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Deciphering Sulfur Amino Acid Metabolism in Developing Seeds of Common Bean

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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ABSTRACT

With increasing food insecurity in the populated world, the number of people affected by chronic undernourishment is also increasing. Alone, protein energy malnutrition is linked to 6 million deaths annually. Despite being a good source of protein and dietary fibre, the quality of bean protein is limited because of sub optimal levels of essential sulfur amino acids: methionine and cysteine. Levels of cysteine and methionine in developing seeds have an inverse relationship with the non-protein sulfur amino acid *S*-methyl-cysteine (*S*-methylCys) and dipeptide γ -glutamyl-*S*-methyl-cysteine (γ -Glu-*S*-methylCys).

One of the strategies to improve protein quality in bean is to redirect sulfur from *S*-methylCys and γ -Glu-*S*-methylCys to the cysteine pool. In this thesis, elucidation of the unknown biochemical pathway of *S*-methylCys synthesis was accomplished using ^{13}C and ^{15}N labelled serine and cysteine, and revealed serine as the precursor of *S*-methylCys biosynthesis. Feeding developing seeds with ^{13}C and ^{15}N labelled methionine also suggested a role for methionine in *S*-methylCys biosynthesis. In the cytosol, methanethiol released during hydrolysis of methionine condensed with *O*-acetylserine to form *S*-methylCys. BSAS4;1, a β -substituted alanine synthase family member plays a key role in this reaction. BSAS4;1 is a cysteine synthase that utilizes *O*-acetylserine as a carbon backbone donor and methionine as a methyl donor to synthesize *S*-methylCys. In the case of γ -Glu-*S*-methylCys, another pathway seems more active, whereby homogluthathione is transformed into *S*-methylhomogluthathione and γ -Glu-*S*-methylCys is synthesized in the presence of a carboxypeptidase. This study identifies BSAS4;1 and methionine γ -lyase (MGL) as candidate enzymes for *S*-methylCys biosynthesis in common bean.

According to the Canadian Food Inspection Agency, only the label “Good source of protein” can be used for beans and other legumes. In future, silencing of the *S*-methylCys pathway candidate genes or development of TILLING lines with high cysteine and methionine levels may change bean from a good source to an “excellent source of protein”. Ultimately, the findings of this thesis could provide a helping hand to overcome the food insecurity in the growing world.

KEYWORDS

Sulfur amino acids; cysteine; methionine; *S*-methyl-cysteine; γ -glutamyl-*S*-methylcysteine; isotope labelling; *S*-methylhomoglutathione; common bean.

CO-AUTHORSHIP STATEMENT

The following thesis contains material from manuscripts either published or in preparation which are co-authored by Jaya Joshi, Justin B. Renaud, Mark W. Sumarah and Frédéric Marsolais.

My supervisor Dr. Frédéric Marsolais provided insight and strategic direction for the projects and edited the manuscripts (Chapters 2 - 5).

Chapter 2. Author's contributions

Jaya Joshi designed the research, performed the experiments and drafted the manuscript. Frédéric Marsolais conceived the study and participated in its design. Jaya Joshi and Frédéric Marsolais edited the manuscript.

Chapter 3. Author's contributions

Jaya Joshi designed the research, performed the experiments, analysed the data and drafted the manuscript. Frédéric Marsolais conceived the study and participated in its design. Justin Renaud helped in running the samples on mass spectrometer and assisted in data analysis under the supervision of Mark Sumarah. A part of this study is published in *Sulfur Nutrition and Assimilation in Higher Plants: Fundamental, Environmental and Agricultural Aspects (2017)*.

Chapter 4. Author's contributions

Jaya Joshi designed the research, performed the experiments, analysed the data and drafted the manuscript. Frédéric Marsolais conceived the study and participated in its design. Justin Renaud helped in running the samples on mass spectrometer and assisted in data analysis.

To my parents

For providing me the thrust to fly

Sushant

For being wind beneath my wings

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Heraclitus said, “No man ever steps in the same river twice, for it's not the same river and he's not the same man”. And how true was he! With every passing moment, human beings evolve, they change, conditioned by the circumstances they face, and the person they meet and that’s what happened with me. Seven different cities in two different continents, heartfelt gratitude to those amazing people who helped me to sail on the river of my dreams.

“If you hang around the barbershop long enough, sooner or later you are gonna get a haircut”, I was fortunate to have been born into a family which asked me to keep dream and perseverance together. Without their support, I certainly wouldn’t have dared to dream about the amazing world of science. I also feel blessed to have got another family, my in-laws, who not only supported me in this journey but also acted as an emotional anchor when things really got rough. This journey would not have been feasible without your presence, Sushant Joshi. Thanks for sailing this boat along with me. Thanks to my whole family for being my ardent supporter. This thesis is dedicated to my family for being the wind beneath my wings.

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LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl-coenzyme A
Ala	Alanine
Asn	Asparagine
At	<i>Arabidopsis thaliana</i>
BSAS	β - cyanoalanine synthase
C	Carbon
CS	cysteine synthase
CAS	Cyanoalanine synthase
Cys	Cysteine
GSH	Glutathione
Gln	Glutamine
Gm	<i>Glycine max</i>
hGSH	homoglutathione
HPLC	High-Performance Liquid Chromatography
HRMS	High resolution Mass Spectrometry
<i>K_m</i>	Michaelis-Menten constant
LB	Luria-Bertani Medium
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LC-DDA	Liquid Chromatography-Data Dependent Acquisition
Lys	Lysine
MES	2-(N-morpholino)ethanesulfonic acid

Met	Methionine
MGL	Methionine- γ -lyase
MS	Mass Spectrometry
N	Nitrogen
OAS-TL	<i>O</i> -acetylserine (thiol) lyase
OD	Optical density
PLP	pyridoxal 5'-phosphate
<i>Pv</i>	<i>Phaseolus vulgaris</i>
Pro	Proline
QTL	Quantitative Trait Loci
ROS	<i>Reactive oxygen species</i>
S	Sulfur
SAM	<i>S</i> -Adenosylmethionine
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Ser	Serine
SERAT	Serine acetyltransferase
<i>S</i> -methylCys	<i>S</i> -methyl-cysteine
SMM	<i>S</i> -methylmethionine
TAIR	The Arabidopsis Information Resource
TILLING	Targeted Induced Local Lesions IN Genomes
Thr	Threonine

Tyr	Tyrosine
V _m	<i>Vigna mungo</i>
V _{max}	Maximum reaction rate
YFP	Yellow Fluorescent Protein

CHAPTER ONE - GENERAL INTRODUCTION

Approximately 870 million of the world's population suffer from chronic undernourishment. Protein energy malnutrition (PEM) is the most prevalent form of undernourishment (Habicht, 2008). In the year 2010 alone, approximately six million deaths were attributed to protein energy malnutrition (<http://www.fao.org/hunger/en/>). Increasing food insecurity with escalating population and number of malnourished people has demanded an alternative solution to deal with quality and quantity of protein in human diet.

To deal with PEM, human diet not only requires enough protein but also supply of some of the amino acids that our body cannot synthesize. These indispensable amino acids are known as essential amino acids. These essential amino acids include lysine, phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine and histidine. In animals another sulfur amino acid, cysteine is derived from methionine and has sparing effect on methionine when included in the diet (Ball *et al.*, 2006). Cysteine is also among conditionally essential amino acids which can be limited under special pathophysiological conditions (Iqbal *et al.*, 2006; Riedijk *et al.*, 2007). Nutritional quality of a protein source is considered as good for humans when it covers the requirements for nitrogen and essential amino acids. The nutritional quality of protein differs widely depending on their essential amino acid composition and digestibility (Schaafsma, 2005).

On a global basis, about 80% of food energy and about 65% of food proteins are supplied by plant foods. Among plant foods legumes are referred to as poor man's meat due to their high protein content (Deshpande, 1992). Though plants are a good source of protein, their quality is affected due to deficiency or suboptimal levels of some essential amino acids (Young and Pellett, 1994). Cereals are deficient in lysine while legumes have suboptimal levels of *S* amino acids: methionine and cysteine. Protein quality could be assessed adequately by expressing the content of the first limiting essential amino acid in a test protein as a percentage of the content of the same amino acid in a reference pattern of essential amino acids (Schaafsma, 2005; Millward *et al.*, 2008) (Table 1).

$$\text{Protein quality (\%)} = \frac{\text{mg of first limiting amino acid in 1 g test protein} \times 100}{\text{mg of the same amino acid in 1 g reference protein}}$$

Table 1.1 presents the amino acid requirements of different age groups provided by Food and Agriculture Organization/World Health Organization (FAO/WHO). Subsequently, the protein quality is corrected to true digestibility and presented as the Protein Digestibility-Corrected Amino Acid Score (PDCAAS) (Schaafsma, 2005)

$$\text{PDCAAS (\%)} = \frac{\text{mg of first limiting amino acid in 1 g test protein} \times \text{true digestibility (\%)} \times 100}{\text{mg of the same amino acid in 1 g reference protein}}$$

Values provided by FAO/WHO for the concentration of essential amino acids are used to calculate the amino acid score in the food proteins (Table 1.1). An amino acid score of 1 is related to optimal amount of essential amino acids in the food while a score smaller than 1 points towards a suboptimal level or deficient level (Millward, 2012).

Table 1.1: Required amino acid quantity (mg per g of protein) in different age group provided by FAO/WHO to calculate amino acid score

Amino acids	Preschool child (1-2 y)*	School child (3-10 y)*	Adult*
Histidine	18	16	15
Isoleucine	31	30	30
leucine	63	61	59
lysine	52	48	45
Methionine+	25	23	22
Cysteine			
Phenylalanine	46	41	38
+Tyrosine			
Threonine	27	25	23
Tryptophan	7	7	6
Valine	41	40	39
Total	310	291	277

*Requirement values (mg per g of protein). Preschool children (1-2 years); School child (3-10 years).

1.1 Common bean- A good source of protein

Among all the legumes produced in the world, *Phaseolus vulgaris* or common bean is the most important edible food legume and considered as one of the best for human consumption (Broughton *et al.*, 2003). It contributes 50% of the total grain legumes consumed worldwide. Fifteen percent of the world protein requirement is fulfilled by common bean (Kalavacharla *et al.*, 2011). Common bean contributes 36% of total protein requirement of people from African and American regions. In some countries such as Mexico and Brazil, beans are the primary source of protein in human diets (Broughton *et al.*, 2003). In the African and Latin American region around 300 million are dependent on bean as staple food, representing 65% of total protein consumed and 32% of energy (Blair *et al.*, 2010). Along with protein, it is a major source of fibre (Geil and Anderson, 1994; De Ron *et al.*, 2015). Apart from macronutrients, beans are rich in several important micronutrients, including potassium, magnesium, folate, iron, and zinc. Due to its health benefits and disease prevention properties common bean has secured its place in human diet (Messina, 2014; Petry *et al.*, 2015). Health benefits from a common bean rich diet includes reduced risk of obesity, diabetes, cardiovascular diseases, and colon, prostate and breast cancer (Correa, 1981; Friedman, 1994; Hangen and Bennink, 2002; Thompson *et al.*, 2009).

1.2 Storage proteins

Dried seeds of *P. vulgaris* are a good source of protein and their nutritional value is largely determined by its seed storage and other seed proteins. Common bean protein is constituted of three kinds of storage proteins. Globulins constitute >50% of common bean storage proteins with 11-20% albumins and 2-4% prolamins. Globulins play a major role as protein storage sink, other seed proteins like lectins and cysteine-rich peptides protect the seed against pathogens or herbivory, while others like the lipoxygenases may impact flavor profile of beans (War *et al.*, 2012). Lectins, an albumin type protein, are the second most abundant seed proteins accounting for 5-10% of total protein (Vitale and Bollini, 1995). Lectins include erythroagglutinating phytohemagglutinin (Pha-E), leucoagglutinating phytohemagglutinin (Pha-L), α -amylase inhibitor and α -amylase inhibitor-like protein (Mirkov *et al.*, 1994).

