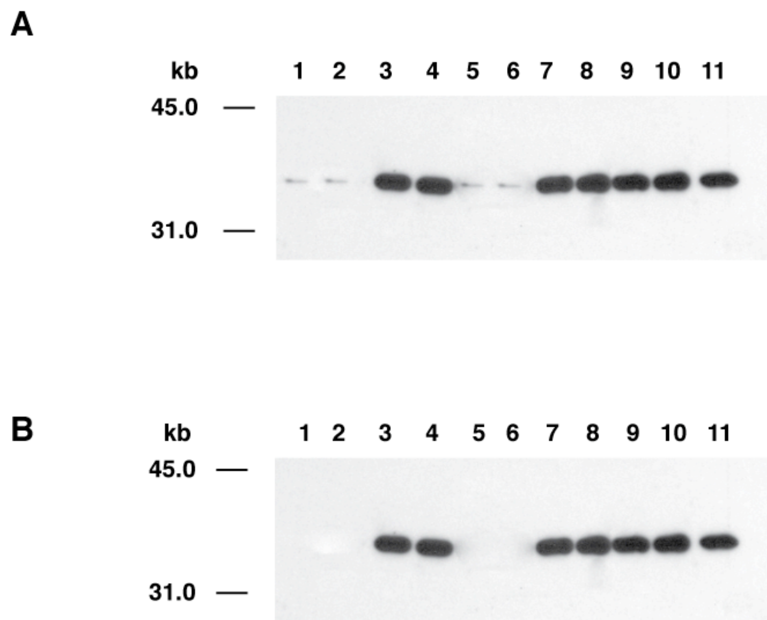


Figure 5: Western blot analysis of transgenic *Arabidopsis* plants transformed with pZSAT1. Lane 1 is wild type *Arabidopsis* (ecotype Columbia) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against **(A)** SAT antibody and **(B)** His antibody.

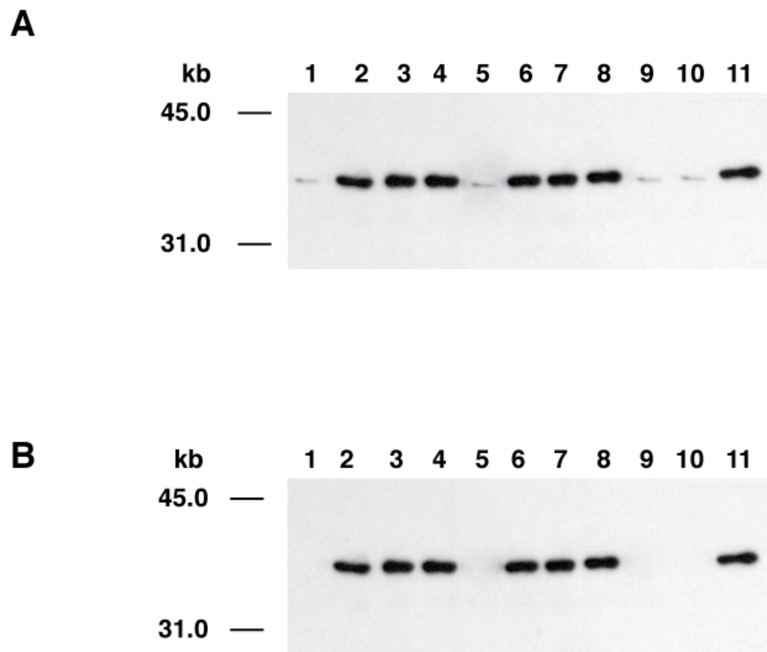


Figure 6: Western blot analysis of transgenic *Arabidopsis* plants transformed with pZSAT5. Lane 1 is wild type *Arabidopsis* (ecotype Columbia) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against (A) SAT antibody and (B) His antibody.

