CHARACTERIZATION OF ENZYMES INVOLVED IN SOYBEAN CYSTEINE SYNTHESIS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

ACKNOWLEDGMENTS

I would like to thank my adviser, Alice Harmon for the opportunity to work in her lab and my committee members for their support. I would like to thank my lab mates especially Mariana Kirst for her guidance and friendship. I would also like to thank the Plant Molecular and Cell Biology Program and the United States Department of Agriculture for funding and all of my professors for a wonderful educational experience. Lastly, I would like to thank my husband, family and friends for their love and advice.

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LIST OF ABBREVIATIONS

CSC	Cysteine synthase complex
CDPK	Calcuim dependent protein kinase
GmOASTL	*Glycine max* OASTL forms
GmSerat	*Glycine max* SAT forms
OASTL	O-acetyl serine (thiol)lyase
OAS	O-acetyl serine
SAT	Serine acetyl transferase

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

CHARACTERIZATION OF ENZYMES INVOLVED IN SOYBEAN CYSTEINE SYNTHESIS

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August 2010

Chair: Alice Harmon
Major: Plant Molecular and Cell Biology

Sulfur metabolism is an essential component necessary for plant growth and production of high nutritional quality food for both human and animal consumption. The cysteine biosynthesis pathway lies directly at the junction between sulfur and nitrogen metabolism and is the location where inorganic sulfur is metabolized into an organic form. In a two step process, the two enzymes serine *O*-acetyltransferase (SAT, E.C. 2.3.1.30) and *O*-acetylserine (thiol)lyase (OASTL, E.C. 4.2.99.8) synthesize cysteine from serine. SAT transfers an acetyl group from acetyl-CoA to the hydroxyl group of serine to form *O*-acetylserine (OAS). The acetyl group of OAS is then replaced with sulfide by OASTL to form cysteine.

Forms of both SAT and OASTL can be found in three different compartments within the cell. Coordination of gene regulation as well as relative contribution of each enzyme form to the overall cysteine levels in the cell is poorly understood. A greater understanding of these processes within the plant could result in an increase in cysteine and other sulfur containing compounds. In staple crops such as soybean, this is of special interest as higher sulfur levels would greatly increase the nutritional quality. Evidence of a unique mechanism of phospho-regulation of a soybean SAT called

GmSerat2;1 by a calcium- dependent protein kinase (CDPK) has been shown. Investigation of the physiological role of phospho-regulation of GmSerat2;1 and its interactions with OASTL are of great interest in elucidating the regulation of cysteine biosynthesis.

Phylogenetic analysis in this study show that OASTL and SAT forms from the same compartments cluster loosely which suggests that gene duplications occurred before species divergence. Several studies have shown that OASTL activity is inhibited in the presence of SAT. This report supports those findings, however, inhibition is not seen to the same extent as in other studies. Additional data suggest that the GmOASTL4 form is subject to lability when incubated in cold conditions. This report also gives an expression analysis during early stages of plant development and compares that to the expression levels of all known soybean OASTL and SAT in specific plant tissues.

CHAPTER 1
INTRODUCTION

Sulfur Metabolism

Plant growth and nutritional quality are highly dependent on sulfur metabolism. The chemical reduction of sulfur in multicellular organisms is limited to the plant and fungi kingdoms making the metabolism of animals dependent on plants as their source for sulfur. The biosynthetic pathway incorporating sulfur into organic compounds ends with the formation of L-cysteine from L-serine. Serine acetyltransferase (SAT) transfers an acetyl group from acetyl-CoA to L-serine making *O*-acetylserine (OAS). The subsequent reaction is catalyzed by *O*-acetylserine (thiol) lyase (OASTL) which replaces the acetyl group from OAS with a sulfhydryl group from hydrogen sulfide to form cysteine and acetate which is cycled back to acetyl-CoA (Droux et al. 1992, Droux et al.2004, Ruffet et al. 1994). As a key component of sulfur metabolism, the synthesis of the amino acid cysteine is the location where inorganic sulfur is metabolized into an organic form.

Assimilation and uptake of sulfate is tightly regulated in plants. The formation of cysteine is one of two important control points in the incorporation of sulfur, the other being the reduction of sulfate to sulfite (Jez 2008). As cysteine is the entry point of organic sulfur containing compounds, it is the major starting material involved in the biosynthesis of sulfur containing amino acids, sulfolipids, oxidative stress compounds and coenzymes (Chronis et al. 2003). The cysteine supply remains a major limitation in the formation of these compounds (Noctor et al. 2002). OAS, the biosynthesis pathway intermediate, is thought to have a regulatory role as a possible regulator of transcription of biosynthetic genes and thus formation of cysteine. OAS has been shown to play a

role in the activation of bacterial genes in the cysteine biosynthesis pathway (Saito et al. 1994). It is suggested that OAS controls the transcript levels of the cysteine regulon by acting as an “internal inducer” (Blaszczyk et al. 2002). Of the genes in the cysteine regulon in bacteria, only the *cysE* encoding SAT is constitutively expressed while the other genes in the regulon are primarily expressed under conditions with reduced amounts of sulfur (Wirtz and Hell 2006). Further, in a microarray study, OAS was shown to de-repress sulfur uptake and assimilation genes in Arabidopsis (Hirai et al. 2003). Because of its location at the merging point of nitrogen and sulfur metabolism, OAS has been suggested as a candidate for coordination of the two pathways (Blaszczyk et al. 2002).

Cysteine synthesis in plants and bacteria is very similar. Because of this, it is possible to complement plant SAT and OASTL cDNAs into *E. coli* that are deficient in the enzymes necessary for cysteine production (Blaszczyk et al. 1999). The reverse is also true and shown in a study over-expressing bacterial SAT under cytosolic and chloroplastic promoters in tobacco in which limitations were indicated on the transport of OAS, cysteine and glutathione between compartments (Blaszczyk et al. 2002). A yeast two-hybrid screen using Arabidopsis cytosolic OASTL and organellar and cytosolic SAT found that binding of the complex components could not distinguish between compartment forms (Bogdanova et al. 1997). In *Spinacea oleracea* for example, the catalytic domains of the different forms of OASTL share 60 to 75% sequence similarity (Saito et al. 1994). However, based on amino acid composition, the spinach chloroplastic form is more similar to the *E. coli* OASTL than to the cytosolic or mitochondrial forms (Droux et al. 1992).

Cysteine Synthesis Enzyme Characteristics

As mentioned, cysteine is synthesized by the enzymes SAT and OASTL. Within the plant, each gene is encoded by a multi-gene family. Different numbers of each gene can be found within in different species. For example, *Arabidopsis thaliana* has at least five known SAT genes and nine known OASTL and OASTL-like genes. In contrast, *Glycine max* has three known SAT genes and seven known OASTL genes. In the context of this report, the functional proteins made from each of the individual genes are called forms. Thus, Arabidopsis has five SAT forms and soybean has three SAT forms. Forms of each enzyme can be found in the three protein producing compartments within the cell: cystosol, mitochondria and plastid. Each compartment that can produce proteins can have its own form of both SAT and OASTL.

Localization

In plants, the enzymes responsible for the reduction of sulfate to sulfite are found in the chloroplast suggesting that incorporation of sulfur into cysteine would occur in this compartment (Saito et al. 1994). However, in spinach, crude cellular extracts were found to have higher SAT and OASTL activities than chloroplasts alone indicating these enzymes were not solely located in the plastid (Droux et al. 1992). Most plants have several nuclear genes encoding different forms of SAT and OASTL (Wirtz and Hell 2006), which are distributed through different subcellular compartments. cDNA clones of several forms were isolated and characterized showing that both SAT and OASTL had localizations in the mitochondria, plastid, and cytosol (Ruffet et al. 1994, Saito et al. 1993).

In a study on the spinach chloroplast OASTL, predictably, the enzyme has a higher expression level in leaves than in roots. The cytosolic form, however, was

expressed constitutively in both leaves and roots. The identification of multiple compartments each with a different form raises questions about their function within the plant. It is suggested that the cytosolic form could be responsible for assimilation and detoxification of sulfide in the cells while the chloroplastic form could be coupled to photosynthesis and uses the compartment's sulfide directly after formation (Saito et al. 1993).

Each compartment involved in protein synthesis appears to require both SAT and OASTL. As yet, no transport of cysteine has been shown between compartments giving one rationalization for the redundancy of SAT and OASTL (Blaszczyk et al. 1999). It has been suggested that because of the reactivity of cysteine's thiol group, it is possible that endomembranes could be impermeable to cysteine transport. If this were the case, each compartment responsible for protein synthesis would require its own machinery to produce cysteine (Lunn et al. 1990). However, independent groups using Arabidopsis knockouts have shown that the plants can compensate for the loss of one or more genes (Riemenschneider et al. 2005, Heeg et al. 2008, Watanabe et al. 2008). In many cases, even multiple gene knockouts produce plants with no visible phenotype. These results suggest that cysteine can be transported between compartments to compensate for the loss of production in one location and by an increased activity in functioning locations.

Enzyme Activity

As each of the three subcellular compartments contains a different form, each compartment was found to have varying levels of enzyme activity. In pea, the mitochondria were found to have the highest SAT activity level at 76% of the total with the cytosol and chloroplast having only 14 and 10% respectively (Ruffet et al. 1995).

OASTL activity levels varied greatly, however, with the predominant activity in the chloroplast and cytosol at 42 and 44% of the total activity, respectively. The mitochondria accounted for only10% of the total SAT activity (Saito et al. 1993, Saito et al. 1994). Large differences in OASTL:SAT ratios estimated at one hundred to three hundred fold OASTL excess occur between compartments because of these varying activity levels. It has been suggested that a higher subcellular OASTL:SAT ratio is critical for maximum cysteine production (Heeg et al. 2008).

Initial isolation of the SAT enzyme was difficult due to its tight association with OASTL. Upon purification of spinach chloroplastic SAT, the enzyme was essentially inactive until incubated with OASTL (Ruffet et al. 1994). A study performed on Arabidopsis cytosolic SAT also showed minimal activity when isolated (Droux et al. 1998). However, a separate study using the mitochondrial form showed stable activity in the absence of OASTL (Berkowitz et al. 2002).

It is widely believed that a large excess of OASTL activity over SAT activity is required to obtain optimal cysteine synthesis *in vitro*. On average, OASTL activity levels were found to be between 100 and 300 times higher than SAT levels *in vitro*. Ratios were found to be similar to those *in vivo* suggesting that the subcellular levels of the enzymes are optimized for cysteine output (Droux et al. 1992, Ruffet et al. 1994). In *E. coli* only 5% of the OASTL activity is associated with SAT in a complex. OASTL $k_{m\ OAS}$ was found to increase 4-fold when isolated from SAT (Ruffet et al. 1994), however, SAT activity is seen almost solely in the complex (Droux et al. 1998). In spinach, similar to *E. coli*, between 3 and 5% of the OASTL activity was found in association with SAT (Droux et al. 1992).