Storage protein globulins are comprised of two main families of storage proteins based on their sedimentation coefficients (S20.w): 7S or vicilin-type globulins (commonly known as vicilins) and the 11S or legumin-type globulins (legumins). Phaseolin, a 7S globulin is the most abundant seed protein in common bean which accounts for 35 to 50% of total seed nitrogen (Sathe, 2002) with characteristic low levels of the 11S globulin legumin, accounting approximately for 3% of seed protein (Muhling *et al.*, 1997). Common bean proteins are rich in essential amino acids except *S*-amino acids. *S*-amino acids methionine and cysteine contribute differently to these storage proteins. Albumins are richer in cysteine than globulins. Among globulins phaseolin has a correlation with methionine concentration while cysteine resides in the non-phaseolin fraction (Gepts and Bliss, 1984).

1.3 Protein quality and metabolites

While high levels of protein, complex carbohydrate, fibre and minerals have defined common beans' importance in the human diet, sub-optimal protein quality due to a low level of sulfur amino acids, methionine and cysteine requires a dedicated scientific approach for improvement. The sum of methionine and cysteine is considered a parameter to define protein quality (FAO, 2013). Major seed proteins in common bean, 7S globulin, phaseolin and lectin phytohaemagglutinin, are low in methionine and cysteine which contributes to low amino acid score for *S* amino acids. On the other hand, common bean accumulates a non-protein amino acid, *S*-methylCys, up to 0.3% of total seed dry weight mainly in the form of γ -Glu dipeptide. This γ -Glu dipeptide accounts for about 35% of total *S*-methylCys in common bean (Giada *et al.*, 1998; Taylor *et al.*, 2008). *S*-methylCys is a non-proteinaceous amino acid and cannot substitute for methionine or cysteine in the diet (Padovese *et al.*, 2001). Another *S*-amino acid derivative *S*-methylhomoglutathione which was previously reported in *Vigna radiata* also accumulates in common bean but at very low levels (Kasai *et al.*, 1986; Liao *et al.*, 2013). Chemical structures of the major sulfur amino acids and their derivatives reported in common bean is presented in Figure 1.1.

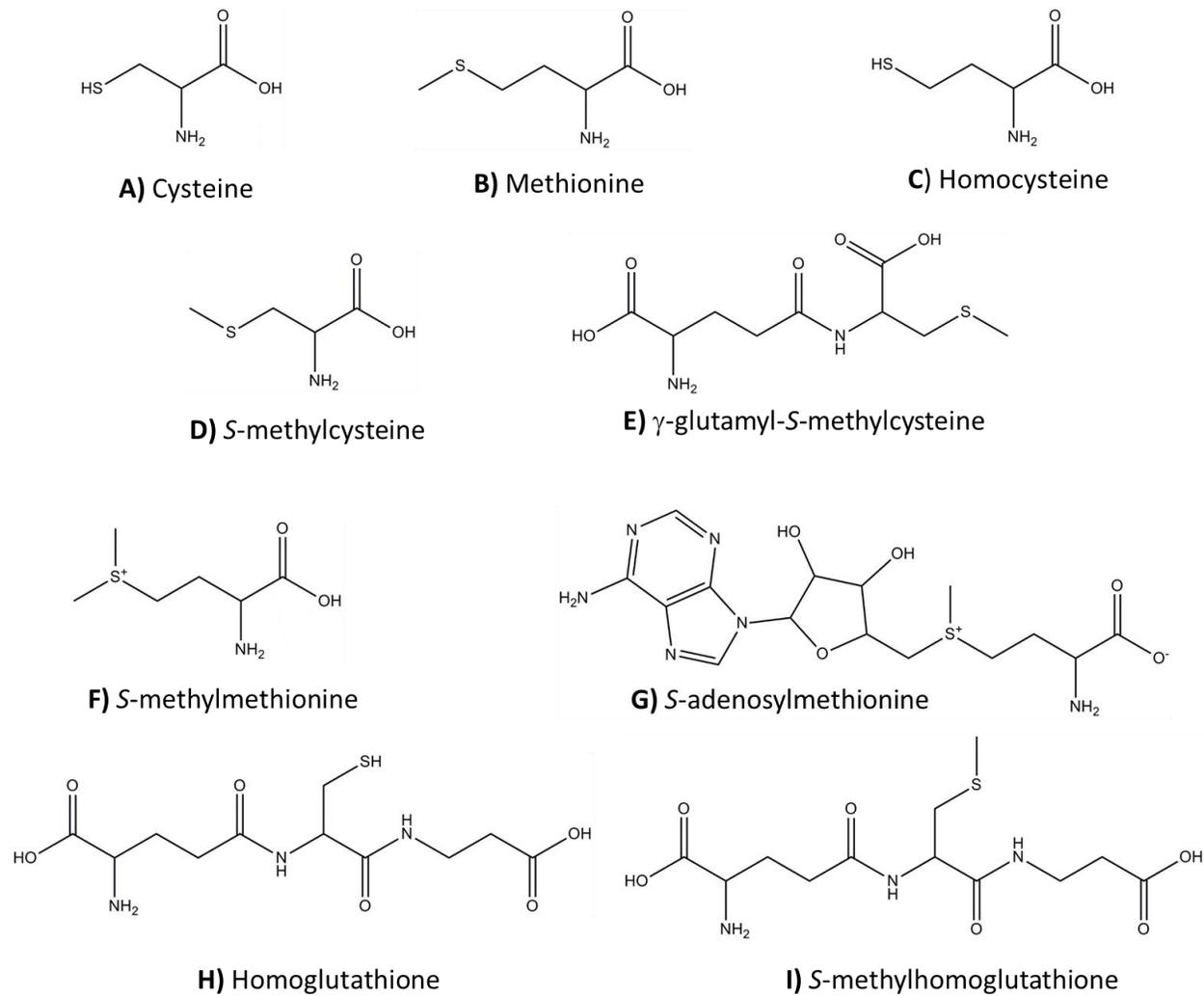


Figure 1.1: Chemical structures of major sulfur containing metabolites (amino acids and S-derivatives) present in *P. vulgaris*.

1.4 Overview of sulfur metabolism in plants

Sulfur metabolism in plants is extensively discussed in *Arabidopsis* and can be divided in two major steps: cysteine biosynthesis and cysteine catabolism.

1.4.1 Sulfate to Cysteine

Sulfate is the primary form of sulfur for the plants. Assimilation of sulfate in plant metabolism is a complex process which can be divided into three steps: (1) Entry and activation of sulfate; (2) Reduction of sulfate; (3) Synthesis of cysteine.

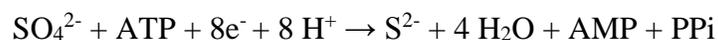
1.4.1.1 Entry and activation of sulfate

Entry of sulfur inside the cell is mediated by sulfate transporters (Takahashi *et al.*, 2011b). In *Arabidopsis* twelve genes are identified which encode sulfate transporters (Takahashi *et al.*, 1996; Takahashi, 2010; Takahashi *et al.*, 2011a). Sulfate transporters are divided into four groups based on their affinity and transporting compartment, such as low affinity transporters, high affinity transporters, vacuole transporters and plastid transporters (Buchner *et al.*, 2004). While high and low affinity transporters help in entry of sulfate in plant tissue, compartment specific transporters maintain local transport within the cell.

In a first reaction, in presence of ATP, sulfate is activated to adenosine 5'-phosphosulfate (APS) with the help of sulfate adenylyltransferase (ATPS, EC 2.7.7.4). Further APS can be phosphorylated and produce adenosine 3'-phosphate 5'-phosphosulfate (PAPS) by an APS kinase (APSK, EC 2.7.1.25) (Mugford *et al.*, 2009) or reduced to sulfite (Figure 1.2A).

1.4.1.2 Reduction of sulfate to sulfide

Reduction of sulfate to sulfide occurs exclusively in plastids in a two-step reaction. In the first reaction, APS reductase (APR) converts APS to sulfite using glutathione (GSH) as a reductant providing two electrons. Ferredoxin-dependent sulfite reductase (SIR) subsequently serves for the second step of reaction where sulfite is reduced to sulfide. (Leustek *et al.*, 2000) (Figure 1.2A). The equation summarizes reduction of sulfate to sulfide.



1.4.1.3 Synthesis of cysteine

In the final step of sulfur assimilation sulfide is incorporated in cysteine. During cysteine biosynthesis, the amino acid backbone is provided by serine metabolism while sulfur comes from sulfide. This reaction involves two enzymes: a serine acetyltransferase (SERAT, EC 2.3.1.30) and an *O*-acetylserine (thiol) lyase (OAS-TL, EC 4.2.99.8) (Figure 1.2A). SERAT catalyzes conversion of serine to *O*-acetylserine using acetyl-CoA. Second enzyme OAS-TL incorporates sulfur in *O*-acetylserine carbon backbone. Cysteine synthase complex is a heterooligomeric macromolecular assembly which coordinates cysteine biosynthesis along with sulfur assimilation. Cysteine synthase complex contains one SERAT hexamer and two OAS-TL dimers. Formation of cysteine synthase complex increases SERAT activity while its dissociation increases OAS-TL activity (Hell *et al.*, 2002; Bonner *et al.*, 2005)

OAS-TL, a cysteine synthase subfamily member, belongs to a large family of enzymes, the β -substituted alanine synthases (BSASs). The BSAS family is comprised of true cysteine synthase (CS/OAS-TL) or cysteine synthase like enzymes. These cysteine synthases like enzymes are classified in another subfamily of BSAS known as cyanoalanine synthase (CAS). CAS subfamily members are involved in formation of the non-protein amino-acid β -cyanoalanine by using available cysteine and toxic cyanide, with the release of sulfide (Hatzfeld *et al.*, 2000; Hell *et al.*, 2002). In *Arabidopsis*, SERAT and BSAS gene families are composed of multiple genes encoding isozymes that are localized in cytosol, plastids and mitochondria, where Cys biosynthesis can be regulated in a subcellular compartment-specific manner (Figure 1.3) (Heeg *et al.*, 2008; López-Martín *et al.*, 2008; Watanabe *et al.*, 2008a; Watanabe *et al.*, 2008b; Birke *et al.*, 2015).

1.4.2 Fate of Cysteine

Cysteine assimilates available sulfur and provides an organic form which is further used as a precursor for several sulfur containing metabolites. Because of this, cysteine is considered as the major source of reduced sulfur for the nutrition of mammals and humans. Some of the fates of

assimilated sulfur in cysteine are methionine, glutathione, iron-sulfur clusters, molybdenum cofactors, vitamins (coenzyme A, lipoic acid, thiamine and biotin) and secondary compounds such as camalexin, glucosinolates and phytochelatins (Noctor *et al.*, 2011; Hell and Wirtz, 2011; Takahashi *et al.*, 2011b) (Figure 1.2 B, C).

1.4.2.1 Cysteine to methionine

Cysteine plays an important role in methionine biosynthesis as sulfur donor that is the reason why cysteine biosynthesis was targeted to improve methionine availability in human diet. Cystathionine- γ -synthase (CGS) catalyzes the first committed step which is considered rate-limiting for methionine biosynthesis. A condensation reaction takes place between *O*-phosphohomoserine and cysteine to synthesize cystathionine. β -Cleavage of cystathionine in the presence of cystathionine- β -lyase (CBL, EC 4.4.1.8) leads to homocysteine production. The last step of methionine biosynthesis is catalyzed by methionine synthase (MS, EC 2.1.1.14). Homocysteine is converted to methionine using N⁵-methyltetrahydrofolate as methyl donor. Although MS is present in the cytosol as well as in the chloroplast, the downstream product of methionine, *S*-adenosylmethionine (SAM) is reported to be synthesized in the cytosol and then transported to plastids by a carrier-mediated facilitated diffusion process (Ravanel *et al.*, 2004). Only 20% of synthesized methionine contributes to synthesis of proteins, and the remaining 80% is accounted for SAM biosynthesis (Giovanelli *et al.*, 1985). SAM acts as a universal methyl donor for transmethylation reaction. SAM also enters in methionine recycling by entering in the Yang cycle (Sauter *et al.*, 2013). Another form of methionine derivative is *S*-methylmethionine (SMM) which is exclusive to higher plants (Ranocha *et al.*, 2001). SMM is a mobile form of S transported from vegetative to reproductive organ *via* phloem and is involved in regeneration of methionine using homocysteine methyltransferases (HMTs) and methionine methyltransferase (MMTs) (Lee *et al.*, 2008) (Figure 1.2B). The SMM cycle plays a vital role in defining methionine levels in seeds (Cohen *et al.*, 2017a). Regulation of methionine biosynthesis differs from cysteine regulation which presents a challenge for improvement of S amino acid content in plants (Hesse *et al.*, 2004; Galili and Amir, 2013).