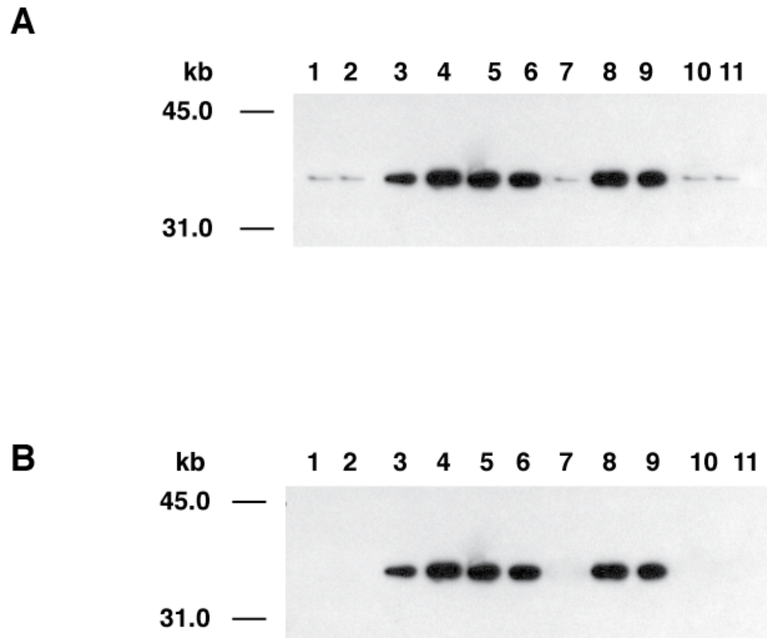
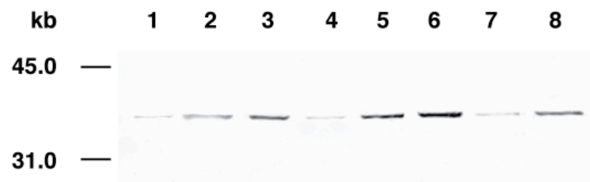


Figure 7: Western blot analysis of transgenic *Arabidopsis* plants transformed with pZSAT6. Lane 1 is wild type *Arabidopsis* (ecotype Columbia) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against (A) SAT antibody and (B) His antibody.

A



B

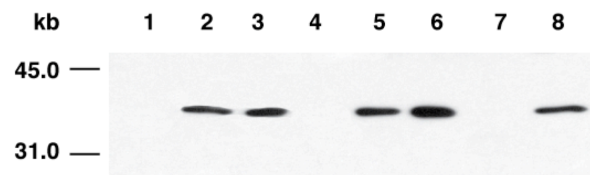
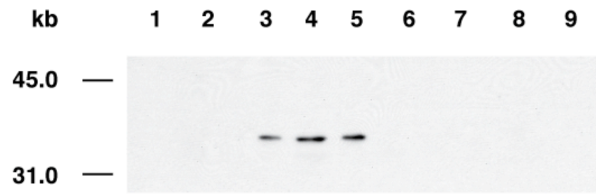


Figure 8: Western blot analysis of transgenic *Arabidopsis* plants transformed with pZCS1. Lane 1 is wild type *Arabidopsis* (ecotype Columbia) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against (A) SAT antibody and (B) His antibody.

A



B

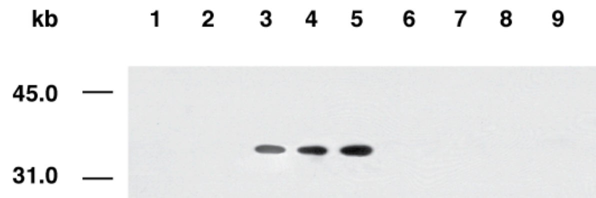
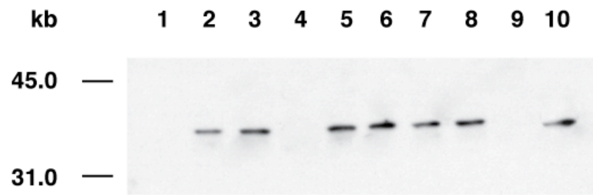


Figure 9: Western blot analysis of transgenic soybean plants transformed with pZSAT1. Lane 1 is wild type soybean (cv. Jack) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against (A) SAT antibody and (B) His antibody.