Expression of Enzymes

Five different forms of SAT, each encoded on one of the five Arabidopsis chromosomes, have been found. Interestingly, of the five Arabidopsis SATs only three are expressed at high levels transcriptionally and each is localized to one of the subcellular compartments involved in cysteine synthesis. The other two are expressed in the cytosol at much lower levels (Kawashima et al. 2005) and will be discussed later on. Arabidopsis has at least ten OASTL-like genes with only four of them being highly expressed. Again, each subcellular compartment has a prominent form of one of the OASTL-like genes, (Wirtz and Hell 2006). In soybean, two of the four known SATs have been studied along with one of the four known OASTLs. Chronis and Krishnan (2004) have identified a cytosolic soybean SAT that is sensitive to feedback inhibition by cysteine (Chronis et al. 2003). However, chloroplastic/cytosolic *Glycine max* SAT 2;1 (GmSerat2;1) contains a serine residue in its C-terminal end that upon phosphorylation blocks feedback inhibition of the enzyme (Liu et al. 2006). This phospho-regulation of inhibition is not universal among species thus making GmSerat2;1 a primary candidate for increasing the cellular thiol content.

Expression patterns of the two enzymes has shown to differ among developmental stage, light conditions and under certain stresses. A watermelon SAT gene transcript level was found to be differentially expressed in etiolated versus green seedlings with a preferential accumulation in etiolated seedlings suggesting an extra-chloroplastic form (Saito et al. 1995). Arabidopsis SAT-A mRNA levels showed a two-fold increase in expression in response to light and sulfate (Bogdanova et al. 1997). However, in the spinach chloroplastic SAT showed little to no difference in mRNA levels of green and etiolated seedlings (Noji et al. 2001). One soybean OASTL gene, is

abundantly expressed in the beginning stages but declines during the later stages of seed development (Chronis et al. 2003).

Of the five Arabidopsis SAT genes, only three are constitutively expressed at detectable levels. The other two, Serat3;1 and Serat3;2, mRNA levels showed distinct expression patterns. The expression levels were highest in later developmental stages. The five genes were also examined under sulfur deficient conditions and under cadmium stress. In both Serat3;1 and Serat3;2, expression levels increased under sulfur deficiency with no effect on the other three genes. Under cadmium stress, all genes were up regulated except Serat3;1 (Kawashima et al. 2005). Based on the data it is easy to infer that other species are likely to have multiple genes that are expressed at very low levels until certain environmental or developmental conditions are met.

Structure of the Cysteine Synthase Complex

Together, SAT and OASTL make up what is called the cysteine synthase complex (CSC). The stoichiometry of the components of the complex has long been debated. Crystal structures for both SAT and OASTL have been elucidated from *Escherichia coli* and *Haemophilus influenzae* respectively. Structures of Arabidopsis forms were also determined and were shown to have similar to the bacterial models. However, to date no crystal structure of both forms together has been produced.

Formerly the bacterial crystal structure data suggested a model for the CSC consisting of two SAT homotrimers of making a “dimer of trimers” (Francois et al. 2006). Using size exclustion chromatography, a new model for the plant cysteine synthase complex (CSC) has been proposed which shows the plant SAT is trimeric with three OASTL homodimer binding to each SAT C-terminal (Kumaran et al. 2009). Crystal

structure data has shown that the ten peptides at the C-terminus of SAT bind into the active site of OASTL (Francois et al. 2006).

Surface plasmon resonance data has shown that negative cooperativity occurs with each OASTL binding event and that formation of the complex “enhances” SAT activity and aids in releasing SAT from feedback and substrate inhibition (Kumaran 2009). The rapid binding of SAT and OASTL is followed by a slow conformational change probably resulting from the closure of OASTL’s active site now bound with SAT’s C-terminal (Salsi 2010). Despite the formation of a complex, kinetic evidence shows that there is no channeling of OAS from SAT to OASTL as the synthesized OAS is released into the bulk solution (Ruffet et al. 1994).

Stability of the Complex

Stability of the CSC is dependent on the subcellular levels of key metabolites in the pathway. Addition of sulfide has been found to stabilize the complex but also lower the activity of free OASTL (Wirtz and Hell 2006) while incubation with OAS is shown to dissociate the complex to free the OASTL and increase activity (Droux et al. 1998). When the sulfide concentration is high, OAS is readily produced and used by the bound and free OASTL until the supply is gone. After the available sulfur is used, a buildup of OAS is seen which triggers the expression of sulfur assimilation genes. The buildup of OAS also causes the complex to dissociate which allows for higher OASTL activity resulting in a lowering of the net concentration of OAS.

Oscillations of the relative sulfur and OAS concentrations in the various compartments cause continuous association and dissociation of the cysteine synthase complex. High cysteine concentrations also appear to have an effect on complex dissociation and often lower SAT activity through feedback inhibition (Droux et al. 2003).

Slight changes in the concentrations of sulfide and OAS *in vivo* are predicted to have significant effects on the equilibrium state of the complex (Berkowitz et al. 2002). It has also been suggested that stability of the complex depends on the rapid association of the complex and the slow dissociation. When examining the rate constants for association, data indicated that the CSC formed a stable macromolecule (Kumaran 2009).

Feedback Inhibition

Several forms of SAT have been found to be feedback inhibited by cysteine. In several studies, the cysteine synthase complex is inhibited to varying degrees by the addition of cysteine as a regulatory mechanism to ensure a steady state level of its end product. The inhibition appears to be specific to L-cysteine. The addition of other products in the pathway or forms of cysteine showed no effect on the enzyme activity levels. The watermelon cytosolic SAT form was inhibited by cysteine in a non-competitive maner at a low concentration with an IC_{50} = 2.9 μM (Droux et al. 2003, Saito et al. 1995).

In Arabidopsis, the forms of SAT show differential cysteine inhibition. The chloroplastic and mitochondrial forms appear to be insensitive to cysteine while the cytosolic form is sensitive (IC_{50} = 1.8 μM) to feedback inhibition (Berkowitz et al. 2002, Noji et al. 1998). A similar study on pea showed a strong inhibition on the chloroplastic form (IC_{50} = 12 μM) while the cytosolic form appeared insensitive. The mitochondrial form showed partial inhibition (IC_{50} = 205 μM). The chloroplastic form of spinach also showed cysteine sensitivity (IC_{50} = 7.6 μM) while the cytosolic form of *Calluna vulgaris* and *Allium tuberosum* were sensitive (Droux et al. 2003). Inhibition of SAT by cysteine can be grouped in the three classes: insensitive, mildly sensitive and, severely

sensitive. It must be noted that the sensitivity to feedback inhibition varies between species and is less dependent on the localization form.

Several studies on using mutagenesis have identified many SAT mutants that are insensitive to cysteine feedback inhibition. Implications suggest that the SAT C-terminus is responsible for conferring feedback sensitivity (Olsen et al. 2004). Mutation of a methionine in the C-terminal end of a watermelon SAT form eliminates cysteine feedback inhibition. Similarly, it is the C-terminal of SAT that binds to OASTL in the CSC. Recent data suggests that formation of the complex, also alleviates feedback inhibition by cysteine (Kumaran 2009).

Similarly, when the soybean GmSerat 2;1 is phosphorylated at its C-terminal serine 378 residue by a calcium dependent protein kinase (CDPK) its feedback inhibition is also eliminated (Francois et al. 2006). Replacing the phosphorylatable serine residue with animo acid residues with a negative charge that mimic phosphorylation also shows a decreased sensitivity to inhibition. Conversely, in the presence of CDPK, when the serine residue was replaced by alanine, a small hydrophobic amino acid, the enzyme has a low IC_{50} at 18 μM because the enzyme was not able to be phosphorylated (Liu et al. 2006). Cysteine is thought to bind to the active site of SAT inhibiting its association with serine and changing the conformation to limit its affinity for acetyl-CoA (Olsen et al. 2004). In addition it is suggested that the phosphorylation of GmSerat2;1 at the serine residue changes the conformation of the complex in such a way as to make it insensitive to competitive binding by cysteine (Liu et al. 2006).

Enzyme Activity Regulation

To date, the activity of the two enzymes involved in cysteine synthesis have been shown to be regulated in three main ways (Sirko et al. 2004). First, as previously mentioned, some SAT forms have feedback sensitivity to certain threshold concentrations of their end product cysteine (Droux et al. 2003, Saito et al. 1995, Berkowitz et al. 2002, Noji et al. 1998). Also, at least one SAT form is known to be sensitive to feedback inhibition based on its phospho-regulation (Liu et al. 2006). Second, sulfate transport is up regulated by a decrease in available sulfide and an increase in OAS. These intermediates have been suggested to be signaling molecules involved in the activation of genes involved in the cysteine synthesis pathway (Saito et al. 1994, Blaszczyk et al. 2002, Hirai et al. 2003).

As previously mentioned, the concentrations of OAS, sulfide and cysteine all contribute to fine balance of complex stability which is tied closely to the third regulation mechanism of complex association and dissociation. Again, some data suggest that SAT is more active in the complex while OASTL is more active independent of the complex. The regulation between enzymes in and out of the complex yields maximum activity and cysteine yield (Hell et al. 2003, Sirko et al. 2004). Understanding the regulation of the enzymes involved in cysteine synthesis, will one day allow for engineering and modification of the pathway to yield higher cysteine and sulfur levels.

CHAPTER 2
MATERIALS AND METHODS

O-Acetylserine(thilol)lyase Recombinant Enzyme

Cloning

Gene specific primers were designed incorporating *NdeI* and *SalI* restriction sites in the 5' and 3' primers respectively and were used to amplify the plastidic GmOASTL4 and cytosolic GmOASTL0 (Table 2-1) from seed soybean cDNAs. The PCR products were then inserted into the pGEM-T easy vector and transformed in the DH5α *E. coli* strain for selection. The plasmids were then digested with the appropriate restriction enzyme and the OASTL sequences were introduced into pET28a+ vectors and again transformed into the DH5α strain. After screening of colonies for correct sequence, the plasmids were isolated and incorporated into the BL21 stain for expression. Accession numbers of cloned genes are GmOASTL0 – AF452451 and GmOASTL4 – EF584900 (Table 2-2). Constructs contain His-tags at their N-terminus which were used for column purification (Figure 1-1 and 1-2).

DNA Sequences

The polymerase chain reaction(PCR) amplified DNA products of GmOASTL0 and GmOASTL4 were prepared as described above with primers listed in Table 2-2 and sequenced at the University of Florida ICBR using the Applied Biosystems Model 3130 Genetic analyzer. DNA Star software was used to analyze the cDNA sequences from Genbank for the arabidopsis and soybean SAT and OASTL genes. ClustalW was used to align and compare the sequences to create a phylogenetic tree. GenBank accession numbers for the genes are listed in Table 2-3.