1.4.2.2 Cysteine to glutathione

Apart from methionine another fate of cysteine is glutathione (GSH; γ -Glu-Cys-Gly) which is a tripeptide and regarded as a modified form of cysteine to avoid oxidation of the sulfhydryl groups. The presence of glutamate and glycine at the side of cysteine results in a shift in redox potential for the cysteine residue and protects the sulfhydryl group against oxidation (Meyer and Hell, 2005; Meyer, 2008). In legumes, glutathione is present as homoglutathione (hGSH; γ -Glu-Cys- β -Ala) (Yi *et al.*, 2010). GSH plays a critical role in homeostasis and cellular defense, including redox status, signal transduction and detoxification (Hell and Wirtz, 2011; Noctor *et al.*, 2011). Glutathione biosynthesis takes place via γ -glutamylcysteine (γ -Glu-Cys) formed by γ -glutamyl cysteine ligase (GCL, EC 6.3.2.2) (Leustek, 2002; Hell and Wirtz, 2011). In the second step, glutathione synthetase (GS; EC 6.3.2.3.) catalyzes the addition of glycine to γ -Glu-Cys to yield glutathione (Figure 1.2C) (Galant *et al.*, 2011). Glutathione is a storage tripeptide which can be further used for synthesis of phytochelatins (PC) (Klapheck *et al.*, 1995) (Figure 1.2C). Phytochelatins (PCs) are cysteine rich polypeptides which bind to heavy metals. The general structure of PCs is (γ -Glu-Cys)_nGly where n is 2-11 (Zenk, 1996). Synthesis of PCs is catalyzed by phytochelatin synthase (Vatamaniuk *et al.*, 1999). In cytosol PCs act as scavengers of toxic metal ions and form complexes with them. Subsequently these complexes are transported into the vacuole to rescue the plant cells from deleterious effect of heavy metals (Oven *et al.*, 2001; Yadav, 2010).

Apart from these metabolites some plant species also accumulate *S*-amino acid derivatives which connect back to cysteine biosynthesis. These derivatives might act as a storage sink for assimilated sulfur. Some of those derivatives are listed in Table 1.2.

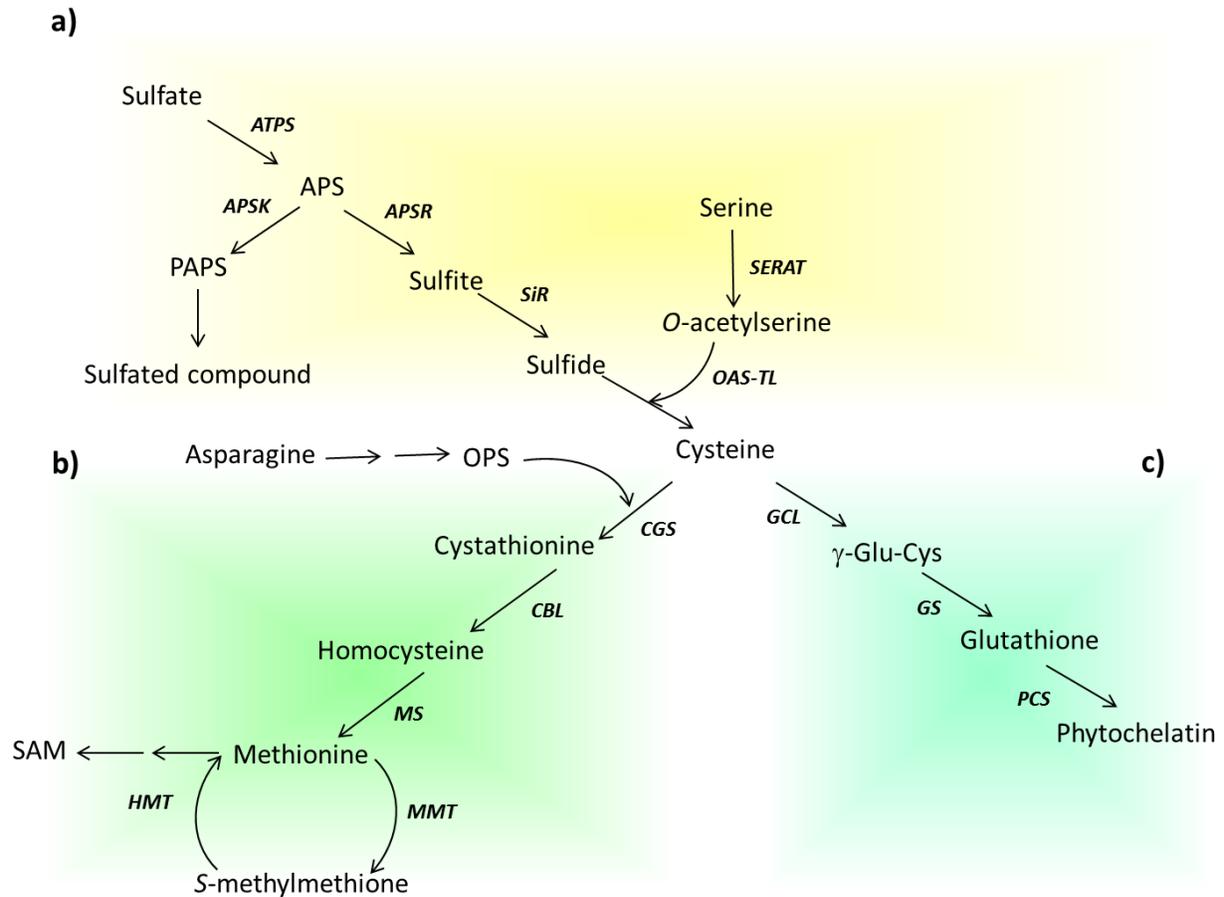


Figure 1.2: General sulfur amino acid metabolism in plants.

The area colored yellow represents cysteine biosynthesis while the two green areas represent the fate of cysteine. Multiple arrows represent more than one step reaction. **a)** Entry, activation and reduction of sulfate. **b)** Methionine biosynthesis. **c)** Glutathione biosynthesis. Abbreviations are as follows. ATP sulfurylase (ATPS): ATP:sulfate adenylyltransferase; APS reductase (APSR): adenosine 5'-phosphosulfate reductase; PAPS: 3'-phosphoadenosine 5'-phosphosulfate; APS kinase (APSK): adenosine 5'-phosphosulfate kinase; SiR: sulfite reductase; SERAT: serine acetyltransferase; OAS-TL: *O*-acetylserine thiol lyase; CGS: Cystathionine- γ -synthase; CBL: Cystathionine- β -lyase; MS: methionine synthase; HMT: Homocysteine methyltransferase; MMT: methionine methyltransferase; GCL: glutamate cysteine ligase; GS: glutathione synthetase; PCS: Phytochelatin synthase

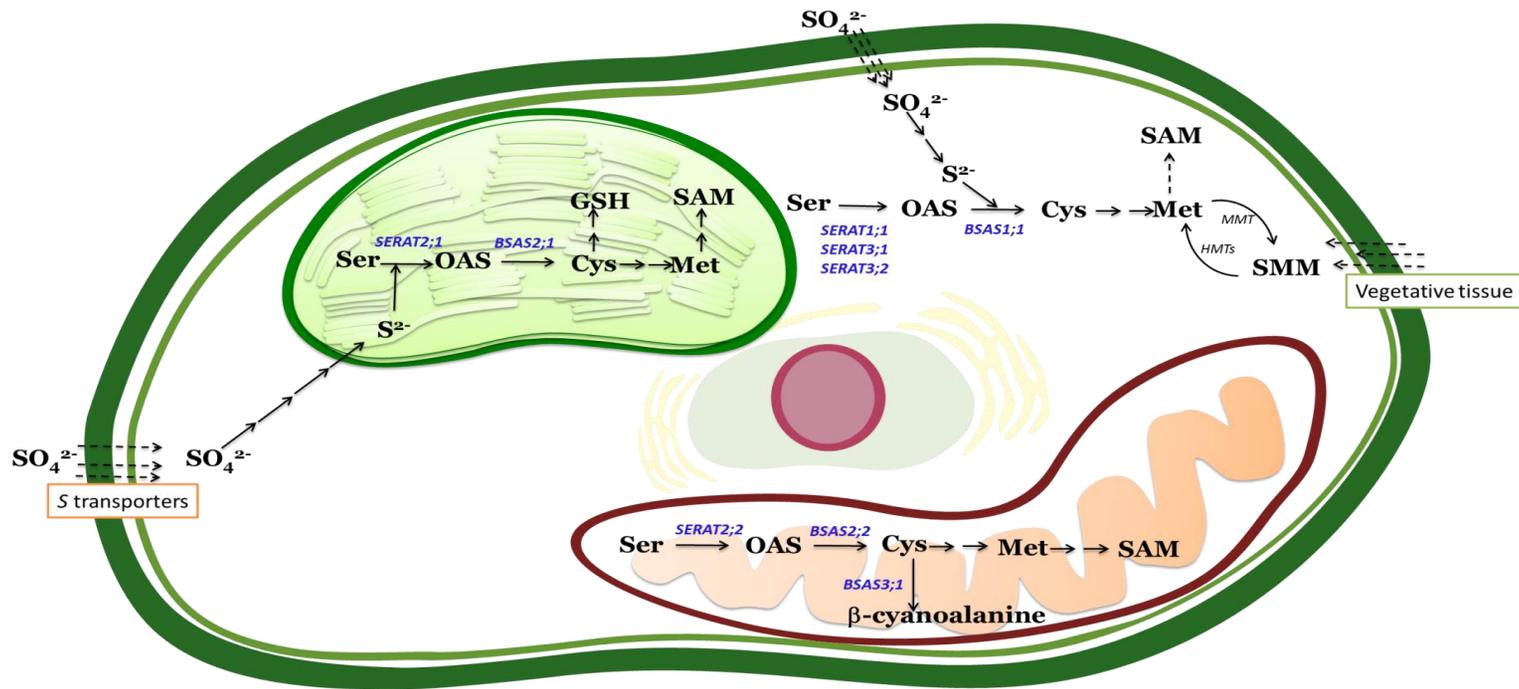


Figure 1.3: Compartment specific role of SERAT and BSAS isoforms in sulfur metabolism.

Cysteine biosynthesis takes place in three compartments: cytosol, plastids and mitochondria. Isoforms of SERAT and BSAS family members characterized in Arabidopsis are italicized. Multiple arrows represent more than one reaction. Dotted arrows represent entry from outside the cell. Abbreviation- SO_4^{2-} : sulfate, S^{2-} : sulfide, Ser: serine, OAS: O-acetylserine, Cys: cysteine, Met: methionine, SAM: S-adenosylmethionine, SMM: S-methylmethionine, GSH: Glutathione.

Table 1.2: Sulfur amino acid derivatives reported in different plant species

Amino acid derivatives	Plant species	References
S-methylCys	<i>Phaseolus vulgaris</i> , <i>Vigna unguiculata</i> , and <i>Vigna radiata</i>	(Baldi and Salamini, 1973; Evans and Boulter, 1975)
S-methylCys sulfoxides	<i>Brassica</i> species	(Fales <i>et al.</i> , 1987; Marks <i>et al.</i> , 1992)
γ -Glu-S-methylCys	<i>P. vulgaris</i> , <i>V. unguiculata</i> , and <i>V. radiata</i>	(Kasai <i>et al.</i> , 1986; Giada <i>et al.</i> , 1998)
γ -Glutamyl-methionine	<i>Vigna mungo</i>	(Otoul <i>et al.</i> , 1975)
γ -Glu-S-ethenylcysteine	<i>Vicia narbonensis</i>	(Arias <i>et al.</i> , 2005; Sanchez-Vioque <i>et al.</i> , 2011)
S-Alk(en)yl-cysteine sulfoxides	<i>Allium</i> species	(Jones <i>et al.</i> , 2004; Rose <i>et al.</i> , 2005; Yoshimoto <i>et al.</i> , 2015b)
S-Methylhomoglutathione	<i>V. radiata</i> and <i>P. vulgaris</i>	(Kasai <i>et al.</i> , 1986; Liao <i>et al.</i> , 2013)

1.5 Strategies to improve protein quality in legumes

Legumes have high potential for nutritional quality improvement and S-amino acids occupy a central position in improvement strategies. The primary focus has been hitherto to improve cysteine and methionine content in seed storage proteins by transgenic development, synthetic protein synthesis or traditional breeding.