A



B

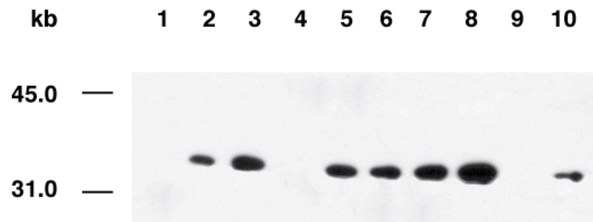
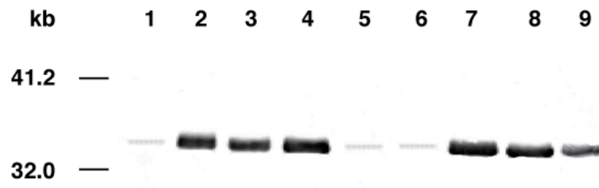


Figure 10: Western blot analysis of transgenic soybean plants transformed with pZSAT5. Lane 1 is wild type soybean (cv. Jack) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against (A) SAT antibody and (B) His antibody.

A



B

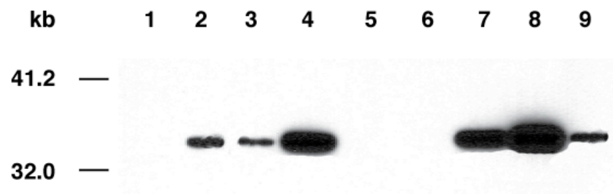


Figure 11: Western blot analysis of transgenic soybean plants transformed with pZCS1. Lane 1 is wild type soybean (cv. Maverick) and lanes 2 to 11 are independent transgenic lines showing resistance to glufosinate ammonium. Total protein leaf samples blotted against **(A)** OAS-TL antibody and **(B)** His antibody.

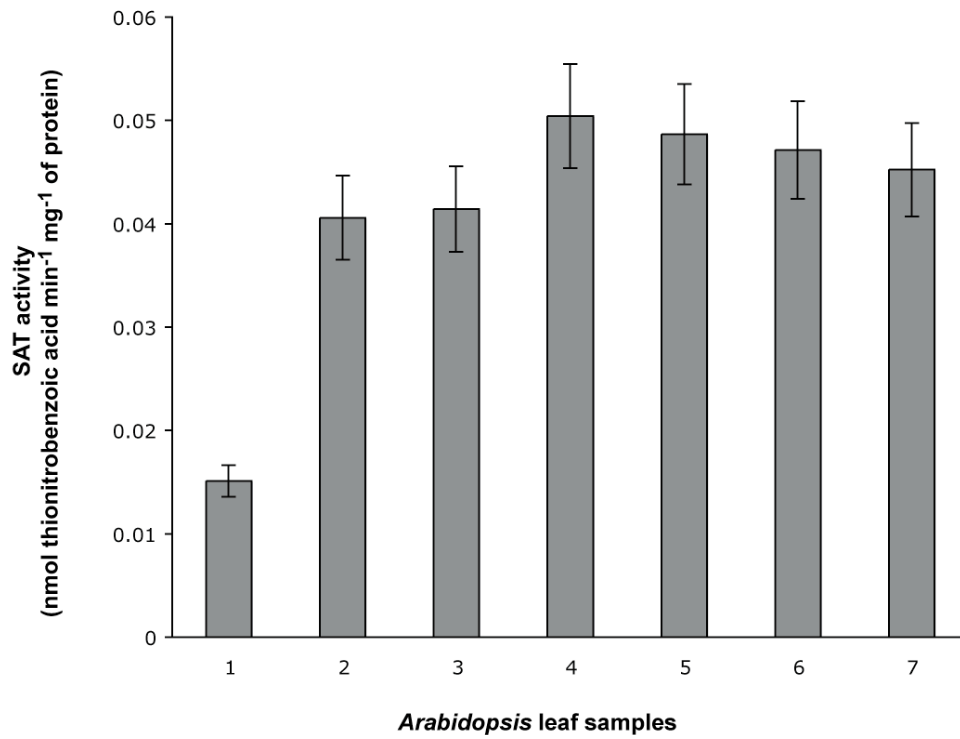


Figure 12: Serine acetyltransferase activity in *Arabidopsis*. Crude protein extracts from *Arabidopsis* wild type leaves (sample 1), and selected transgenic plants (samples 2 and 3 carrying the pZSAT1 plasmid; 4 and 5 carrying the pZSAT5 plasmid; 6 and 7 carrying the pZSAT6 plasmid) were used to determine SAT activity. The production of thionitrobenzoic acid was followed spectrophotometrically at 412 nm. Bars represent the standard error of the mean.