Expression of OASTL Enzymes

To obtain recombinant proteins, cultures were grown in LB broth with kanamycin. Once an A_{600} of approximately 0.5 was reached, induction was carried out for 3-4 hours at 22ºC. Final concentrations of 1 mM IPTG, 0.02% arabinose, 15 µM thiamine and 10 µM pyridoxine were added to the culture flasks.

Purification of OASTL Enzymes

Cell lysis was carried out by resuspending bacterial pellets in extraction buffer containing 50 mM Tris-HCl pH 8, 25 mM imidazole, 500 mM NaCl, 10% glycerol and 1% Tween-20. Samples were then sonicated three times for 30 seconds. Following sedimentation of cellular debris, recombinant proteins were purified by affinity chromatography in nickel affinity gel. Following binding to gel, proteins were washed with 10 mM OAS to break any interaction between native *E coli* proteins and the recombinant protein of interest. The column was then washed with a second buffer containing 50 mM Tris-HCl pH 8, 25 mM imidazole, 500 mM NaCl, and 10% glycerol. Proteins were then eluted from the column using an elution buffer containing 50 mM Tris-HCl pH 8, 250 mM imidazole, 500 mM NaCl, and 10% glycerol. Protein amounts were quantified using Bradford assay and BSA as standard.

OASTL Activity Assays

OASTL activity was determined by measuring the formation of cysteine using the ninhydrin assay (Gaitonde 1967). The standard reaction mixture contained 50 mM Tris-Hydrochloride pH 7.5, 5 mM DTT, 10 mM O-acetylserine (OAS), 1 mM sodium sulfide and 0.2 µg of enzyme mixture in a final volume of 100 µL. The reaction was initiated by addition of the enzyme to the reaction mixture. After 5 minutes at 25ºC, the reaction was

stopped with 50 µL 20% TCA. 200 µL of ninhydrin solution (containing 250 mg ninydrin in 6 mL glacial acetic acid 4 mL hydrochloric acid) and 100 µL glacial acetic acid was added to each reaction tube and mixture was then boiled for 10 minutes. Tubes were cooled on ice and 600 µL of 95% ethanol was added for a final volume of 1ml to stabilize the cysteine and protect against degradation. Absorption was measured at 560 nm. For determining Serine acetyltransferase effect of OASTL 6 µg of SAT enzyme mixture was used in each assay. SAT and OASTL were incubated for 30 minutes at room temperature prior to being added to reaction mixture. Cysteine quantification was extrapolated by comparison to a cysteine standard curve. Cysteine concentrations in the standard curve were zero to 30 µM. Fisher's Cysteine-HCl in a sodium phosphate buffer was used to create curve.

Sulfide and OAS Incubation Assays

OASTL enzyme was incubated in the presence of SAT and varied concentrations of either substrate for one hour. The assay was initiated by the addition of the remaining substrate as well as more of the tested substrate to bring all samples up to equal concentrations of each substrate. The assay was stopped after 15 minutes by the addition of 50 µL 20% TCA. The ninhydrin solution and acetic acid were added as described above and the sample was boiled for 10 minutes. After the sample was cooled, 100 µL was added to 900 µL of 95% ethanol. The OD560 was then obtained.

OASTL Localization

Full length cDNA clones of GmOASTL0 and GmOASTL4 were inserted into the pGEM-EZ plasmid. Plasmid samples were sent to Jung Youn Lee, a collaborator at the University of Delaware. Transient expression of GFP-fusion proteins were made with full length sequences in tobacco leaf cells.

Plant Genotyping

Six different T0 soybean transgenic lines (5159, 5160, 5186, 5187, 5193, and 5194) were produced by collaborator Byung-Chun Yoo, DuPont Crop Genetics, Wilmington, Delaware. Red fluorescent protein 540 T0 seeds, 90 seeds from each line) were germinated in the growth chamber and then grown to seed in a greenhouse to screen for homozygosity. Leaf material from the 5193 line of these plants was harvested and analyzed for the expression of the transgene using western blotsT1 seeds of positive plants from Western blots were then screened under UV light to detect plants that contained all seeds fluorescing indicating homozygous lines. Homozygous lines were then used for plant genotype analysis. The other five lines were assigned to lab-mates and assistance was given in analyzing them using semi-quantitative PCR and western blots.

Expression Analysis

Post-Germination Expression Analysis

Post-Germination expression analysis was performed using vector only control seedlings as the plant material. Plant tissue was collected at nine time points: new shoot, unopened cotyledon, opened cotyledon, plumule, new leaf, first trifoliate, second trifoliate, stem of plant with open cotyledon and root of plant with open cotyledon. RNA was extracted from 2 replicates of each sample using the Bio-Rad Total RNA extraction kit. Complementary DNA was then synthesized using the Invitrogen Superscript III Reverse Transcriptase enzyme kit. Semi-quantitative PCR analysis was performed on each sample using the primer sets from Table 2-3. Each primer set was tested for optimization at 22 to 34 cycles and optimal cycles numbers are listed in Table 2-3.

Cadmium Stress

Four transgenic lines were grown (5159, 5160, 5187 and 5193) for stress analysis (Table 2-4). Eighteen seedlings of each line were grown in two trays of vermiculite, nine seedlings for each treatment. Seedlings were imbibed for 1 hour before planting and then given MS media every three days. At three weeks old, control plants were given 20 ml of MS media per plant while stress treatment plants were given 20 ml of MS media with 25 µM $CdCl_2$ as used by Kawashima et al 2005. Plant tissue was collected at 0, 24, and 72 hours. Replicate experiment was performed with a three day lag. RNA was extracted from tissue using the BioRad Total RNA extraction kit. Analysis using real-time PCR was done by Heidi Wang.

Nucleotide Sequence of GmOASTL0 Clone:

GAAGGAGATATACCATGGGCAGCAGC***CATCATCATCATCATCAT***AGCAGCGGCCT
GGTGCCGCGCGGCAGCCAT**ATG**ATGGCTGTTGAAAGGTCCGGAATTGCCAAAGAT
GTTACGGAATTGATTGGTAAAACCCCATTAGTATATCTAAATAAACTTGCGGATGGT
TGTGTTGCCCGGGTTGCTGCTAAACTGGAGTTGATGGAGCCATGCTCTAGTGTGA
AGGACAGGATTGGGTATAGTATGATTGCTGATGCAGAAGAGAAGGGACTTATCACA
CCTGGAAAGAGTGTCCTCATTGAGCCAACAAGTGGTAATACTGGCATTGGATTAGC
CTTCATGGCAGCAGCCAGGGGTTACAAGCTCATAATTACAATGCCTGCTTCTATGA
GTCTTGAGAGAAGAATCATTCTATTAGCTTTTGGAGCTGAGTTGGTTCTGACAGATC
CTGCTAAGGGAATGAAAGGTGCTGTTCAGAAGGCTGAAGAGATATTGGCTAAGAC
GCCCAATGCCTACATACTTCAACAATTTGAAAACCCTGCCAATCCCAAGGTTCATTA
TGAAACCACTGGTCCAGAGATATGGAAAGGCTCCGATGGGAAAATTGATGCATTTG
TTTCTGGGATAGGCACTGGTGGTACAATAACAGGTGCTGGAAAATATCTTAAAGAG
CAGAATCCGAATATAAAGCTGATTGGTGTGGAACCAGTTGAAAGTCCAGTGCTCTC
AGGAGGAAAGCCTGGTCCACACAAGATTCAAGGGATTGGTGCTGGTTTTATCCCT
GGTGTCTTGGAAGTCAATCTTCTTGATGAAGTTATTCAAATATCAAGTGATGAAGCA
ATAGAAACTGCAAAGCTTCTTGCGCTTAAAGAAGGCCTATTTGTGGGAATATCTTC
CGGAGCTGCAGCTGCTGCTGCTTTTCAGATTGCAAAAAGACCAGAAAATGCCGGG
AAGCTTATTGTTGCCGTTTTTCCCAGCTTCGGGGAGAGGTACCTGTCCTCCGTGCT
ATTTGAGTCAGTGAGACGCGAAGCTGAAAGCATGACTTTTGAGCCCTGAGTCGAC

Amino Acid Sequence of GmOASTL0 Clone:

RRYTMGSSHHHHHHSSGLVPRGSHMMAVERSGIAKDVTELIGKTPLVYLNKLADGCV
ARVAAKLELMEPCSSVKDRIGYSMIADAEEKGLITPGKSVLIEPTSGNTGIGLAFMAAAR
GYKLIITMPASMSLERRIILLAFGAELVLTDPAKGMKGAVQKAEEILAKTPNAYILQQFEN
PANPKVHYETTGPEIWKGSDGKIDAFVSGIGTGGTITGAGKYLKEQNPNIKLIGVEPVES
PVLSGGKPGPHKIQGIGAGFIPGVLEVNLLDEVIQISSDEAIETAKLLALKEGLFVGISSG
AAAAAAFQIAKRPENAGKLIVAVFPSFGERYLSSVLFESVRREAESMTFEP

Figure 1-1. Nucleotide and amino acid sequence of GmOASTL1. Cloned gene expressed in the pet28α+ plasmid and expressed amino acid sequence shown. Sequencing performed at the University of Florida ICBR using the Applied Biosystems Model 3130 Genetic analyzer. DNA Star software used to predict the amino acid sequence from the nucleotide sequence. Bold and itlaic font indicate site of N-terminal His-tag. Bold and underlined font indicates start codon. Bases in red indicate sites different from published clone; this clone and published clone have 99% sequence identity.