Traditional breeding approaches rely on mutant lines where storage protein deficiency is compensated through a mechanism of proteome rebalancing, resulting in improvement of essential amino acid levels (Herman, 2014; Wu and Messing, 2014). The maize *opaque-2* mutant provides such an example where the mutation affected expression of α -zeins but a two fold increase was observed in lysine, a limiting amino acid in grains (Gibbon and Larkins, 2005). *opaque-2* mutants are key players in the development of “Quality Protein Maize” for better nutritional quality maize in developing countries. In the case of soybean, a mutant line resistant to ethionine had 20% improvement in methionine and cysteine levels (Imsande, 2001). In soybean QTLs associated with sulfur amino acids methionine and cysteine were identified (Panthee *et al.*, 2006a; Panthee *et al.*, 2006b) which were later used to identify QTLs related to protein and oil content of soybean seeds (Van and McHale, 2017).

Transgenic approaches focus on either creating an additional storage sink by expressing a protein rich in sulfur amino acids and/or manipulation of amino acid biosynthesis pathways (Amir and Tabe, 2006; Shewry, 2007; Ufaz and Galili, 2008; Tabe *et al.*, 2010; Nguyen *et al.*, 2012). For example, transgenic expression of Brazil nut 2S albumin (Townsend and Thomas, 1994) or 15 kDa δ -zein has increased methionine and cysteine concentration in soybean (Dinkins *et al.*, 2001). In common bean, the expression of Brazil nut 2S albumin increased methionine concentration by 20% (Aragão *et al.*, 1999). In *Vicia narbonensis*, co-expression of Brazil nut 2S albumin with a bacterial feedback-insensitive asparagine kinase increased methionine and cysteine concentrations by 100% and 20%, respectively. The increase in cysteine and methionine was as a result of decreases in the concentration of γ -Glu-S-ethenyl-Cys and free thiols, particularly γ -Glu-Cys and glutathione (Demidov *et al.*, 2003). However, allergenic properties of

Brazil nut 2S albumin make it a non-preferred agent for seed protein improvement (Nordlee *et al.*, 1996). Recently a synthetic protein MB-16 was produced in soybean, which resulted in an increase in methionine and cysteine, by 16.2 and 65.9%, respectively (Zhang *et al.*, 2014). In order to manipulate the biosynthetic pathway a seed specific transgenic expression of *Arabidopsis* plastidic SERAT in lupin resulted in an increased concentration of free cysteine but no change in free methionine concentration, leading to a hypothesis of separate regulation of methionine and cysteine biosynthesis (Tabe *et al.*, 2010). In soybean, constitutive over-expression of a cytosolic form of *O*-acetylserine thiol lyase (OAS-TL), led to sustained enzymatic activity at the late stages of seed development and resulted in a 70% increase in total cysteine concentration in mature seed (Kim *et al.*, 2012). This remarkable increase in total cysteine content was associated with enhanced levels of the Bowman–Birk protease inhibitor. Expression of methionine-insensitive form of *Arabidopsis* cystathionine γ -synthase (AtD-CGS), a first committed enzyme of methionine biosynthesis in soybean led two fold increase in methionine content (Song *et al.*, 2013).

Undoubtedly extensive knowledge of sulfur metabolism, its regulation and QTL related *S*-metabolism helped to develop strategies to improve protein quality in legumes. In the near future shifting *S*-pool from non-proteinaceous *S*-derivatives to *S*-amino acids could be an effective strategy to improve protein quality of legumes. One such target metabolite in common bean can be *S*-methylCys.

1.6 *S*-methylCys as an analogue to *S*-amino acids - A potential target for protein quality

S-methylCys is a cysteine derivative which shares lower homology with methionine. Being an analogue to cysteine and methionine it replaces cysteine and methionine as substrate in various enzymatic reactions in animal and plant metabolism. In nature, it is present as *S*-methylCys and its sulfoxide and is reported to be present in *Leguminosae*, *Brassicaceae* and *Alliaceae* family members (Maw, 1982; Padovese *et al.*, 2001; Taylor *et al.*, 2008; Edmands *et al.*, 2013; Kodera *et al.*, 2017). A selenium derivative of cysteine, *Se*-methylselenocysteine was also reported in the genus *Astragalus* and is analogous to *S*-methylCys (Sors *et al.*, 2005; Sors *et al.*, 2009). In legumes, *S*-methylCys and its dipeptide γ -Glu-*S*-methylCys are present in relatively large

quantities as compared with other families. In *P. vulgaris* around 10-15 $\mu\text{mol/g}$ *S*-methylCys was reported in dry seeds (Zacharius, 1970; Evans and Boulter, 1975) which is higher than any other legumes. Taxonomic analysis of presence of *S*-methylCys reveals that all *Phaseolus* and several *Vigna* species except black gram (*Vigna mungo*) and adzuki bean (*Vigna angularis*) synthesize *S*-methylCys (Baldi and Salamini, 1973; Kasai and Larsen, 1980; Kasai *et al.*, 1986). Along with pipercolic acid, *S*-methylCys was reported as a biomarker of common bean rich diet in human and mouse studies (Perera *et al.*, 2015)

S-MethylCys and γ -Glu-*S*-methylCys are non-proteinaceous amino acids, and accumulate exclusively in the seeds (Watanabe *et al.*, 1971). Reviews on *S*-methylCys and its sulfoxide have discussed potential antidiabetic, anti-carcinogenic and antioxidant effect of *S*-methylCys on animals (Edmands *et al.*, 2013; Akash *et al.*, 2014; Hsia and Yin, 2015). Human bronchial epithelial cells treated with *S*-methylCys had reduced ROS generation when treated with H_2O_2 which helped in maintenance of glutathione redox cycle in cells (Hsia and Yin, 2015). On the other hand, many earlier studies have reported anti-nutritional effects of *S*-methylCys (Benevenga *et al.*, 1976; Case and Benevenga, 1976; Padovese *et al.*, 2001). Rats fed with casein and *S*-methylcys supplemented diets had reduced food intake and weight gain which was associated with enlargement of the kidney (Benevenga *et al.*, 1976; Padovese *et al.*, 2001).

In common bean, *S*-methylCys and γ -Glu-*S*-methylCys content share inverse relationship with cysteine and methionine content. Two genetically related lines SMARC1-PN1 and SMARC1N-PN1 are deficient in major storage proteins, phaseolin and lectin while SARC1 has a variant of arcelin-1 (Osborn *et al.*, 2003). Storage protein deficiency is correlated with increased cysteine and methionine content in SMARC1-PN1 and SMARC1N-PN1. In these genetically related lines, increases in cysteine and methionine by 70% and 10%, respectively, were observed at the expense of the non-protein amino acid *S*-methylCys (Taylor *et al.*, 2008).

Despite recent progress towards elucidating the biosynthetic pathway of cysteine and its fate in plant tissue, little is known about the biosynthesis of *S*-methylCys or γ -Glu-*S*-methylCys. *Allium* genus members, onion (*Allium cepa*) and garlic (*Allium sativum*) accumulate *S*-methylCys as a sulfoxide and biosynthesis is initiated by *S*-alkylation of GSH (Turnbull *et al.*, 1980; Lancaster *et*

al., 1989; Jones *et al.*, 2007; Yoshimoto *et al.*, 2015a; Yoshimoto *et al.*, 2015b). Production of *S*-methylCys in *Arabidopsis* cell cultures was reported to be associated with methionine catabolism (Giovannelli *et al.*, 1980; Rebeille *et al.*, 2006). In radish, the relatively high recovery of radioactivity in methyl cysteine sulfoxide after radiolabelled cysteine or methyl-labelled methionine application indicated that *S*-methylCys is formed by direct methylation of cysteine (Thompson and Gering, 1966). In *Astragalus* species the synthesis of *Se*-methylselenocysteine is activated by *Se*-methyltransferase (SMT) by using either *S*-adenosylmethionine (SAM) or *S*-methylmethionine (SMM) as methyl sources (Sors *et al.*, 2009).

1.7 Candidate genes for *S*-methylCys biosynthesis in common bean

Liao *et al.* (2012) published a detailed comparative gene expression analysis between SARC1 and SMARC1N-PN1 lines. Up-regulation of *SERAT1;1* and *-1;2* gene expression in SMARC1N-PN1 revealed an activation of cytosolic *O*-acetylserine biosynthesis for cysteine synthesis, while down-regulation of plastidic *SERAT2;1* in SMARC1N-PN1 suggested a spatial separation of cysteine and *S*-methylCys biosynthesis in the cytosol and plastid, respectively (Figure 1.2). This analysis shed some light on candidate genes involved in *S*-methylCys biosynthesis in common bean seeds. Another comparative transcriptome profiling of sulfur metabolism related genes in *P. vulgaris* and *V. mungo*, pointed out some candidate genes associated with *S*-methylCys (Liao *et al.*, 2013). In this study, expression of the cytosolic *SERAT1;1* and *-1;2* was antagonistic to predicted plastidic *SERAT2;1*, which was similar to the previous analysis (Liao *et al.*, 2012). Transcript levels of *SERAT1;1* and *-1;2* were four-fold higher in *P. vulgaris*, while expression of the *SERAT2;1* was two-fold higher in *V. mungo*. Among *BSAS* family members, *BSAS4;1* and *BSAS3;1* were more highly expressed in *P. vulgaris* than in *V. mungo*. The expression of *BSAS3;1* encoding a β -cyanoalanine synthase (Watanabe *et al.*, 2008a) was nine fold higher in *P. vulgaris* than *V. mungo*. Recently, published RNA-Seq based gene expression atlas also identifies *BSAS3;1* and *BSAS4;1* as seed specific β -cyanoalanine synthases (O'Rourke *et al.*, 2014). Based on these transcriptome profiling data I predicted a possible role of *BSAS* family members in *S*-methylCys biosynthesis in common bean seeds.

1.8 Objectives of Study

My long-term objective of this study is to introduce candidate gene(s) for high cysteine synthesis into common bean cultivars and improve nutritional quality of bean. In common bean the suboptimal level of essential sulfur amino acids, methionine, and cysteine is predicted due to a shift of sulfur from the cysteine pool to the non-protein amino acid, *S*-methylCys. The major aim of this study is to understand *S*-methylCys biosynthesis in common bean developing seeds. Previously, efforts were made to fully understand sulfur metabolism in *Arabidopsis* and some other crops but no such effort was made to decode sulfur metabolism in common bean. In today's world, due to the advent of next generation sequencing, the genome of common bean has been fully sequenced and extensively annotated (Schmutz *et al.*, 2014; Vlasova *et al.*, 2016). This provides us an extra edge to understand key pathway enzymes of sulfur metabolism in common bean.

Therefore, the objectives of this study are as follows:

- 1. To study the common bean BSAS family to determine its role in cysteine biosynthesis. Further, investigate the role of cysteine synthase family members in *S*-methylCys biosynthesis.**

This study will include an evolutionary relationship of common bean BSAS family members with *Arabidopsis* and soybean, characterization of seed specific cysteine synthases and their role in *S*-methylCys biosynthesis and, investigate subcellular localization of candidate cysteine synthases to learn about the role of subcellular compartmentation in *S*-methylCys biosynthesis

- 2. To elucidate the biochemical pathway of *S*-methylCys synthesis in developing seeds of common bean.**

This study will focus on identifying the precursor amino acid for *S*-methylCys by using isotope tracking method. A combination of targeted and non-targeted high resolution mass spectrometry (HRMS) will be used to track movement of labelled carbon (^{13}C) and labelled nitrogen (^{15}N) in developing common bean seeds.