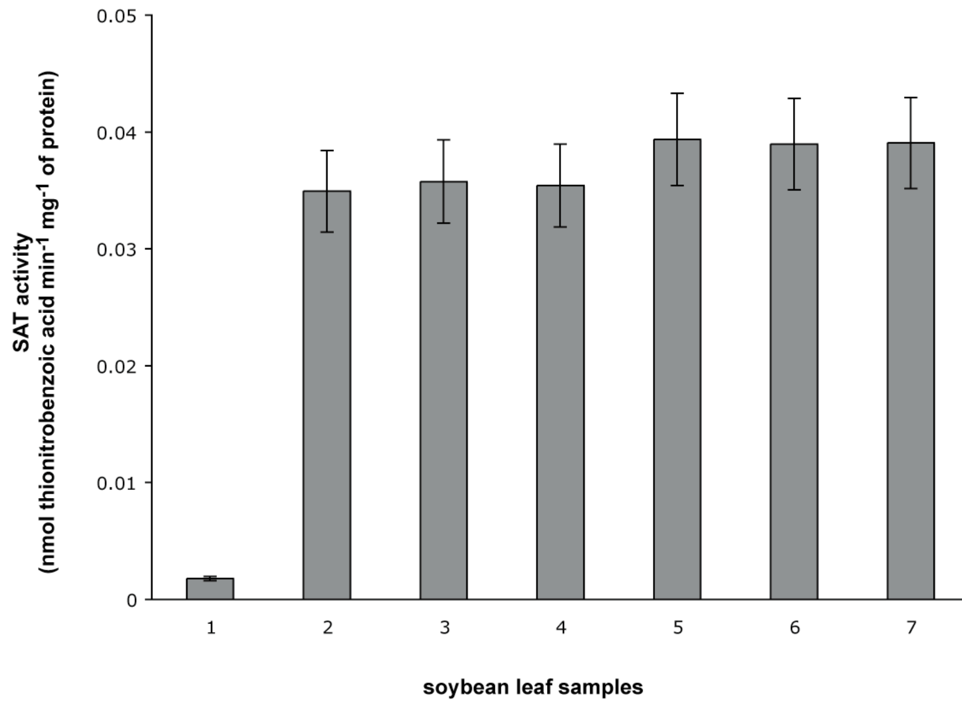


Figure 13: Serine acetyltransferase activity in soybean plants. Crude protein extracts from soybean wild type leaves (sample 1), and selected transgenic plants (samples 2 to 4 carrying the pZSAT1 plasmid; 5 to 7 carrying the pZSAT5 plasmid) were used to determine SAT activity. The production of thionitrobenzoic acid was followed spectrophotometrically at 412 nm. Bars represent the standard error of the mean.