Nucleotide Sequence of GmOASTL4 Clone:

GAAGGAGATATACCATGGGCAGCAGC***CATCATCATCATCATCATC***AGCAGCGGCC
TGGTGCCGCGCGGCAGCCAT**ATG**GTTTACCTGAACAATATCGTGAAGGGTTCTGT
GGCCAATATTGCTGCCAAGCTTGAGATTATGGAGCCCTGTTGTAGTGTAAAGGACA
GGATTGGTTTTAGTATGATAAATGATGCTGAGCAGAGAGGAGCTATAACACCTGGG
AAGAGTATATTGGTGGAACCTACTAGCGGCAACACCGGAATTGGTCTTGCCTTTAT
AGCAGCTTCAAGAGGTTATAAACTCATTTTGACAATGCCTGCTTCAATGAGTTTAGA
AAGACGAGTTCTCTTGAAAGCATTTGGGGCCGAGCTTGTTTTGACTGATGCAGCAA
AGGGCATGAATGGAGCAGTACAAAAAGCTGAAGAAATTTTAAAAAGTACCCCCAAT
TCATACATGCTTCAACAATTTGATAATCCTTCAGATCCTAAGGTTCATTATGAGACA
ACTGGCCCGGAGATATGGGAAGATACTAAAGGAAAGATAGATATTTTAGTTGCAGG
AATTGGAACCGGTGGAACTGTTTCTGGAGCTGGCCAATTCTTAAAACAACAAAATC
GGAAAATACAGGTTATTGGCGTAGAGCCTCTGGAAAGCAACATACTTACAGGTGGA
AAGCCAGGACCTCACAAAATTCAGGGTATTGGGGCTGGTTTTGTGCCCAGGAATTT
GGATCAAGATGTGCTTGATGAAGTTATAGCGATATCAAGTGATGAAGCAGTTGAAA
CTGCAAAGCAATTAGCACTCCAGGAAGGCTTGCTGGTGGGAATCTCTTCAGGAGC
TGCAGCTGCAGCTGCTTTGAAGGTTGGAAAGAGGCCTGAGAATGCAGGAAAGCTT
ATTGGGGTTGTCTTCCCAAGCTTTGGTGAAAGATATTTGTCAACTATTCTTTTCCAG
TCAATACGGGAAGAGTGCGAGAAAATGCAACCTGAGCCATGA

Amino Acid Sequence of GmOASTL4 Clone:

RRYTMGSSHHHHHHSSGLVPRGSHMVYLNNIVKGSVANIAAKLEIMEPCCSVKDRIGF
SMINDAEQRGAITPGKSILVEPTSGNTGIGLAFIAASRGYKLILTMPASMSLERRVLLKAF
GAELVLTDAAKGMNGAVQKAEEILKSTPNSYMLQQFDNPSDPKVHYETTGPEIWEDTK
GKIDILVAGIGTGGTVSGAGQFLKQQNRKIQVIGVEPLESNILTGGKPGPHKIQGIGAGF
VPRNLDQDVLDEVIAISSDEAVETAKQLALQEGLLVGISSGAAAAAALKVGKRPENAGK
LIGVVFPSFGERYLSTILFQSIREECEKMQPEP

Figure 1-2. Nucleotide and amino acid sequence of GmOASTL4. Cloned gene expressed in the pet28α+ plasmid without transit peptide. Expressed amino acid sequence. Sequencing performed at the University of Florida ICBR using the Applied Biosystems Model 3130 Genetic analyzer. DNA Star software used to predict the amino acid sequence from the nucleotide sequence. Bold and Italic font indicate site of N-terminal His-tag. Bold and underlined font indicates start codon. Bases and amino acids in red indicate sites differing from published clone; this clone and published sequence have 98% sequence identity.

Full Length Nucleotide Sequence of GmOASTL4:

TTGTCCCTTTACCTTCCAAATACACTTCTTCTATTGCGACCCATTTTTCCAATCCACA
ATCGTGGGAGTGAGCGAACAACTACAAAGGGATATTAGATACTATTGTGAGTGAGT
GCGTGCGATTCATTGTGTTTGTGTAGTAGTGACTTTGGGTTGGGTTTCGTCTCAAC
TCAACAAACGTCGTCGTTCTGCATTTCCCAACCTTCCTCCAATGGCTTCTGCTTCCT
TAATCAACTCGTTGACTTGTTCTTCGCGTGCTCCACTCAGCACTGTTCCACTTTCAC
CCGCACCACCGCCGCCACGTCACTGAGACAATTTAACAGCCACTGTCGGAGACCA
CTCGCGACGAGGATCTCGCCGCCTTCCACCGTTGTCTGCAAAGCTGTCTCCGTTA
AGCCCCAAACCGAAATCGAAGGTCTCAACATTGCTGAAGATGTCACCCAGCTCATA
GGGAAAACCCAATGGTTTACCTGAACAATATCGTGAAGGGTTCTGTGGCCAATATT
GCTGCCAAGCTTGAGATTATGGAGCCCTGTTGTAGTGTAAAGGACAGGATTGGTTT
TAGTATGATAAATGATGCTGAGCAGAGAGGAGCTATAACACCTGGGAAGAGTATAT
TGGTGGAACCTACTAGCGGCAACACCGGAATTGGTCTTGCCTTTATAGCAGCTTCA
AGAGGTTATAAACTCATTTTGACAATGCCTGCTTCAATGAGTTTAGAAAGACGAGTT
CTCTTGAAAGCATTTGGGGCCGAGCTTGTTTTGACTGATGCAGCAAAGGGCATGAA
TGGAGCAGTACAAAAAGCTGAAGAAATTTTAAAAAGTACCCCCAATTCATACATGCT
TCAACAATTTGATAATCCTTCAGATCCTAAGGTTCATTATGAGACAACTGGCCCGGA
GATATGGGAAGATACTAAAGGAAAGATAGATATTTTAGTTGCAGGAATTGGAACCG
GTGGAACTGTTTCTGGAGCTGGCCAATTCTTAAAACAACAAAATCGGAAAATACAG
GTTATTGGCGTAGAGCCTCTGGAAAGCAACATACTTACAGGTGGAAAGCCAGGAC
CTCACAAAATTCAGGGTATTGGGGCTGGTTTTGTGCCCAGGAATTTGGATCAAGAT
GTGCTTGATGAAGTTATAGCGATATCAAGTGATGAAGCAGTTGAAACTGCAAAGCA
ATTAGCACTCCAGGAAGGCTTGCTGGTGGGAATCTCTTCAGGAGCTGCAGCTGCA
GCTGCTTTGAAGGTTGGAAAGAGGCCTGAGAATGCAGGAAAGCTTATTGGGGTTG
TCTTCCCAAGCTTTGGTGAAAGATATTTGTCAACTATTCTTTTCCAGTCAATACGGG
AAGAGTGCGAGAAAATGCAACCTGAGCCATGAATTGACTTTGCTTGTTAATGTTTC
ATACTCATTTTCCACAAGTATCTGTATGGAATGACAGTGTCAGATGTCCATGTTATG
CCCGTGTTTCTCATTTTTGACACTCAAGAAATTAGATAACTGTGTACAGGTCATTGA
TTTGACTTACAACACAGATTAATGACCCTTGATTGAAGAAATTTTAGATGTAATAATT
ATTGGCATTGATGACAACGTAGCAAATTTCTGCCTAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAA

Amino Acid Sequence GmOASTL4:

MASASLINSLTCSSRAPTQHCSTFTRTTAATSLRQFNSHCRRPLATRISPPSTVVCKAV
SVKPQTEIEGLNIAEDVTQLIGKTPMVYLNNIVKGSVANIAAKLEIMEPCCSVKDRIGFSM
INDAEQRGAITPGKSILVEPTSGNTGIGLAFIAASRGYKLILTMPASMSLERRVLLKAFGA
ELVLTDAAKGMNGAVQKAEEILKSTPNSYMLQQFDNPSDPKVHYETTGPEIWEDTKGK
IDILVAGIGTGGTVSGAGQFLKQQNRKIQVIGVEPLESNILTGGKPGPHKIQGIGAGFVP
RNLDQDVLDEVIAISSDEAVETAKQLALQEGLLVGISSGAAAAAALKVGKRPENAGKLIG
VVFPSFGERYLSTILFQSIREECEKMQPEP

Figure 1-3. Full length nucleotide and amino acid sequence of GmOASTL4. Full length coding region including transit peptide which GmOASTL4 clone was derived. Accession number EF584900. Underlined amino acid is start of mature peptide.

Table 2-1. List of genes names

Gene Name	Species	Localization	Reference
AtOASTL1;1	Arabidopsis	Cytosol	Hell et al. 1994
AtOASTL1;2	Arabidopsis	Cytosol	Jost et al. 2000
AtOASTL2;1	Arabidopsis	Plastid	Jost et al. 2000
AtOASTL2;2	Arabidopsis	Mitochondria	Hesse et al. 1999
AtOASTL3;1	Arabidopsis	Mitochondria/CAS-like	Hatzfeld et al. 2000
AtOASTL4;1	Arabidopsis	Cytosol	Hatzfeld et al. 2000
AtOASTL4;2	Arabidopsis	Cytosol	Hatzfeld et al. 2000
AtOASTL4;3	Arabidopsis	Cytosol	Watanabe et al. 2008
AtOASTL5;1	Arabidopsis	Plastid	Nakamura et al. 1997
AtSerat1;1	Arabidopsis	Cytosol	Noji et al. 1998
AtSerat2;1	Arabidopsis	Cytosol/Plastid	Noji et al. 1998
AtSerat2;2	Arabidopsis	Mitochondria	Noji et al. 1998
AtSerat3;1	Arabidopsis	Cytosol	Kawashima et al. 2005
AtSerat3;2	Arabidopsis	Cytosol	Kawashima et al. 2005
GmOASTL0	Soybean	Cytosol	Chronis and Krishnan 2003
GmOASTL1	Soybean	Cytosol	Zhang et al. 2008
GmOASTL2	Soybean	Cytosol	Zhang et al. 2008
GmOASTL3	Soybean	Mitochondria/CAS-like	Zhang et al. 2008
GmOASTL4	Soybean	Plastid	Zhang et al. 2008
GmOASTL6	Soybean	Cytosol	Zhang et al. 2008
GmOASTL7	Soybean	Cytosol	Zhang et al. 2008
GmSerat1;1	Soybean	Cytosol	Chronis and Krishnan 2004
GmSerat2;1	Soybean	Cytosol/Plastid	Liu et al. 2006
GmSerat2;2	Soybean	Mitochondria	

Table 2-2. List of primers used for gene cloning

Gene Name	Gene Accession Number	Orientation	Primer Sequence
GmOASTL0	AF452451	Forward	5’ CATATGATGGCTGTTGAAAGGTCCGG 3’
		Reverse	5’ GTCGACTCAGGGCTCAAAAGTCATGC 3’
GmOASTL1	EF433420	Forward	5’ CATATGAATATGGCGGTTGAGAAG 3’
		Reverse	5’ GTCGACCATATTCAAGGCTCGAAG 3’
GmOASTL2	EF433421	Forward	5’ CATATGATCATGGCAGTTGAAAAGTC 3’
		Reverse	5’ GTCGACATCCACCACTTGAAATCAG 3’
GmOASTL3	EF584898	Forward	5’ CATATGTCAATGGCTTCCCTCATG 3’
		Reverse	5’ GTCGACTTAATCAACTGCTACTGG 3’
GmOASTL4	EF584900	Forward	5’ CATATGATGGCTTCTTCTGCTTCCTT 3’
		Reverse	5’ GTCGAGTCATGGCTAGGTTGCATT 3’
GmOASTL6	EF433422	Forward	5’ CATATGAACATGGAGCCACAGTG 3’
		Reverse	5’ GTCGACGTTCTTAATCAAATGTC 3’
GmOASTL7	EF433423	Forward	5’ CATATGAGCATGGAGGAGCCTCAA 3’
		Reverse	5’ GTCGACCACCTTAATCAAATGTC 3’