- 3. To characterize the function of gene(s) and enzyme(s) responsible for the biosynthesis of *S*-methylCys in common bean**

Based on *S*-methylCys pathway elucidation from isotopic label tracking, the function of candidate genes and enzymes in *S*-methylCys biosynthesis will be determined by performing recombinant enzyme assays and using precursor(s) as substrate(s).

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CHAPTER TWO - CHARACTERIZATION OF *Phaseolus vulgaris* BSAS3;1 AND ITS POSSIBLE ROLE IN S-METHYLCYSTINE BIOSYNTHESIS

2.1 Introduction

In nature, sulfur is present in both inorganic and organic forms. Sulfur enters plant metabolism in the form of inorganic sulfate; the primary organic acceptor molecule is *O*-acetylserine, which yields the amino acid cysteine. Cysteine is the central metabolite of *S*-metabolism pathway and is used for synthesis of various *S*-containing metabolites. Free cysteine content in plants is reported to be very low but its flux is quite high. High flux of cysteine in the plant cell summarizes quick utilization of cysteine in cell for the methionine, glutathione and protein biosynthesis (Leustek *et al.*, 2000; Hell and Wirtz, 2011). Cysteine biosynthesis takes place in the various compartments of the plant cell where *O*-acetylserine provides the carbon backbone while sulfur is provided in the form of sulfide. Integration of sulfide into the carbon skeleton requires two reactions. In first step biosynthesis of *O*-acetyl serine is catalyzed by (SERAT; EC 2.1.3.30) by utilizing serine and acetyl coenzyme A. Subsequently a β -replacement reaction is catalyzed by *O*-acetylserine (thiol) lyase (OAS-TL) [β -substituted alanine synthase (BSAS); EC 2.5.1.47] which replaces acetyl moiety with sulfide forming cysteine.

Cysteine synthases (CS/OAS-TL) constitute a subfamily of β -substituted alanine synthase (BSAS) family (Hatzfeld *et al.*, 2000). β -Substituted alanine synthase (BSAS) family members contain the characteristic pyridoxal-5'-phosphate (PLP) cofactor (Alexander *et al.*, 1994). In plants, various isoforms of OAS-TL are present and are involved in cysteine biosynthesis. Some of the BSAS family members are cysteine synthase-like proteins but are not involved in cysteine biosynthesis. One such member is β -cyanoalanine synthase (CAS), which utilizes cysteine to detoxify cyanide synthesized in the cell during ethylene biosynthesis, and forms a separate subfamily of BSASs (Yu *et al.*, 2012). Apart from these two major reactions BSAS isoforms are

also involved in some secondary reactions depending on developmental stage and light conditions (Burandt *et al.*, 2001).

One important characteristic which plays a major role in defining the activity of BSAS family members is subcellular compartmentation. Cysteine biosynthesis in a plant cell is reported to be under compartment-specific regulation (Heeg *et al.*, 2008). Cysteine biosynthesis takes place in three compartments within the cell; plastid, mitochondria and cytosol (Watanabe *et al.*, 2008a; Birke *et al.*, 2013). Among all BSAS family members *Arabidopsis* BSAS1;1, BSAS2;1, and BSAS2;2 which are localized in cytosol, plastids, and mitochondria respectively, were reported as major CSases for cysteine biosynthesis (Hell *et al.*, 1994; Wirtz *et al.*, 2004). Using reverse genetics approach, redundancy of three SERAT and OAS-TL was reported in *Arabidopsis* which suggests traffic between these compartments for sulfide, *O*-acetylserine and cysteine (Watanabe *et al.*, 2008a; Watanabe *et al.*, 2008b; Lee *et al.*, 2013a). In combination with the corresponding SERAT, BSAS isoforms perform different reactions, depending on environmental pH and availability of H₂S within the compartment (Romero *et al.*, 2014).

The cytosol is the prime compartment for cysteine biosynthesis. In *Arabidopsis*, various BSAS family members perform cysteine synthase activity by forming a cysteine synthase complex with SERAT family members, and regulate cysteine biosynthesis which is limited by *O*-acetylserine availability rather than sulfide (Wirtz and Hell, 2006; Romero *et al.*, 2014). In *Arabidopsis* while cytosolic AtBSAS1;1 was reported to be the most contributing cysteine synthase in roots and leaves, other cytosolic CS AtBSAS4;1, AtBSAS4;2 and AtBSAS4;3 had less contribution in cysteine biosynthesis (Watanabe *et al.*, 2008a). These BSAS family members were recently shown to encode cysteine desulfhydrases (DES, EC 4.4.1.1) in the cytosol. To maintain cysteine homeostasis Cys desulfhydrase catalyzes the degradation of cysteine to pyruvate, ammonia, and sulfide (Álvarez *et al.*, 2010).

In plastids which are the exclusive compartment for sulfate reduction, AtBSAS2;1 was reported to be responsible for cysteine synthesis (Jost *et al.*, 2000; Birke *et al.*, 2012). However, another BSAS member in plastids, *AtBSAS5;1* encodes sulfocysteine synthase activity and is essential for chloroplast activity and redox regulation (Bermúdez *et al.*, 2010; Birke *et al.*, 2015). The

sulfocysteine synthase activity of BSAS family member acts as a protective protein sensor to detect the accumulation of thiosulfate. Formation of thiosulfate in the plant cell is not very well understood but predicted to be catalyzed by a sulfurtransferase from sulfite and persulfide (Krübel *et al.*, 2014). Under excess light conditions, thiosulfate produced due to the presence of reactive oxygen species becomes a substrate for *S*-sulfocysteine synthesis catalyzed by AtBSAS5;1 in plastids. This reaction triggers protection mechanisms of the photosynthetic apparatus and *S*-sulfocysteine can be metabolized by reductive conversion to cysteine and sulfate (Bermúdez *et al.*, 2010).

In mitochondria, due to excess accumulation of cyanide during ethylene production (Yang and Hoffman, 1984; Yip and Yang, 1988; Seo *et al.*, 2011), one of the BSAS family members plays a major role in cyanide detoxification rather than cysteine biosynthesis (Hatzfeld *et al.*, 2000; Yamaguchi *et al.*, 2000). Another AtBSAS2;2 acts as CS in mitochondria. In the case of *Arabidopsis*, spinach (Warrilow and Hawkesford, 1998) and soybean (Yi and Jez, 2012), a CAS subfamily member, BSAS3;1, catalyzes quenching of cyanide molecule by cysteine and synthesis of β -cyanoalanine (Garcia *et al.*, 2010). β -cyanoalanine is channelled back to amino acid synthesis pathway via asparagine (Asn) biosynthesis (Castric *et al.*, 1972) or conjugated to synthesize γ -glutamyl- β -cyano-alanine in *Arabidopsis* and cyanogenic plants such as *Vicia* and *Lathyrus* species (Ressler, 1962; Watanabe *et al.*, 2008a). γ -Glutamyl- β -cyano-Ala as a dipeptide serves as storage molecule in the case of *Arabidopsis*, while in the case of *Vicia sativa* and *Lathyrus sylvestris* it was reported as a possible neurotoxin (Harper and Arscott, 1962; Ressler *et al.*, 1969)

Apart from these known capabilities of BSAS family members, BSAS enzymes can utilize other substrates in supplementary reactions and synthesize other *S*-amino acid derivatives (Table 1.2). As discussed in Section 1.1 common bean (*Phaseolus vulgaris*) and several *Vigna* species accumulate *S*-methyl-cysteine (*S*-methylCys) and its dipeptide γ -glutamyl-*S*-methylCys (γ -Glu-*S*-methylCys). To gain understanding of *S*-methylCys biosynthesis in *P. vulgaris*, a comparative transcriptome analysis was performed between *P. vulgaris* and *Vigna mungo*. Compared to *V. mungo* which does not accumulate γ -Glu-*S*-methylCys, *P. vulgaris* had two BSAS family

members upregulated. In *P. vulgaris* BSAS3;1 was highly expressed with approximately nine fold more ESTs than *V. mungo* (Liao *et al.*, 2013). In *Arabidopsis* and soybean, BSAS3;1 was reported as a CAS family member involved in cyanide detoxification using a thiol exchange reaction (Watanabe *et al.*, 2008a; Yi *et al.*, 2012). My hypothesis was that PvBSAS3;1 can catalyze a thiol exchange reaction using methanethiol and cysteine as substrates, forming *S*-methylCys. To test this hypothesis, I used phylogenetic, physical and biochemical methods to characterize BSAS family members in common bean and their role in *S*-methylCys biosynthesis.

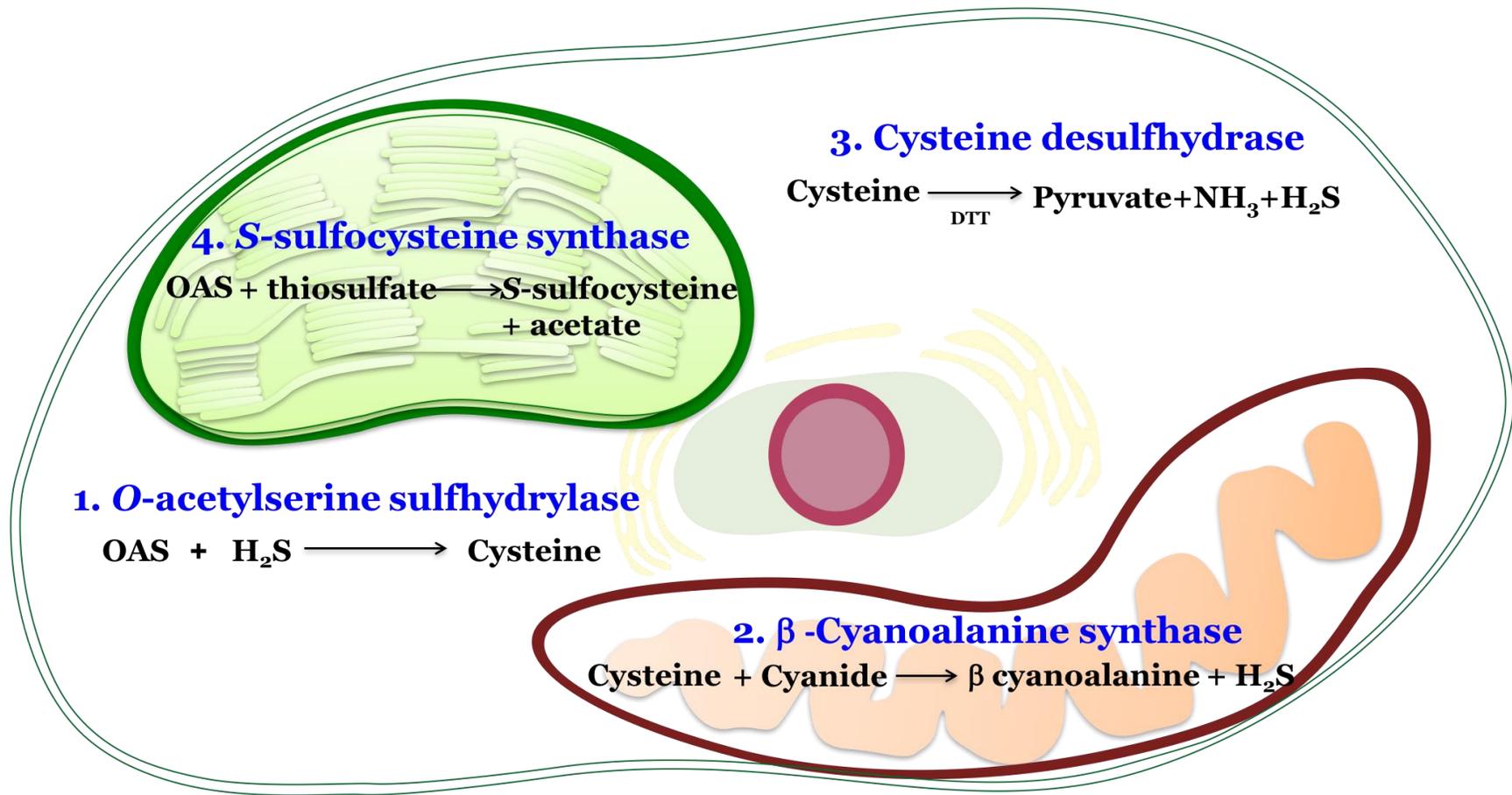


Figure 2.1: Various reactions of BSAS family members in three major subcellular compartments.