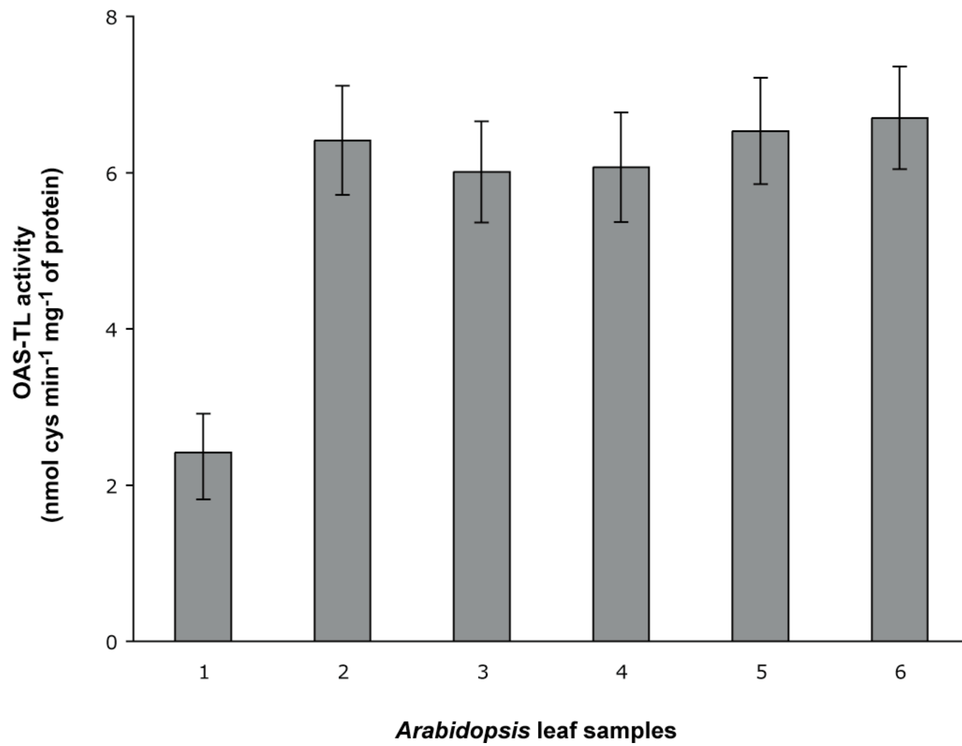


Figure 14: *O*-acetylserine (thiol) lyase activity in *Arabidopsis*. Crude protein extracts from *Arabidopsis* wild type leaves (sample 1), and selected transgenic plants (sample 2 to 7) were used to determine OAS-TL activity. Formation of cysteine was determined with an OAS-ninhydrin assay. Bars represent the standard error of the mean.

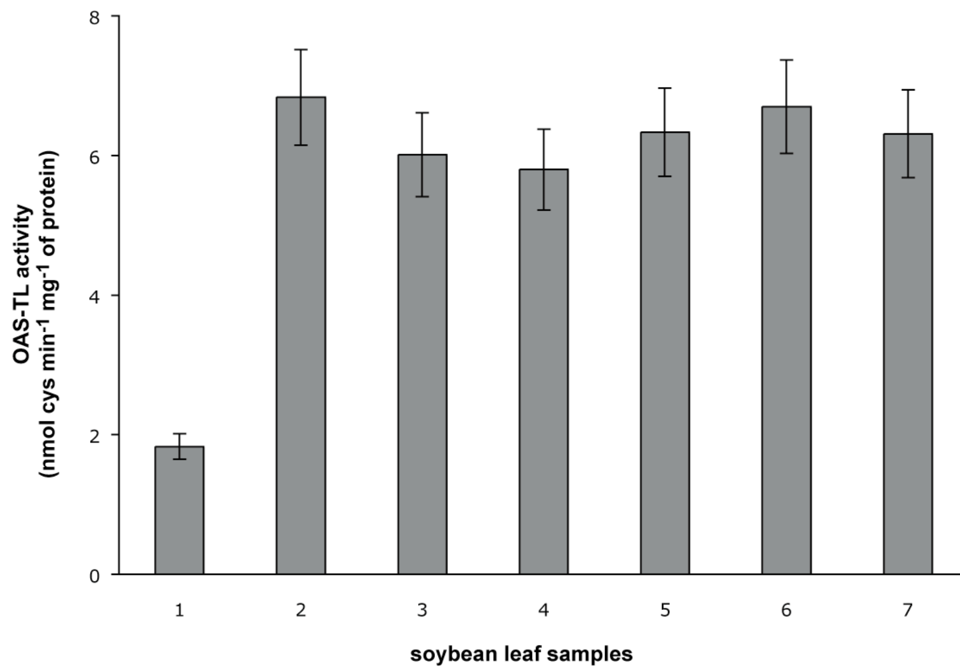


Figure 15: *O*-acetylserine (thiol) lyase activity in soybean. Crude protein extracts from soybean wild type leaves (sample 1), and selected transgenic plants (sample 2 to 9) were used to determine OAS-TL activity. Formation of cysteine was determined with an OAS-ninhydrin assay. Bars represent the standard error of the mean.