Table 2-3. List of real-time PCR primers used for semi-quantitative PCR

Gene Name	Gene Accession Number	Direction	Primer Sequence	Cycle #
GmOASTL0	AF452451	Forward	5’ GCCAATCCCAAGGTTCATTA 3’	28
		Reverse	5’ GGTTCCACACCAATCAGCTT 3’	
GmOASTL1	EF433420	Forward	5’ CGGGTGAAAGTGTCCTCATT 3’	24
		Reverse	5’ TGCCTTCTGAACAGCTCCTT 3’	
GmOASTL2	EF433421	Forward	5’ AAAATCGCAGACGGTTCTGT 3’	30
		Reverse	5’ CCCTTCTTCTCTGCATCAGC 3’	
GmOASTL3	EF584898	Forward	5’ CCTAAGGACCTCCCTGGAAC 3’	32
		Reverse	5’ TGCTGGTCTGTCTTTGATGC 3’	
GmOASTL4	EF584900	Forward	5’ ATGTCACCCAGCTCATAGGG 3’	30
		Reverse	5’ CCGCTAGTAGGTTCCACCAATA 3’	
GmOASTL6	EF433422	Forward	5’ AGCCACAGTGTGCAATCAAG 3’	34
		Reverse	5’ CCGGTGTAAGAGGCCTTTA 3’	
GmOASTL7	EF433423	Forward	5’ CAGGTGACTATGCCGTCTTACG 3’	32
		Reverse	5’ CGAGTCCCTCCAAATCTCAG 3’	
GmSerat1;1	AF452452	Forward	5’ AAAGGTTGGTGCTGGTTC 3’	32
		Reverse	5’ CTCAGAGATAAAGGAAGTATGG 3’	
GmSerat2;1	AY422685	Forward	5’ TTGGTTGATGATGGAGATGATGAC 3’	34
		Reverse	5’ TGAGCAATCCTATGAGCCTGAC 3’	
GmSerat2;2	AK286410	Forward	5’ ATGTGAAACGGGTGAGGTTG 3’	34
		Reverse	5’ TGAGGTTGATTGCAAGGTGA 3’	

Table2-4. List of transgenic soybean lines

Transgenic Line	Description
5159	GmSerat2;1 overexpressor
5160	GmSerat2;1serine to alanine mutant
5186	CDPK RNAi
5187	Vector only control
5193	GmSerat2;1 serine to aspartate mutant
5194	GmSerat2;1RNAi mutant

CHAPTER 3
RESULTS

Rationale for Study

The incorporation of sulfur into an organic form by plants is a vital step in the nutrition of not only the plant but of animals as well. Plants and bacteria are the only organisms that are capable of performing this process and thus a large number of organisms rely on plants as their source of sulfur. Sulfur metabolism in plants has been studied for many years. However, conclusive mechanisms for regulation remain elusive. By studying the interactions of serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OASTL) the mechanisms of regulation and expression of cysteine synthesis will be better understood.

In the short term, the research is vital to understand how SAT and OASTL interact with one another in soybean. In many plant pathways, mechanisms of regulation and enzyme characteristics are fairly similar. However, in the case of cysteine synthesis, many differences between species have been found. For example, localization of SAT forms sensitive to feedback inhibition have not been consistent among species. In addition, some species' feedback inhibition, such as soybean, have been shown to be phospho-regulated. These differences make it important to study several species in order to get a larger picture and a better understanding of the dynamics of the pathway.

In the long term, once a greater understanding of the enzyme regulation and expression is reached, engineering of the pathway could result in an increase in cysteine and other sulfur containing compounds. In staple crops such as soybean, this is of special interest as higher sulfur levels would greatly increase the nutritional quality.

Also, several studies have shown that crops with an increased amount of cysteine can increase the concentration of other sulfur containing metabolites such as glutathione which would aid the plant in defense against oxidative stress (Blaszczyk et al. 1999, Reimenschneider et al. 2005).This thesis describes the characterization of two soybean OASTL enzymes and the expression analysis of soybean's cysteine synthesis genes. The specific aims of this study were:

1. To characterize soybean OASTL proteins and their interactions with GmSerat2;1.
2. To examine the expression levels of soybean OASTL and SAT genes *in vivo.*

Specific Aim 1: OASTL Characterization

Multigene Families

To date, three soybean genes encoding SAT and seven encoding of OASTL have been identified while Arabidopsis has five and nine genes of each, respectively (Figure 3-1 and 3-2). When phylogeny of these two species is studied, amino acid sequences of expressed enzymes each in each of the three subcellular compartments loosely cluster indicating that the increase in gene number occurred before species differentiation. Several groups have examined the Arabidopsis SATs, however little work has been done on the soybean SATs. GmSerat1;1 is a cytosolic enzyme and has been studied in detail (Chronis and Krishnan 2003), GmSerat2;1 has a cytosolic/plastidic localization and has a serine phosphorylation site at amino acid position 378 in its C-terminus (Liu 2006). Thus far no published data is available for GmSerat2;2 which is thought to be located in the mitochondria based on PSORT, a protein sorting software.

Through the use of the recently published soybean genome, Zhang et al. (2008) published an overview of all seven known soybean OASTLs. Of the seven forms, all but

two, GmOASTL3 thought to be mitochondrial and GmOASTL4 thought to be plastidic, were predicted to be localized to the cytosol. Of the seven OASTLs, only GmOASTL0 had been previously studied (Chronis and Krishnan 2004). The naming system employed in this study for the OASTLs comes from that of the Zhang group (Zhang et al. 2008). Localization studies done by collaborator Jung Youn Lee confirm the cytosolic localization of GmOASTL0 and GmOASTL4 in the chloroplast by fusions of each gene with GFP (data not shown).

The goal of this work was to characterize enzymes involved in cysteine synthesis in soybeans including OASTL forms and their interactions with SAT forms.

OASTL Activity Assays

Several studies have shown SAT activity to increase in the presence of OASTL (Berkowitz et al. 2002, Droux et al. 1998, Ruffet et al. 1995), the most recent study showing a two-fold increase in SAT activity (Kumaran 2009). However, OASTL activity has been shown to decrease in the presence of SAT despite large excesses of OASTL in comparison to SAT (Droux et al. 1992, Droux et al. 1998, Kumaran 2009). In addition, stability of the cysteine synthase complex is dependent on the subcellular levels of key metabolites in the pathway. Addition of sulfur has been found to stabilize the complex but also lower the activity of free OASTL (Wirtz and Hell 2006) while incubation with OAS is shown to dissociate the complex to free OASTL and increase activity (Droux et al. 1998). For this reason, during the purification of both SAT and OASTL enzymes, affinity columns were washed with an OAS solution before other washes to ensure that only the bound protein was being purified and not the other proteins in the complex.

To determine how the activity of purified recombinant enzymes compared to reported activities, OASTL activity assays were preformed. Initiation of the reaction was

generally by the addition of the enzyme to a mix of the two substrates, sulfide and OAS, in a buffer mix. Cytosolic GmOASTL0 and plastidic GmOASTL4 were determined to have similar specific activity when assayed (Figure 3-3). When comparing enzyme activity to the published activities seen for these enzymes, Zhang et al. reported the activity of GmOASTL4 to be 724.5 µmol cys min^{-1} mg^{-1} while ours was considerably lower at 313.9 µmol cys min^{-1} mg^{-1}. The activity for GmOASTL0 was higher than the reported value (Kumaran 2009) of 1400 µmol min^{-1} mg^{-1} with our activity at 321.74 µmol cys min^{-1} mg^{-1}.

Despite several methods of activity optimization, measured activities for the OASTL enzymes tested remained lower than the reported averages. Several reasons could account for these discrepancies including inconsistencies in standardization and calculation of activity. Poorer quality enzyme could have been a factor as an unknown inhibitor could have been present in the enzyme preparation. Sequence of the clone is not likely to be a factor as comparison to published clones is 99%identical in GmOASTL0 and 97% identical in GmOASTL4. In addition, the Km_{OAS} = 0.04 mM and $Km_{Sulfide}$ =0.087 mM for GmOASTL4 while reported values for three Arabidopsis forms were Km_{OAS} = 0.3-0.7 mM and $Km_{Sulfide}$ = 3-6 µM (Wirtz and Hell 2004). Indicating that Arabidopsis enzymes have a higher affinity for sulfide than GmOASTL4 but a lower affinity for OAS.

To compare the inhibition of OASTLs by SAT, recombinant enzymes were added in a 25:1 trimer to dimer excess. OASTL and SAT enzymes were incubated for 30 minutes prior to assay to ensure enzymes had the opportunity to come in contact with one another. When GmSerat2;1 wild-type and GmSerat2;1S-D were added in excess

during activity assays, OASTL activity was seen to drop to about sixty percent of its normal activity. Both forms of GmSerat2;1 affected both GmOASTL in a similar manner with GmOASTL4 showing slightly less inhibition (Figure 3-4). The serine to aspartate mutant seemed to reduce activity slightly more than wildtype in both instances but was not statistically significant.

Substrate Incubation Assays

The substrates of OASTL are sulfide and OAS. The proposed mechanisms of regulation of complex formation by the concentrations of these substrates have been discussed previously in this thesis (page 20). Substrate incubation assays were done to assess how addition of either substrate during the incubation time with SAT prior to an assay affected GmOASTL4. With various concentrations of OAS in the presence of a constant amount of both OASTL and SAT, it would be hypothesized that less enzyme would be in a complex and thus OASTL activity would be higher. Conversely, with higher sulfide concentrations and in the presence of SAT, more OASTL would be found in the complex and less activity would be seen.

Because of this OAS incubation, it is predicted that all curves would be approximately equal with the presence of SAT having little to no effect on OASTL activity because of lack of complex formation. This prediction is supported by the data as the curves with no SAT and 1 mM SAT are very similar (Figure 3-4). During the sulfide incubation, it is hypothesized that the activity of OASTL would be lower in the presence of SAT. This is seen in the data, however, the difference in the activity is not large.

Interestingly, during the incubations it was noticed that longer incubations with substrates and SAT before the start of the assay had lower overall OASTL activity. In

both OAS and sulfide incubations, the activity of OASTL was much lower with lower concentrations of either substrate regardless of the presence of SAT (Figure 3-4). As the concentration of the substrate increased, the activity of OASTL also increased.

OASTL was incubated in the cold in varying concentrations of sulfide in similar conditions to the previous assay. In this situation, the incubation length was varied at seven time points. As shown in Figure 3-5, the longer the incubation period the more inactivity the enzyme had during the normal assay. However, when higher concentrations of sulfide were added the enzyme recovered activity. It is hypothesized that the enzyme is cold labile and does loose activity when incubated in the cold but the presence of either substrate protects the enzyme from the harm done by the cold conditions.