Two major substrates for BSAS family members are *O*-acetylserine (OAS) (reaction 1 and 4) or cysteine (reaction 2 and 3). Products of different reactions catalyzed by BSAS family members include cysteine, β -cyanoalanine, *S*-sulfocysteine and pyruvate.

2.2 Materials and methods

2.2.1 Plant materials

Common bean (*P. vulgaris*) genotype BAT93 and black gram (*V. mungo*) cultivar Barimash-2 (Afzal et al., 2002) plants were grown in growth cabinets (Environmental Growth Chambers, Chagrin Falls, OH, USA) under 16 h light (300-400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 8 h dark at a temperature cycling between 18 and 24 °C as described by Pandurangan et al. (2012). Seeds were germinated in vermiculite and 12-day old seedlings were transplanted to pots (17 × 20 cm) containing Promix BX soil (Premier Tech Horticulture, Québec, Canada) and regularly fertilized with a nitrogen, phosphorous, and potassium (20-20-20) mixture.

For subcellular localization studies, *Nicotiana benthamiana* plants were grown in a growth cabinet at 22 °C with a 16 h photoperiod (110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Seeds were germinated in Promix BX soil and after two weeks, plants were transferred to pots (10.5 x 9.0). Plants were fertilized once a week with mixture of nitrogen, phosphorous, and potassium (20-20-20) at 0.25 g/L. Leaves from six-week-old plants were infiltrated for transient expression.

2.2.2 Phylogenetic analysis

To identify putative common bean BSAS family enzymes, eight *Arabidopsis* sequences with accession numbers At4g14880, At2g43750, At3g59760, At3g61440, At5g28020, At3g04940, At3g03630, and At5g28030 (Watanabe et al., 2008a) were used as query to perform BLASTP-search of *P. vulgaris* v2.1 database available at www.phytozome.net. Multiple sequence alignment using the eight common bean, 16 soybean and eight *Arabidopsis* BSASs was prepared with CLUSTALW2 and transferred to MEGA7 for phylogenetic analysis (Kumar et al., 2016). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987; Kumar et al., 2016) based on the JTT matrix-based model using 1000 bootstrap value (Jones et al., 1992).

2.2.3 Cloning of BSAS3;1 for recombinant protein expression

A truncated form of β -substituted alanine synthase (BSAS) gene was amplified from both common bean (*P. vulgaris*) line BAT93 and black gram (*V. mungo*) cultivar Barimash-2. RNA was extracted from developing seeds as described by Wang and Vodkin (1994), and quantified by spectrophotometry with a NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). The quality of RNA was evaluated from $A_{260/280}$ ratio and by 1% agarose gel electrophoresis. One μ g of total RNA treated with amplification-grade DNase I (Life Technologies, Burlington, ON, Canada) was used for cDNA synthesis using qScript™ cDNA SuperMix (Quanta Biosciences).

The coding region of *PvBSAS3;1* without the putative signal peptide (the N-terminal 58 amino acids, encoded by the first 174 bp from the ATG) was amplified by polymerase chain reaction (PCR) using Pfx50 DNA polymerase (Life Technologies, Burlington, ON). To design primers for amplification 454 pyrosequencing data available at the Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI) under the accession numbers SRS360251 for *P. vulgaris* and SRS360666 for *V. mungo* was used (Liao et al., 2013). The primer sequences were PvBSAS3;1 (Fw: 5'-GGTGATGATGATGACAAGACCAATATCAAAAAGCATGTG-3' and Rvs: 5'-GGAGATGGGAAGTCATTAATCAACTGCTACTGGCTGC-3') and VmBSAS3;1 (Fw: 5'-GGTGATGATGATGACAAGACAAACATCAAGAAGCATGTG-3' and Rvs: 5'-GGAGATGGGAAGTCATTAGTCAACTGCTACTGGCTGC-3'), containing the vector-specific sequences (underlined). The amplification conditions were 94 °C for 30 s; followed by 35 cycles of 55 °C for 30 s, 68 °C for 60 s. The PCR products were gel purified and subcloned into the expression vector, pLATE51 with an N-terminal His-tag as per the manufacturer's protocol [aLICator LIC Cloning and Expression Kit 2 (N-terminal His-tag/EK), #K1251, Thermo Scientific]. Positive recombinant clones selected on ampicillin (100 μ g/mL) plates were confirmed by restriction digests (*EcoRV* and *HindIII*) and DNA sequencing using the LIC sequencing primers. The constructs were then transformed into One Shot BL21 (DE3) cells (Invitrogen, cat # C6000-03).

2.2.4 Protein purification

One Shot BL21 (DE3) cells were grown in NZY media with ampicillin (100 µg/mL) as antibiotic at 37 °C. At OD of 0.6, cells were induced with 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) and allowed to grow at room temperature for 14-16 hours. Cells were harvested by centrifugation (6,000 × g, 30 min at 4 °C) and suspended in binding buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl and 0.01% Tween®-20). Suspended cells were treated with 1 mg/ml lysozyme for 30 min, lysed with French press and supernatant was collected after centrifugation at 18,000 × g for 30 minutes. Supernatant was applied to Cobalt-based Dynabeads® His-Tag Isolation & Pulldown beads (Thermo Fisher Scientific) and incubated on a roller for 2 h at 4 °C. Beads were collected after placing the tubes on magnet and supernatant was discarded. To remove all unbound protein, beads were washed six times with wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl and 0.01% Tween®-20) and His-tagged protein was eluted by using 2.5 mL of elution buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 300 mM imidazole and 0.01% Tween®-20. Eluted protein was buffer exchanged with 50 mM Tris-HCl pH 9.0 using a PD-10 column (GE Healthcare Life Sciences) and concentrated using the Amicon Ultra-15 Centrifugal Filter Unit (Millipore, Etobicoke, ON). Purified protein was flash frozen using liquid nitrogen with 10% glycerol and stored at -80°C for biophysical and biochemical assays.

2.2.5 Size exclusion chromatography

Size exclusion chromatography was performed on ÄKTApurifier system (GE Healthcare) using HiLoad Superdex 200 prep grade column with FPLC buffer (20 mM Tris-HCl buffer, pH 9.0, 150 mM sodium chloride). Molecular weight of eluted protein was calculated based on a standard curve generated with Gel Filtration Standard (Bio-Rad). The protein quantification was performed using the Bio-Rad Protein Assay solution (Mississauga, ON), and bovine serum albumin (BSA) as standard. Purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using a 12% polyacrylamide gel, and the protein bands were visualized by Coomassie staining as described (Laemmli, 1970).

2.2.6 Enzyme assays

To determine the CAS activity of PvBSAS3;1 and VmBSAS3;1, a reaction mixture of 500 μ L containing 50 mM Tris-HCl (pH 9.0) buffer, 1 mM cysteine, 2 mM KCN and 50 ng of protein was incubated at 25 °C for 10 min. The reaction was stopped by adding 50 μ l of 30 mM FeCl₃ in 1.2 N HCl and 50 μ l of 20 mM N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) in 7.2 N HCl. Production of methylene blue due to reaction of one molecule of produced H₂S with two molecules of DMPD was spectrophotometrically determined at 670 nm using PowerWave XS plate reader with Gen5.5 software (BioTeK Instruments, Winooski, VT). CAS activity was expressed as the amount (moles) of H₂S produced by 1 mg protein in 1 sec (katal mg⁻¹). To test *S*-methylCys formation, KCN was substituted with sodium thiomethoxide. To determine the kinetic parameters of PvBSAS3;1, various concentrations of substrates including cysteine (0-2 mM), KCN (0-1 mM) and sodium thiomethoxide (0-1 mM) were used in the enzyme assay reaction. Multiple concentrations of Na₂S were used to generate a standard curve to determine H₂S released in the reaction. Steady state kinetic parameters K_m and V_{max} were determined by nonlinear fitting of initial velocity versus substrate concentration to the Michaelis-Menten equation.

2.2.7 Cloning BSAS3;1 for subcellular localization

For the subcellular localization study, *PvBSAS3;1* was amplified by PCR using attB1 and attB2 site-containing Gateway primers. The band corresponding to *PvBSAS3;1* size was purified from gel using DNA Gel Extraction Kit (Qiagen, USA). *PvBSAS3;1* amplicon with attB1 and attB2 sites was recombined with the entry vector pDONR-Zeo (Invitrogen, USA) using BP clonase reaction mix (Invitrogen, USA) following Gateway cloning methods. The BP reaction product was transformed into *E. coli* XL10 Gold and grown on zeocin (50 μ g/mL) supplemented LB medium. Colonies were screened by restriction digest. Plasmid DNA with *PvBSAS3;1* was extracted using High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd, Taiwan) and sent for sequencing by using M13 forward and reverse primer. After confirmation of *PvBSAS3;1* in pDONR-Zeo with restriction digestion and sequencing, a LR recombination reaction was performed using entry clone pDONR-Zeo-PvBSAS3;1 and destination vector pEarleyGate101

(Invitrogen, USA). *E. coli* strain XL10-Gold (Agilent Technologies, Mississauga, ON) was used to transform cell with destination clone. Cells were grown on kanamycin (50 µg/mL) supplemented LB medium and recombinants were screened by restriction digest (*Pci*I). The plasmid DNA carrying the ‘destination clone’, pEG101-PvBSAS3;1 was transformed into *Agrobacterium tumefaciens* strain GV3101 for infiltration.

2.2.8 Plant transformation

To monitor the transient expression of fused PvBSAS3;1 the construct (pEG101-PvBSAS3;1) in *A. tumefaciens* strain GV3101 was transiently expressed into *N. benthamiana* leaf epidermal cells by infiltration (Sparkes *et al.*, 2006). Briefly, a single colony was used to inoculate a medium (LB broth containing 10 mM 2-(N-morpholino) ethanesulfonic acid [MES] pH 5.6, and 100 µM acetosyringone) supplemented with kanamycin (50 µg/mL), rifampicin (25 µg/mL), and gentamycin (50 µg/mL) and grown at 28 °C with shaking (250 rpm) until the OD₆₀₀ reached 0.5-0.6. The culture was centrifuged in a microcentrifuge tube at 3,000 g for 30 minutes at room temperature. The pellet was re-suspended in Gamborg’s solution (3.2 g/L Gamborg’s B5 medium with vitamins, 20 g/L sucrose, 10 mM MES pH 5.6, and 200 µM acetosyringone) to a final OD₆₀₀ equal to 1 and incubated at room temperature for 1 h with gentle agitation to activate the virulence gene required for transformation. To verify subcellular localization of PvBSAS3;1 a translationally fused mitochondrial protein (cytochrome oxidase with CFP) was mixed with pEG101-PvBSAS3;1 in a 1:1 ratio and coexpressed in *N. benthamiana* leaves (Nelson *et al.*, 2007).

The leaves of 4-5-week-old *N. benthamiana* plants were transformed using a 1 mL syringe. Bacteria were slowly injected into the abaxial side of the leaf. The infiltrated leaves were labelled and plants were returned to the growth room under normal conditions as described in section 2.2.1. Protein expression was visualized by confocal microscopy after 48 h.

2.2.9 Confocal microscopy

Epidermal cell layers of *N. benthamiana* leaves were visualized using an OLYMPUS confocal microscope. A 60 × water immersion objective was used at excitation wavelengths of 514 and

458 nm, and emission spectra of 530-560 nm and 470-500 nm, for YFP and CFP, respectively. A ‘Sequential Scan Tool’, which records fluorescence in a sequential fashion, was used for studying co-localization of PvBSAS3;1 with marker protein.

2.3 Results

2.3.1 BSAS family members

To identify all the members of the BSAS family in *P. vulgaris*, previously identified *Arabidopsis* (Watanabe *et al.*, 2008b) and *P. vulgaris* (Liao *et al.*, 2013) sequences were used as a BLAST search query against the protein database of *P. vulgaris* v2.1 available in Phytozome (www.phytozome.net). In total, eight *PvBSAS* genes are present across eleven chromosomes of *P. vulgaris*. Table 2.1 presents detailed information on gene location, gene length and prediction of subcellular localization of various BSAS family members in the common bean genome.