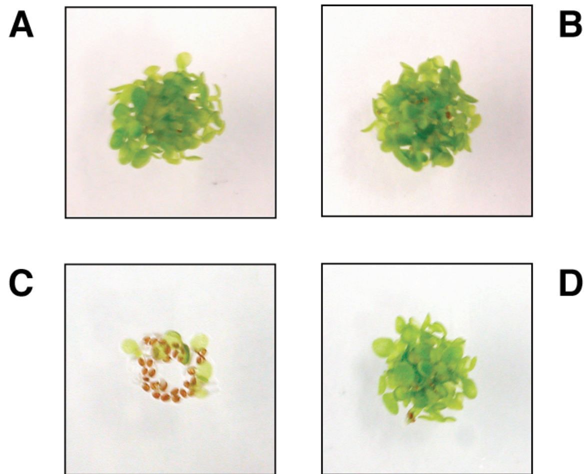


Figure 16: Cadmium chloride tolerance of *Arabidopsis* seedlings. **(A)** wild type *Arabidopsis* grown on MS medium without cadmium chloride; **(B)** transgenic *Arabidopsis* carrying the pZCS1 plasmid grown on MS medium without cadmium chloride; **(C)** wild type *Arabidopsis* grown on MS medium containing 250 μM $CdCl_2$; **(D)** transgenic *Arabidopsis* carrying the pZCS1 plasmid grown on MS medium containing 250 μM $CdCl_2$.

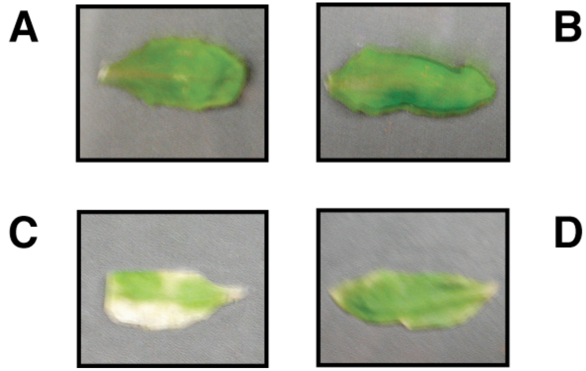


Figure 17: Effect of oxidative stress on *Arabidopsis* plants after 24 hr incubation with methyl viologen. **(A)** wild type *Arabidopsis* (control); **(B)** transgenic *Arabidopsis* carrying the pZCS1 plasmid (control); **(C)** wild type *Arabidopsis* incubated with 2 μM methyl viologen; **(D)** transgenic *Arabidopsis* carrying the pZCS1 plasmid incubated with 2 μM methyl viologen.

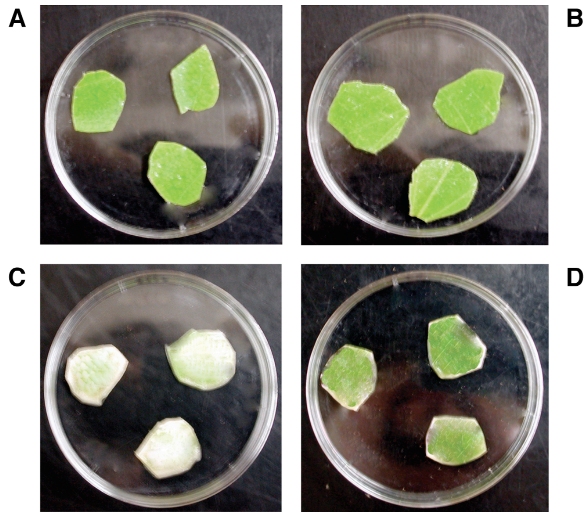


Figure 18: Effect of oxidative stress on soybean plants after 48 hr incubation with methyl viologen. **(A)** wild type soybean (control); **(B)** transgenic soybean carrying the pZCS1 plasmid (control); **(C)** wild type soybean incubated with 2 μM methyl viologen; **(D)** transgenic soybean carrying the pZCS1 plasmid incubated with 2 μM methyl viologen.

Table I: Kinetic parameters of native and recombinant SATs

	K_m		Inhibition (K_i) by L-cysteine	
	L-Serine	**Acetyl-CoA**	**L-Serine**	**Acetyl-CoA**
	mM		mM	
SAT	2.26	0.31	11.2 (noncompetitive)	7.6 (competitive)
SAT3†	2.45	0.95	-*	-
SAT4	2.92	0.60	-	-
SAT5	2.99	0.44	-	-
SAT6	1.89	0.41	-	-

* no inhibition by L-cysteine
† SAT3, Gly substitution; SAT4, His substitution; SAT5 Gly and His substitution; SAT6, complete elimination of the allosteric site

LITERATURE CITED

Barroso C., Vega J.M., Gotor C. (1995) A new member of the cytosolic *O*-acetylserine (thiol) lyase gene family in Arabidopsis thaliana. FEBS Lett. **363:** 1-5