Specific Aim 2: Expression Analysis

To date only two SATs and one OASTL have been studied in any detail (Chronis and Krishnan 2003, Chronis and Krishnan 2004, Liu et al. 2006). From a study preformed in Arabidopsis, it is known that all SAT genes are not equally expressed. Of the five SAT genes only three were found to be constitutively expressed while the other two were expressed at much lower levels and thought to be induced by various stresses. This study also suggested that genes were expressed differentially throughout tissues and developmental stages (Kawashima et al. 2005). Additionally, evidence suggests differential expression in etiolated versus light-grown plants in some species while some species show no difference in expression (Bogdanova et al. 1997, Noji et al. 2001, Saito et al. 1995). It is unknown whether these differences in expression of transcript levels equate to similar differences in protein levels in the plant.

By examining the expression levels of each form, it is easier to understand their function and be able to properly characterize their role in regulation of cysteine synthesis. Based on Arabidopsis data, it is to be expected that each form will have its own expression fingerprint that can be used to determine when, where and under what conditions it is most likely to be expressed. This will aid in determining promoters and elements likely to induce expression of each form. This comparative data will help define each gene's relation relative to other enzymes involved in cysteine metabolism.

Post-Germination Expression Analysis

As yet, extracting native protein has been extremely difficult due to very low levels of the desired protein in the whole cell extracts. Knowing the stage of development that has the highest expression can help to gain higher efficiency during extraction. Through a preliminary test performed by Mariana Kirst, it was found that the native protein was found at its highest levels during the earliest stages of plant growth. With this data, an expression analysis of the first stages of plant development was employed. Nine stages of development were studied and include:

- New shoot
- Unopened cotyledon
- Opened cotyledon
- Plumule
- New leaf
- First trifoliate
- Second trifoliate
- Stem of plant with open cotyledon
- Root of plant with open cotyledon

Using gene specific primers, semi-quantitative PCR was run on each tissue sample to study the expression of four different genes, two SATs and two OASTLs. The cytosolic form GmSerat1;1 was found to be expressed in low levels but was at its

highest in the root and stem of a seedling with opened cotyledons (Figure 3-6). The cytosolic/plastidic form GmSerat2;1 was found to be expressed fairly evenly in all tissue types except roots. Expression was highest in the plumule and stem in the cytosolic GmOASTL0 while the plastidic form GmOASTL4 showed the highest expression earlier at the unopened and opened cotyledon stage. Aside from giving information as to protein harvesting, this information hints about the relation of expression of the different enzymes from the same compartment. This idea will be in more detail later.

Comparative Expression

Tissue from the first true leaves of soybean seedlings was used for comparing all known soybean SAT and OASTL forms. Specific primers (Table 2-3) for each gene were used to determine from a single tissue type how each form's expression compared to the others. Figure 3-7 shows the resulting data in the form of a gel. It must be noted that each gene is amplified by a different primer set and thus has a different band size. From this comparison, it appears that SAT genes are expressed at higher levels than the OASTL genes with two of the OASTL genes (GmOASTL2 and GmOASTL6) having no detectable expression. This comparative analysis could be representative of relative expression levels of each form, however, from the data seen in Figure 3-6 it has been shown that this is more likely a snapshot as each tissue type has differential expression levels.

Cadmium Stress

Four genotypes of transgenic soybean seedlings (Table 2-4) were grown for gene expression analysis: vector only control, GmSerat2;1 serine to alanine mutant, GmSerat2;1 serine to aspartate mutant, and GmSerat2;1 over-expresser. After three weeks of growth, seedlings were given MS media with 25 mM $CdCl_2$. A previous study

done with arabidopsis showed an increase in specific SAT genes that were expressed at very low levels under control conditions (Kawashima et al. 2005). The expression analysis of this study has yet to be done and will be examined later in the discussion section. It is expected, however, that a few of the soybean OASTL and SAT forms will have an increased expression level when under heavy metal stress.

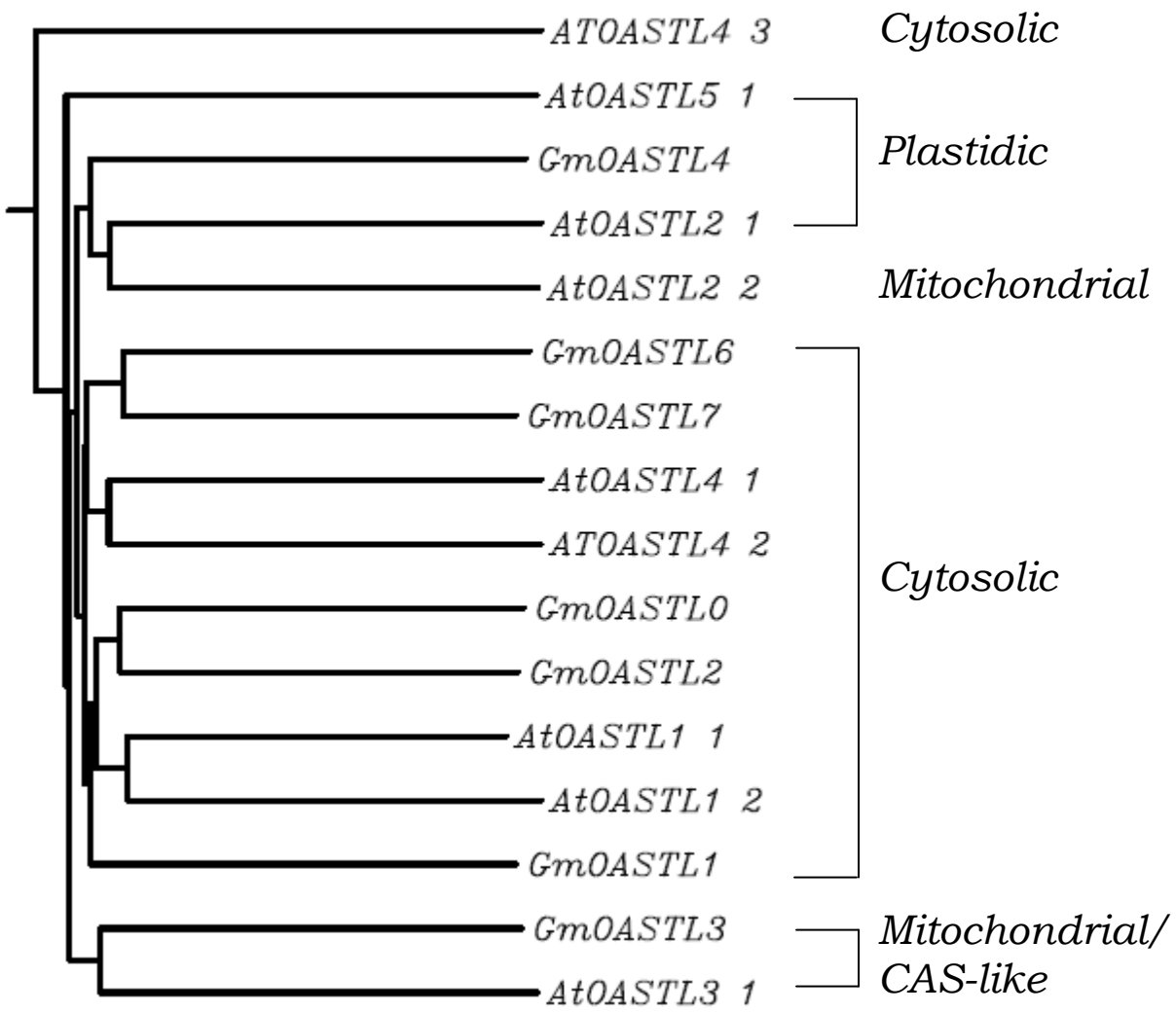

Figure 3-1. OASTL phylogenetic tree. OASTLs are encoded by gene families and they are present in the cytosol, plastid and mitochondria. GmOASTL0 and GmOASTL4 cluster with cytosolic and plastidic proteins, respectively, and their localization has been confirmed in vivo (Jung Youn Lee, unpublished data). OASTL genes are numbered according to Zhang 2008 and Wantanabee 2008.

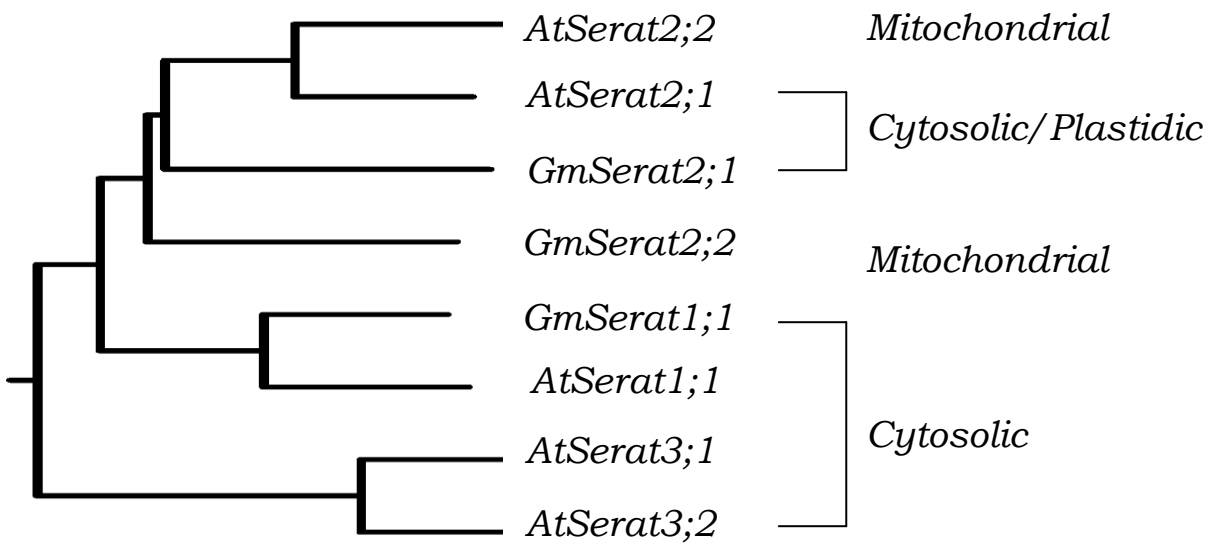

Figure 3-2. SAT phylogenetic tree. GmSerat1;1 clusters with cytosolic proteins and GmSerat2;1 and GmSerat2;2 cluster with Arabidopsis plastidic and mitochondrial proteins. In vivo localization studies show that GmSerat2;1 is present both in the cytosol and plastids. Full length amino acid sequences were aligned and compared using Clustal W.