The eight common bean BSASs share greater than 50% amino acid sequence identity with each other. These eight members are present on six different chromosomes and predicted to be having cysteine synthase or cysteine synthase like activity (Table 2.1). Out of eight *PvBSAS*s, three of the members, *PvBSAS3;1*, *PvBSAS2;1* and *PvBSAS5;1* contain around 50 amino acid long putative transit peptide sequences at the N-termini and are predicted to be localized in subcellular organelles. Other five *PvBSAS*s do not have transit peptides and are predicted to be cytoplasmic in nature. According to WOLF pSORT prediction (<http://www.genscript.com/wolf-psort.html>) (Horton *et al.*, 2007), *PvBSAS2;1*, *PvBSAS3;1* and *PvBSAS5;1* are localized in the chloroplast. The theoretical molecular weight of six of the members is around 34 kDa while proteins containing transit peptide have molecular weight of 41 kDa. Among all the BSAS family members *PvBSAS3;1* is predicted to have CAS activity while *BSAS4;1* and *BSAS4;2* are may be bifunctional in nature (both CS and CAS activity).

All the BSAS family members in common bean are highly conserved and contain the characteristic pyridoxal 5'-phosphate (PLP) binding domain. The PLP binding domain has a lysine residue which binds PLP through a Schiff base linkage (Bonner *et al.*, 2005;Lai *et al.*,

2009). Sequence alignment of all the BSAS family members reveals the conserved Lys residue at position 102 in relation with PvBSAS3;1 sequence (Figure 2.2).

For cysteine biosynthesis, OAS-TL interacts with SERAT to make the cysteine synthase complex. Presence of the β 8A- β 9A loop (Lys²¹⁷ to Phe²³⁰ in AtBSAS1;1) was predicted to be potential interaction site for OAS-TL and SERAT interaction (Bonner *et al.*, 2005). In case of PvBSAS family this loop is highly conserved.

Table 2.1: Molecular and genetic characteristics of the *Phaseolus vulgaris* BSAS gene family

Gene Name	Locus name	Gene Location	Coding sequence	Predicted protein		
				molecular weight (kDa)	Subcellular* localization	Subfamily
PvBSAS1;1	Phvul.002G045200	Chr02:4196019..4202149	978	34.26	Cytoplasmic	CS
PvBSAS1;2	Phvul.006G099100	Chr06:20983629..20987672	978	34.36	Cytoplasmic	CS
PvBSAS1;3	Phvul.007G057600	Chr07:4847776..4852447	978	34.06	Cytoplasmic	CS
PvBSAS2;1	Phvul.003G060200	Chr03:8251298..8265078	1164	35.11	Chloroplast	CS
PvBSAS3;1	Phvul.008G061100	Chr08:5554616..5558227	1143	34.80	Mitochondria/	CAS
					Chloroplast	
PvBSAS4;1	Phvul.007G185200	Chr07:30508330..30514006	975	34.51	Cytoplasmic/	CS/CAS
					Chloroplast	
PvBSAS4;2	Phvul.007G185100	Chr07:30495856..30503923	972	34.31	Glyoxysomal	CS/DES
					Mitochondria/	
PvBSAS5;1	Phvul.001G107500	Chr01:22505318..22510009	1116	35.22	Chloroplast	SCS

*Subcellular localization of isoforms was predicted using WoLF PSORT a protein subcellular localization prediction tool. kDa: kilodalton CS: Cysteine synthase, CAS: β -cyanoalanine cysteine synthase, DES: cysteine desulfhydrase; SCS: sulfocysteine synthase

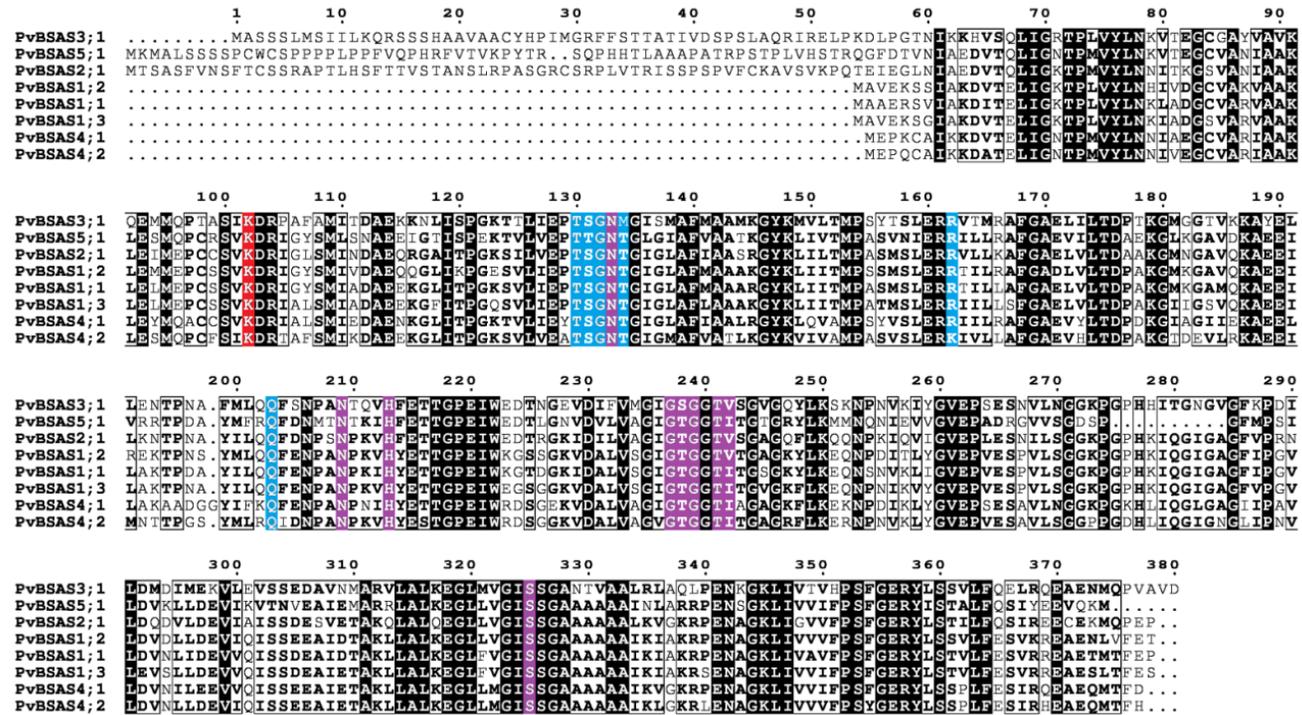


Figure 2.2: Multiple alignment of deduced amino acid sequences of the common bean BSAS gene family.

Black shade indicates conserved sequence. Residues in boxes indicate a variation from the highly-conserved sequence. The Lys¹⁰² highlighted in red is required for binding PLP with enzyme. Residues in the PLP binding sites are highlighted in purple while amino acid binding sites are highlighted in blue.

Table 2.2: Pairwise comparison of deduced amino acid sequences and coding DNA of the *P. vulgaris* BSAS family

	Amino Acid							
	PvBSAS3;1	PvBSAS5;1	PvBSAS2;1	PvBSAS4;1	PvBSAS1;2	PvBSAS1;1	PvBSAS1;3	PvBSAS4;2
PvBSAS3;1		51.3	53.4	58.5	60.6	58.8	58.2	56.2
PvBSAS5;1	58.4		54.6	61.0	62.7	63.3	62.4	63.4
PvBSAS2;1	60.8	67.3		60.1	69.3	68.6	69.7	59.9
PvBSAS4;1	61.5	63.6	66.7		58.5	71.8	74.0	77.7
PvBSAS1;2	63.9	64.3	68.8	72.4		85.5	84.6	71.7
PvBSAS1;1	60.8	66.4	69.9	70.9	78.5		87.1	71.4
PvBSAS1;3	61.4	65.8	68.0	71.4	78.6	84.8		72.1
PvBSAS4;2	60.0	62.9	64.5	78.7	71.1	69.1	70.0	
	Nucleotide							

Sequence identity (%) between eight BSAS family members at the amino acid (upper triangle) and nucleotide (lower triangle) levels. Nucleotide sequences correspond to the coding region sequences and amino acid sequences, acquired from the phytozome; nucleotide and amino acid sequences were aligned by ClustalW and percentage identities were calculated from the subsequent multiple sequence alignment. Protein name and their corresponding accession number are listed in Appendix A

2.3.1.1 Phylogenetic analysis

Previously, several studies used phylogenetic analysis of BSAS sequences in diverse plants species to predict catalytic activity and localization of CS and CAS enzymes (Hatzfeld *et al.*, 2000; Jost *et al.*, 2000; Maruyama *et al.*, 2001; Han *et al.*, 2007; Lai *et al.*, 2009). BSAS family members of common bean group together with *Arabidopsis* and soybean BSAS in five separate clades. Each clade representing distinct catalytic functions of BSAS subfamilies CS and CAS (Figure 2.4).

Clade 1 contains the extensively studied cytosolic OAS-TL (BSAS1) group from *Arabidopsis* and soybean. Three of the common bean BSAS1 isoforms, PvBSAS1;1, PvBSAS1;2 and PvBSAS1;3 are located in this clade. These three PvBSAS1 isoforms share around 80-85% identity in amino acid sequence. Duplicated block data of *P. vulgaris* downloaded from the PGDD (<http://chibba.agtec.uga.edu/duplication/>) (Lee *et al.*, 2013b) with a threshold score greater than 300 suggests that PvBSAS1;3 arose from a duplication of PvBSAS1;1. The three PvBSAS1 isoforms share greater than 80% identity with cytosolic OAS-TL from *Arabidopsis* and lack N-terminal transit peptide for subcellular localization suggesting that the three BSAS isoforms are the major cytosolic OAS-TLs in common bean.

Clade 2 represents BSAS4 family members which include CS enzymes from *Arabidopsis* and soybean which have an auxiliary function with respect to the major cytosolic OAS-TL (Hatzfeld *et al.*, 2000; Yamaguchi *et al.*, 2000). These isoforms were reported to have DES activity during high cysteine availability conditions (Álvarez *et al.*, 2010; Yi and Jez, 2012). Two of the PvBSAS isoforms, PvBSAS4;1 and PvBSAS4;2 are grouped in this clade and predicted to be cytosolic in nature. Duplicated block database reports PvBSAS4;2 as a duplication event from PvBSAS1;2 which supports auxiliary function of BSAS4 enzymes as cysteine synthase to cytosolic BSAS1 isoforms.

Clade 3 and 4 represent the enzymes which are located in subcellular compartments like chloroplasts and mitochondria. Clade 3 has *Arabidopsis* BSAS2;1 and BSAS2;2 which are reported to be having dominant cysteine synthase activity in plastids and mitochondria

respectively (Barroso *et al.*, 1995; Jost *et al.*, 2000; Watanabe *et al.*, 2008a). PvBSAS2;1 is the only common bean sequence in this clade. According to WoLF PSORT, PvBSAS2;1 is predicted to be in chloroplast. Another plastidic BSAS, PvBSAS5;1 has an N-terminal transit peptide sequence like PvBSAS2;1 and is present in clade 4. ATBSAS5;1 has been reported to have cysteine synthase and sulfocysteine activity during H₂S exposure (Watanabe *et al.*, 2008a; Birke *et al.*, 2015).

Clade 5 which branches out from the rest of the BSAS family has one *Arabidopsis*, two soybean and one common bean BSAS. AtBSAS3;1 and GmBSAS3;1 are exclusive cyanoalanine synthases (CAS) in BSAS family. Presence of PvBSAS3;1 in this clade and transit peptide at the N terminal end suggests PvBSAS3;1 is a cyanoalanine synthase in common bean.

2.3.1.2 Sequence analysis of PvBSAS3;1 and other CASs reported in other crops

To confirm PvBSAS3;1 as a cyanoalanine synthase, amino acid sequences of known cyanoalanine synthases from different crops were compared with the PvBSAS3;1 sequence (Figure 2.3). Sequence analysis reveals that this putative CAS contains a highly conserved PLP-binding domain (P--SV/IKDR) as do other reported CAS members. Compared to AtOAS-TL, all CAS members have a 50-60 amino acids long transit peptide. Amino acid residues required for SERAT interaction (Lys²¹⁷, His²²¹ and Lys²²² in AtOAS-TL) are not conserved in CAS subfamily members when compared to *Arabidopsis* CS (AtOAS-TL) (Lai *et al.*, 2009).