Bogdanova N., Hell R. (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. **11:** 251-262

Brander K.A., Owttrim G.W., Brunold C. (1995) Isolation of a cDNA encoding a putative chloroplastic isoform of cysteine synthase from maize. Plant Physiol. **108:** 1748

Burnett W.N. (1981) Western blotting: electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiodinated protein A. Anal. Biochem. **112:** 195-203

Chronis D., Krishnan H.B. (2003) Sulfur assimilation in soybean: Molecular cloning and characterization of *O*-Acetylserine (thiol) lyase (cysteine synthase). Crop Sci. **43:** 1819-1827

Chronis D., Krishnan H.B. (2004) Sulfur assimilation in soybean (*Glycine max* [L.] Merr.): Molecular cloning and characterization of a cytosolic isoform of serine acetyltransferase. Planta **218:** 417-426

Clough S.J., Bent A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735-743

Dominguez-Solis J.R., Gutierrez-Alcala G., Romero L.C., Gotor C. (2001) The cytosolic *O*-Acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. J. Bio. Chem. **276(12):** 9297-9302

Droux M., Ruffet M.L., Douce R., Job D. (1998) Interactions between serine acetyltransferase and *O*-acetylserine (thiol) lyase in higher plants: structural and kinetic properties of the free and bound enzymes. Eur. J. Biochem. **255:** 235-245

Harms K., von Ballmoos P., Brunold C., Hofgen R., Hesse H. (2000) Expression of bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine glutathione. Plant J. **22(4):** 335-343

Hell R., Bork C., Bogdanova N., Frolov I., Hauschild R. (1994) Isolation and characterization of two cDNAs encoding for compartment specific isoforms of *O*-acetylserine (thiol) lyase from *Arabidopsis thaliana*. FEBS Lett. **351:** 257-262

Hell R., Schuster G., Gruissem W. (1993) An *O*-acetylserine (thiol) lyase cDNA from spinach. Plant Physiol. **102:** 1057-1058

Hesse H., T. Altmann T. (1995) Molecular cloning of a cysteine synthase cDNA from *Arabidopsis thaliana*. Plant Physiol. **108:** 851-852

Hinchee M.A.W., Connor-Ward D.V., Newell C.A., McDonnell R.E., Sato S.J., Gasser C.S., Fischhoff D.A., Re D.B., Fraley R.T., Horsch R.B. (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Bio/ Technology **6:** 915-922

Hoffmann A, Roeder RG (1990) Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acid Res. **19:** 6337-6338

Hopkins L., Parmar S., Blaszczyk A., Hesse H., Hoefgen R., Hawkesford M.J. (2005) *O*-acetylserine and the regulation of expression of genes encoding components for sulfate uptake and assimilation in potato. Plant Physiol. **138:** 433-440

Inoue K., Noji M., Katagiri T., Shinozaki K., Saito K. (2000) Subcellular localization and feedback inhibition of serine acetyltransferase. Sulfur nutrition and sulfur assimilation in higher plants, Bern, Switzerland, pp. 327-329.

Kim W-S., Krishnan H.B. (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 paranchyma cells. Plant Biotech Journal **2:** 199-210

Kredich N.M., Tompikns G.M. (1966) The enzymatic synthesis of L-cysteine in *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. **21:** 4955-4965

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685

Noji M., Inoue K., Kimura N., Gouda A., Saito K. (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J. Biol. Chem. **273:** 32739-32745

Noji M., Murakoshi I., Saito K. (1994) Molecular cloning of a cysteine synthase cDNA from *Citrullus vulgaris* (watermelon) by genetic complementation in an Escherichia coli Cys auxotroph. Mol. Gen. Genet. **244:** 57-66

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., Aoono M., Saji H., Saito K. (2001) Cysteine synthase overexpression in tobacco confers tolerance to sulfur-conatining environmental pollutants. Plant Physiol. **126:** 973-980

Nordlee J.A., Taylor S.L., Townsend J.A., Thomas L.A., Bush R.K. (1996) Identification of Brazil-nut allergen in transgenic soybeans. N. Engl. J. Med. **334:** 688-692