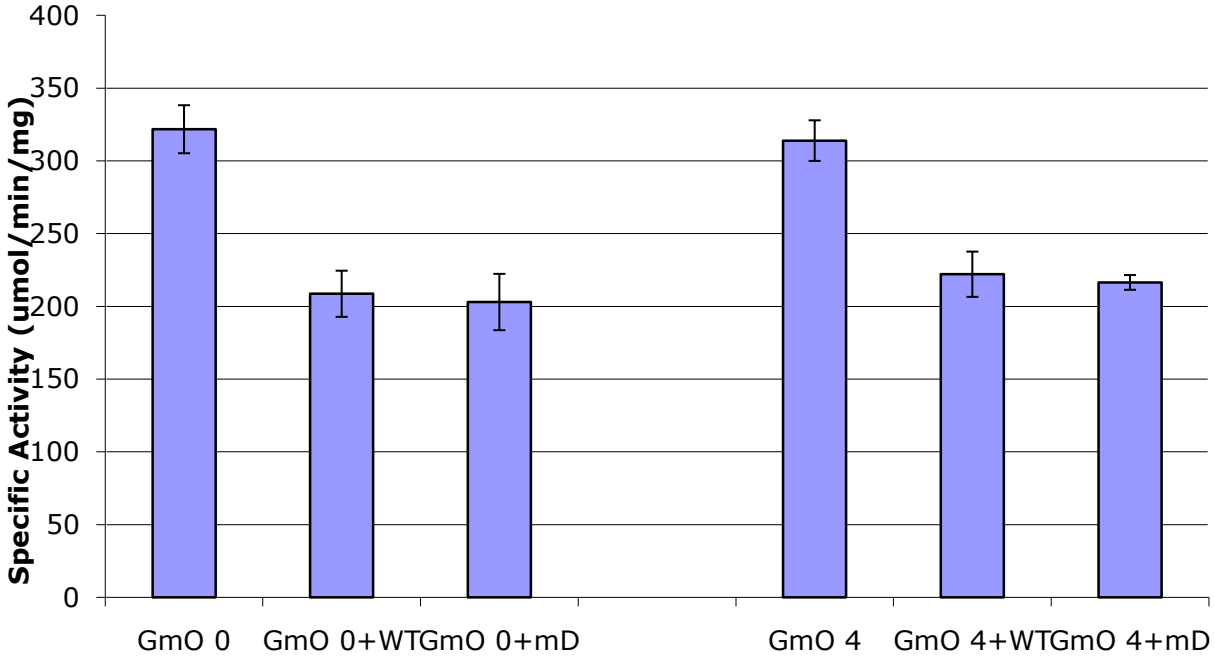

Figure 3-3. OASTL activity assays. Activity of GmOASTL0 (GmO 0) and GmOASTL4 (GmO 4) shown alone and with 25:1 trimer to dimer excess of both forms of GmSerat2;1 wildtype (WT) and the phosphomimic (mD). Recombinant proteins were used and each sample represents a minimum of four replicates.

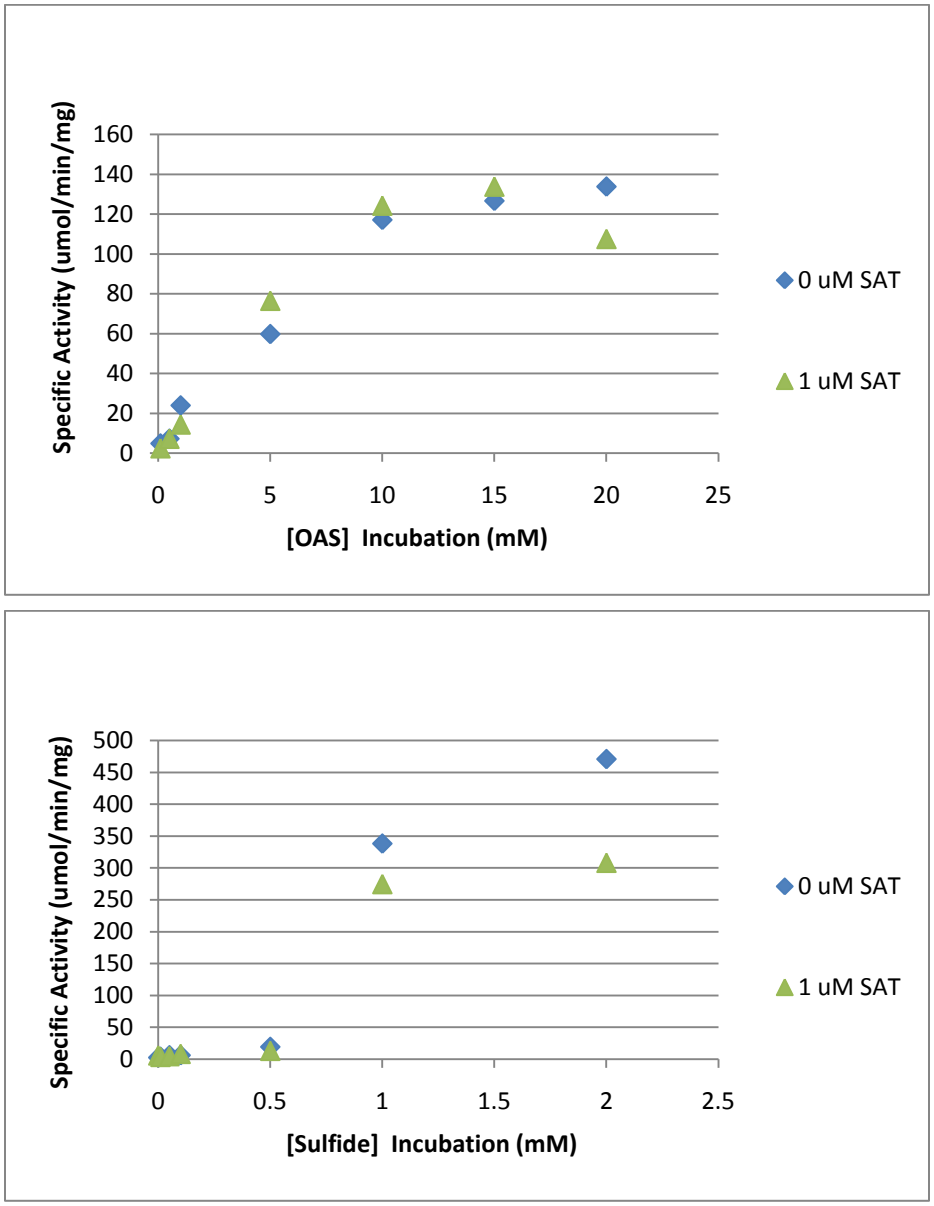

Figure 3-4. Substrate incubation assays. One hour incubations of GmOASTL4 performed in the presence of SAT. Varyious concentrations of both OAS (A) or Sulfide (B) were added prior to activity assay. The assay was initiated by adding a solution to each sample that brought the varied substrate to equal concentrations in all samples and by the addition of the remaining substrate. Blue diamonds represent samples that were free of SAT. Green triangles represent samples that contained 1µM GmSerat2;1.

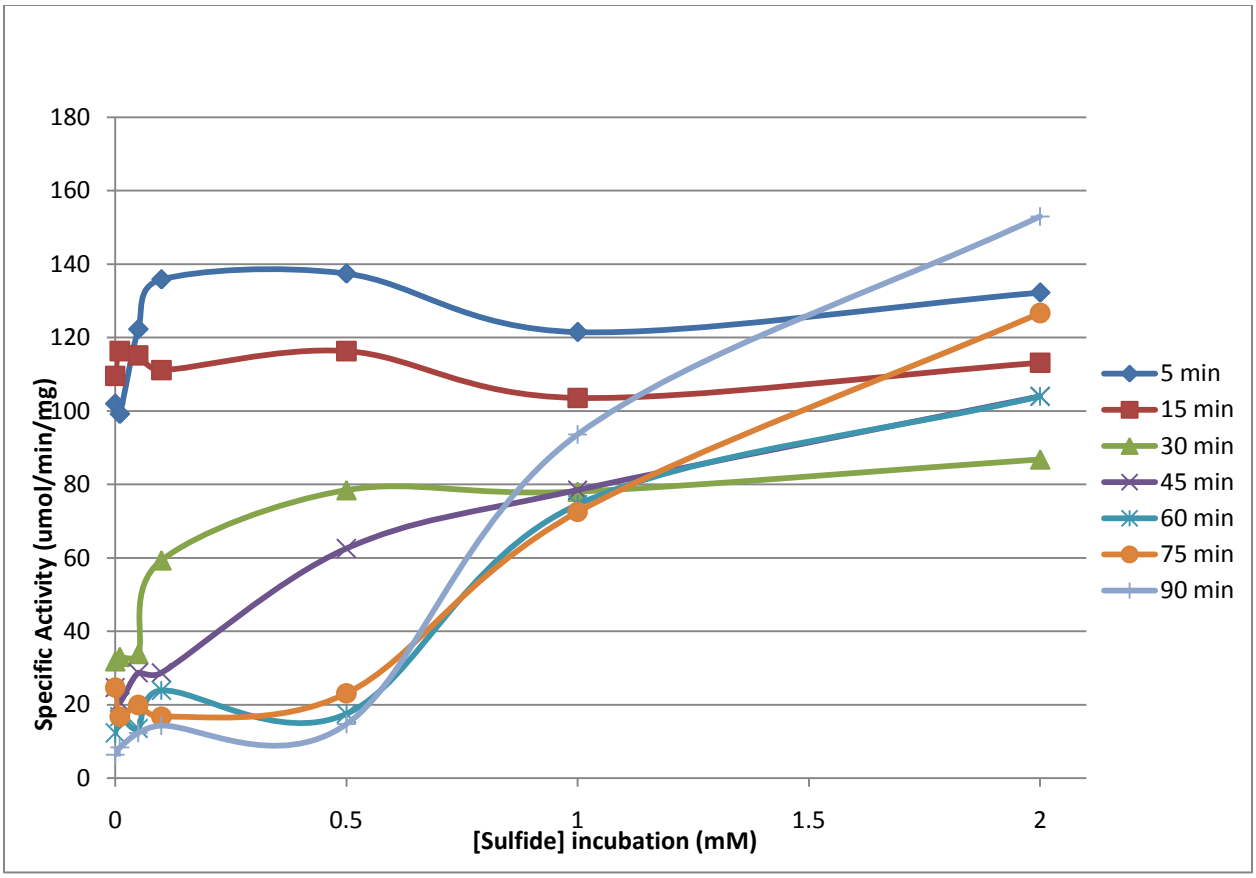

Figure 3-5. OASTL activity with varying time for sulfide incubation. GmOASTL4 was incubated with sulfide for varying lengths of time at varying concentrations.