Riemenschneider A., Riedel K., Hoefgen R., Papenbrock J., Hesse H. (2005) Impact of reduced *O*-aceytlserine(thiol)lyase isoform contents on potato plant metabolism. Plant Physiol. **137:** 892-900

Rolland N., Droux M., Lebrun M., Douce R. (1993) *O*-Acetylserine (thiol) lyase from spinach (*Spinacia oleracea* L) leaf: cDNA cloning, characterization, and over-expression in *Escherichia coli* of the chloroplast isoform. Arch. Biochem. Biophys. **300:** 213-222

Romer S., d'Harlingue A., Camara B., Schantz R., Kuntz M. (1992) Cysteine synthase from *Capsicum annuum* chromoplasts. J. Biol. Chem. **267:** 17966-17970

Saito K., Miura H., Yamazaki M., Hirano H., Murakoshi I. (1992) Molecular cloning and bacterial expression of cDNA encoding plant cysteine synthase. Proc. Natl. Acad. Sci. USA **89:** 8078-8082

Saito K., Takahashi H., Noji M., Inoue K., Hatzfeld Y. (2000) Molecular regulation of sulfur assimilation and cysteine synthesis. Sulfur nutrition and sulfur assimilation in higher plants, Bern, Switzerland, pp 59-72

Saito K., Tatsuguchi K., Murakoshi I., Hirano H. (1993) cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacia oleracea*. FEBS Lett. **324:** 247-252

Saito K, Tatsuguchi K., Takagi Y., Murakoshi I. (1994) Isolation and characterization of cDNA that encodes a putative mitochondrion-localizing isoform of cysteine synthase [*O*-acetylserine (thiol)-lyase] from *Spinacia oleracea*. J. Biol. Chem. **269:** 28187-28192

Saito K., Yokoyama H., Noji M., Murakoshi I. (1995) Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. J. Biol. Chem. **270:** 16321-16326

Tabe L., Higgins T.J.V. (1998) Engineering plant protein composition for improved nutrition. Trends Plant Sci. **3:** 282-286

Takahashi H., Saito K. (1996) Subcellular localization of spinach cysteine synthase isoforms and regulation of their gene expression by nitrogen and sulfur. Plant Physiol. **112:** 273-280

Townsend J.A., Thomas L.A. (1994) Factors which influence the Agrobacterium-mediated transformation of soybean. J. Cell. Biochem. Suppl. **18A:** 78

Urano Y., Manabe T., Noji M., Saito K. (2000) Molecular cloning and functional characterization of cDNAs encoding cysteine synthase and serine acetyltransferase that may be responsible for high cellular cysteine content in *Allium tuberosum*. Gene **257:** 269-277

Warrilow A.G.S., Hawkesford M.J. (1998) Separation, subecellular location and influence of sulphur nutrition on isoforms of cysteine synthase in spinach. J. Exp. Bot. **49:** 1625-1636

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

Youssefian S., Nakamura M., Orudgev 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

Youssefian S., Nakamura M., Sano H. (1993) Tobacco plants transformed with the *O*-acetylserine (thiol) lyase gene of wheat are resistant to toxic levels of hydrogen sulphide gas. Plant J. **4:** 759-769

Zhang Z., Xing A., Staswick P., Clemente T.E. (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. Plant Cell Tissue Organ Cult. **56:** 37-46

VITA

Demosthenis (aka Demos) Chronis was born on a winter night in 1975. He grew up and went high school in Sparta, Greece. During his early years in high school he was fascinated by biology and mainly the cellular aspect of biology. Coming from an agricultural area, he wanted to explore the world of plants and combine his interest with molecular biology.

The opportunity came when he decided to leave Greece in 1995 and study abroad. He end up in Scotland and joined the Institute of Cell and Molecular Biology (ICMB) at the University of Edinburgh. After four years of study at ICMB he graduated with a BSc in Molecular Biology, with his thesis research focusing on the calcium-signaling pathway in *Nicotiana tabacum*.

In the fall of 1999, he joined the University of Missouri - Columbia and did an orientation for a year. During that period, he worked with Dr. Joe Polacco on the urea pathway. At the end of the orientation, he was sure that plant molecular biology was the career he wanted to pursue. Thus he joined the laboratory of Dr. Hari Krishnan and started his work with soybeans.