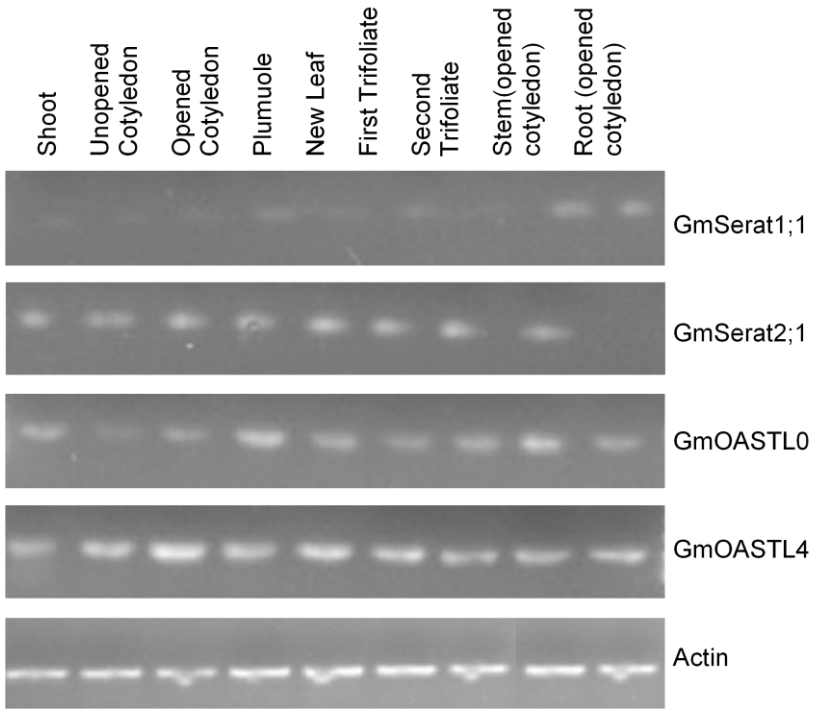

Figure 3-6. Germination expression analysis. A semi-quantitative expression analysis was performed on cDNA from nine stages of postgerminative growth (shoot of 2 day old seedling, unopened cotyledon, opened cotyledon, plumuole, first true leaves, first and second trifoliate, stem and roots from a seedling with an opened cotyledon) on GmSerat1;1 (cytosolic, 32 cycles), GmSerat2;1 (cytosolic/plastidic, 34 cycles), GmOASTL0 (cytosolic, 28 cycles) and GmOASTL4 (plastidic, 30 cycles) soybean genes. Cycle numbers were optimized for each gene. Expression of the plastidic genes appears to be more uniformly expressed throughout germination while the cytosolic genes are more localized to specific tissue types.

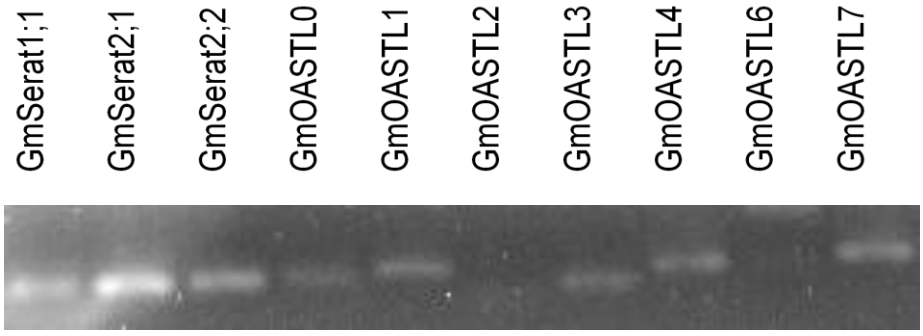

Figure 3-7. Comparative expression of soybean OASTLs and SATs. Semi-quantitative PCR was performed on the first true leaves of seedlings for 34 cycles. Specific primer sets for three SATs and seven OASTLs were used.

CHAPTER 4
DISCUSSION

Specific Aim 1: OASTL Characterization

Despite having been studied for over fifty years, many aspects of cysteine synthesis are still unclear. With each study, new models are proposed and more insight is gained into the regulation of the system. The last decade has seen an explosion in understanding and yet many questions remain. Currently there are several theories as to the presence of multiple forms of each gene, however, no one theory has yet been proven correct. When phylogeny of Arabidopsis and soybean O-acetylserine(thiol)lyase (OASTL) and serine acetyltransferase (SAT) genes is studied, forms of the genes in each of the three subcellular compartments loosely cluster. This data indicates that the genes were duplicated before species differentiation. The fact that no plants have yet been found that have lost the duplication suggests that multiple copies of both genes are important for the plant development.

As previously mentioned, one theory for the duplication in gene number is that cysteine could not be transported between compartments (Lunn et al. 1990). Several independent groups using Arabidopsis knockouts have shown that the plants can compensate for the loss of one or more forms of either enzyme (Riemenschneider et al. 2005, Heeg et al. 2008, Watanabe et al. 2008). In many cases, even multiple gene knockouts produce plants with no visible phenotype. These findings cast doubt that cysteine cannot be transported between compartments and show that compensation for the loss of production in one location is seen along with an increased activity in functioning locations.

Another question raised by the redundancy of SAT and OASTL forms is how each form or compartment contributes to the net cysteine synthesis of the cell or plant as a whole. When looking at SAT single mutants, studies have found no significant up regulation in expression of the other SATs forms (Wantanabe et al. 2008, Haas et al. 2008). When similar experiments are done using OASTL knockouts the findings are consistent; no up regulation in expression is seen in the remaining forms (Heeg et al. 2008, Wantanabe et al. 2008, Riemenschneider et al. 2005). It could be possible that there is a standard pool of RNA transcripts of each of these forms that are translationally regulated by the metabolite levels in the cell. However no evidence has yet been found. Not only is OAS an intermediate between SAT and OASTL, but it has also been predicted to be a signaling molecule for many genes in the sulfur assimilation pathway (Hirai et al. 2003, Maruyama-Nakashita et al. 2005, Haas et al. 2008). When OAS, sulfide, serine or another unknown metabolite reaches certain threshold values in the various compartments, it could trigger translation.

This study showed a decrease in OASTL activity when incubated with SAT as shown in other studies. The data here show a forty percent loss in activity (Figure 3-3), however, when compared to data for GmOASTL0 from other groups a ninety-seven percent loss is seen (Kumaran et al. 2009). Differences in loss of activity are significant and could be due to the presence of the His-tag on the recombinant proteins in this study which could also contribute to the difference in activity without SAT present. In the future, tag cleavage needs to be done in order to discern why there is such a discrepancy in activity. Also, in the future more OASTL forms can be analyzed to get a broader picture of the pathway in soybean.

To further elucidate the characteristics of soybean cysteine synthesis, native enzymes should also be examined. As previously mentioned, extraction of high yields of native protein is very difficult. The development of a method for extracting and purifying this protein would be extremely important in characterizing the pathway. Further analysis of *in vivo* enzymes also needs to be done and six transgenic lines have been developed for this purpose (Table 2-4).

Specific Aim 2: Expression Analysis

Understanding how the regulation of each form of SAT and OASTL is expressed has proven to be a challenge. In Arabidopsis, the mitochondria have been shown to be the source of the majority of SAT activity at roughly seventy-five percent (Ruffet et al. 1995, Watanabe et al. 2008). However, multiple studies show very little OASTL activity, about ten percent, is from mitochondria whereas activity in the plastid, forty-two percent, and cytosol, forty-four percent, are roughly equal (Saito et al. 1993, Saito et al. 1994, Heeg et al 2008). Two current studies in Arabidopsis conclude that despite the mitochondria contributing less to the overall OASTL activity, they are still the most important compartment for OASTL activity (Watanabe et al. 2008 ref for 2nd study).

With these large differences in the relative activities between compartments, it is interesting to see that the mitochondria is responsible for the majority of SAT activity and needed for proper OASTL functioning. This study showed that the predicted mitochondrial OASTL and SAT forms were not expressed any higher than other OASTL and SAT forms (Figure 3-7) which is similar to data seen in other studies. The expression levels of the mitochondrial genes do not appear to be significantly higher, however, it is responsible for a larger portion of the activity. No data has shown that the mitochondrial forms have higher activity on a molar basis which again points to a

standard pool size of transcripts with an unknown signal triggering translation of the different forms. To answer this question in the future, a side by side comparison of *in vivo* protein and transcript levels could be done. It would then be possible to determine if one compartment contained significantly more protein copies which would explain the large difference in mitochondrial activity.

Future Work

To further elucidate the regulatory methods involved in this pathway, much work is still needed. Further characterization of the interaction between the remaining OASTL and SAT forms is required to determine if all enzymes in the system are universally affected in a similar manner. Further characterization OASTLs' interactions with both wild-type and the phosphomimic of GmSerat2;1 is needed to determine how phosphorylation affects OASTL activity among the different forms.

In addition to studying recombinant enzymes, native protein complexes should also be studied. Purification using an antibody specific for the protein is needed because of the natural low abundance of SAT. Antibodies specific to the N-terminal exposed portion of GmSerat2;1 will need to be produced as the C-terminus should be bound to OASTL. Analysis on the complex can also be performed with HPLC detection of OAS and cysteine production. Some *in vivo* protein analysis has been performed by Mariana Kirst and Dhiraj Vijas using salt stresses, HPLC metabolite analysis and photosynthetic tests on the different lines (data unpublished), however, more work needs to be done elucidating the characteristics of GmSerat2;1 and the other coordinating enzymes.

Individualized real-time PCR primers have been designed (Table 2-3) for each known SAT and OASTL gene as well as for other sulfur metabolizing. Plants can be

exposed to various stresses including metabolite stress, oxidative stress and heavy metal stress. Comparing transcript levels of each gene of plants under the stress verses controls can be done to determine if gene expression is inducible or constitutively expressed. Antibodies specific for each SAT and OASTL form can also be generated to analyze *in vivo* protein expression levels. In conjunction with a transcript analysis, protein levels can be examined from samples taken at various developmental stages. Expression levels of each gene can then be inspected to determine whether there is any evidence of spatial expression patterns.

From previous studies, it was shown that specific SAT genes are expressed at very low levels under control conditions but are up regulated when stressed (Kawishima et al. 2005). As mentioned previously, a cadmium stress experiment was started that will help to determine how soybean genes are affected by heavy metal stress. When the results are available, the data will be able to indicate if soybean genes are regulated similar to Arabidopsis. This data would give an insight into the method of regulation on a compartment by compartment basis.

All molecules containing a reduced sulfur moiety have cysteine as a common precursor making the formation of cysteine an important pathway in plant metabolism. The regulation of the enzymes involved in cysteine synthesis is a complex process and the pathway to understanding its complexities have proven to be interesting and will continue to be.

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BIOGRAPHICAL SKETCH

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