Figure 2.3: Multiple alignment of deduced amino acid sequences of the common bean BSAS3;1 with CASs from other plant species.

AtOAS-TL from *Arabidopsis* gene family is used as CS for comparison. CASs from other crops include *Os*: rice, *So*: spinach, *At*: *Arabidopsis*, *St*: potato, *Bp*: birch, *Gm*: soybean and *Pv*: common bean. Black shade indicates conserved sequence. Dotted line indicates the absence of a target peptide in AtOAS-TL. The Lys⁴⁶ residue highlighted in red is required for binding PLP. Conserved residues participating in the PLP binding site are marked with a blue line. The green line represents the β 8A- β 9A loop (Lys²¹⁷ to Phe²³⁰ in AtBSAS1;1) responsible for interacting with SERAT which is conserved in the plant and bacterial kingdoms. Orange boxes represent amino acid residues required for the interaction between SERAT and OAS-TL.

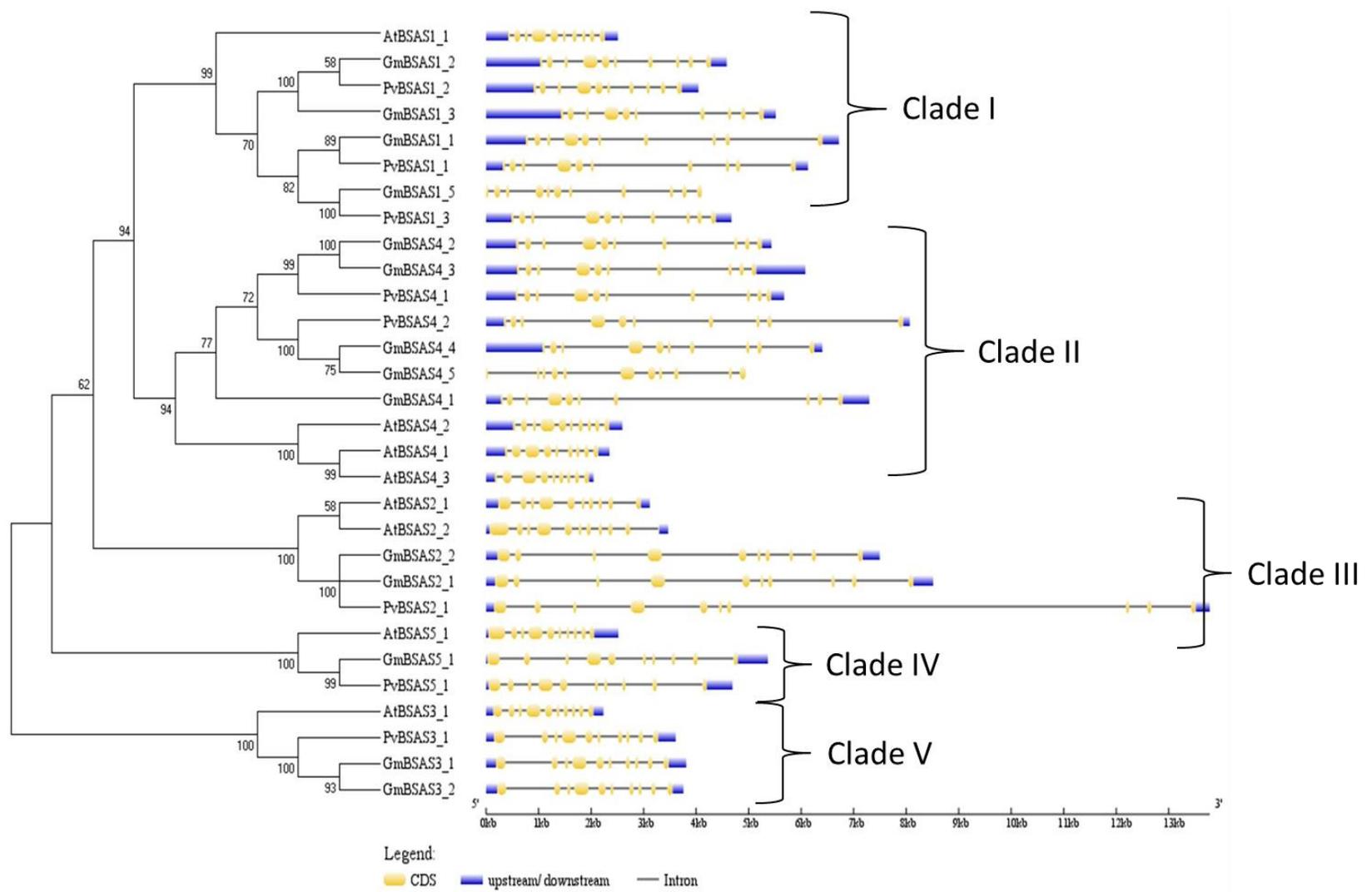


Figure 2.4: Evolutionary relationships and gene structure of BSAS isoforms in *Arabidopsis*, soybean and common bean using the neighbor-joining method.

The percentage of replicate trees in which the associated proteins clustered together in the bootstrap value (1000 replicates) is shown next to the branches. CDS coding DNA sequence. Protein name and their corresponding accession number are listed in Appendix A

2.3.2 Tissue expression pattern of PvBSAS3;1

RNA-Seq based gene expression atlas of the common bean published in 2014 revealed that PvBSAS3;1 is predominantly expressed in seeds in early developmental stage (O'Rourke *et al.*, 2014) (Figure 2.5). Based on phylogenetic analysis and expression profile, BSAS3;1 is predicted as a CAS which may prefer cysteine and methanethiol as substrates in a secondary reaction and produce *S*-methylCys during seed development stage.

2.3.3 Biochemical characterization of PvBSAS3;1

2.3.3.1 Size-exclusion chromatography

To determine the role of PvBSAS3;1 as CAS, a truncated sequence (without transit peptide) was cloned and expressed in *E. coli* as a His-tagged recombinant protein. Based on comparative transcriptomic data, PvBSAS3;1 had higher expression than VmBSAS3;1 in developing seeds. To determine the role of PvBSAS3;1 in *S*-methylCys biosynthesis, a truncated version of VmBSAS3;1 was also cloned and expressed in *E. coli* for recombinant protein production. Comparison of the elution profile of PvBSAS3;1 and VmBSAS3;1 with known molecular weight markers suggested a homodimeric nature of BSAS3;1 in *P. vulgaris* and *V. mungo* (Figure 2.6). The denatured protein on SDS gel migrated as a single band of 37 kDa while calculated molecular masses of PvBSAS3;1 and VmBSAS3;1 after size-exclusion chromatography were approximately 72 kDa.

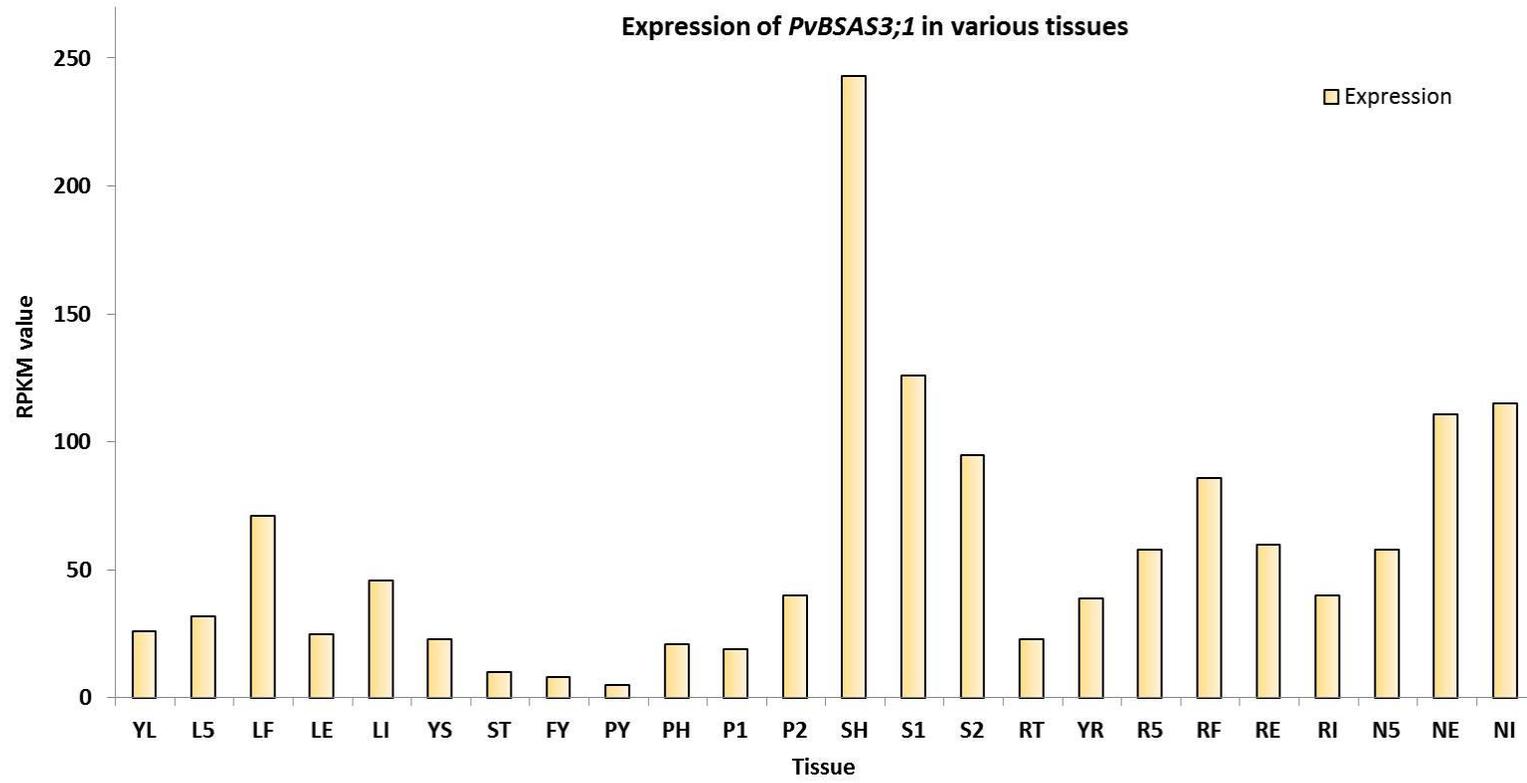


Figure 2.5: Expression profile of the *PvBSAS3;1* in various plant tissues.

Descriptions of tissues are as follows: YL- Fully expanded 2nd trifoliolate leaf tissue; L5- Leaf tissue collected 5 days after rhizobium inoculation; LF- Leaf tissue from fertilized plants collected at the same time of LE and LI; LE- Leaf tissue collected 21 days after rhizobium inoculation; LI- Leaf tissue collected 21 days after plants were inoculated with ineffective rhizobium; YS- All stem internodes above the cotyledon collected at the 2nd trifoliolate stage; ST- Shoot tip, including the apical meristem, collected at the 2nd trifoliolate stage; FY- Young flowers, collected prior to floral emergence; PY- pods containing globular stage embryos (1-4 days after fertilization); PH- pods containing heart stage seeds; P1- pods associated with stage 1 seeds (pods only); P2- pods associated with stage 2 seeds (pods only); SH- heart stage seeds (ca. 7 mg); S1- stage 1 seeds (ca. 50 mg); S2- stage 2 seeds (ca. 150 mg); RT- Root tips; YR- Whole roots, including root tips; R5- Whole roots separated from 5 day old pre-fixing nodules; RF- Whole roots from fertilized plants collected at the same time as RE and RI; RE- Whole roots separated from fix nodules collected 21 days after inoculation; RI- Whole roots separated from fix- nodules collected 21 days after inoculation; N5- Pre-fixing (effective) nodules collected 5 days after inoculation; NE- Effectively fixing nodules collected 21 days after inoculation; NI- Ineffectively fixing nodules collected 21 days after inoculation (O'Rourke *et al.*, 2014).

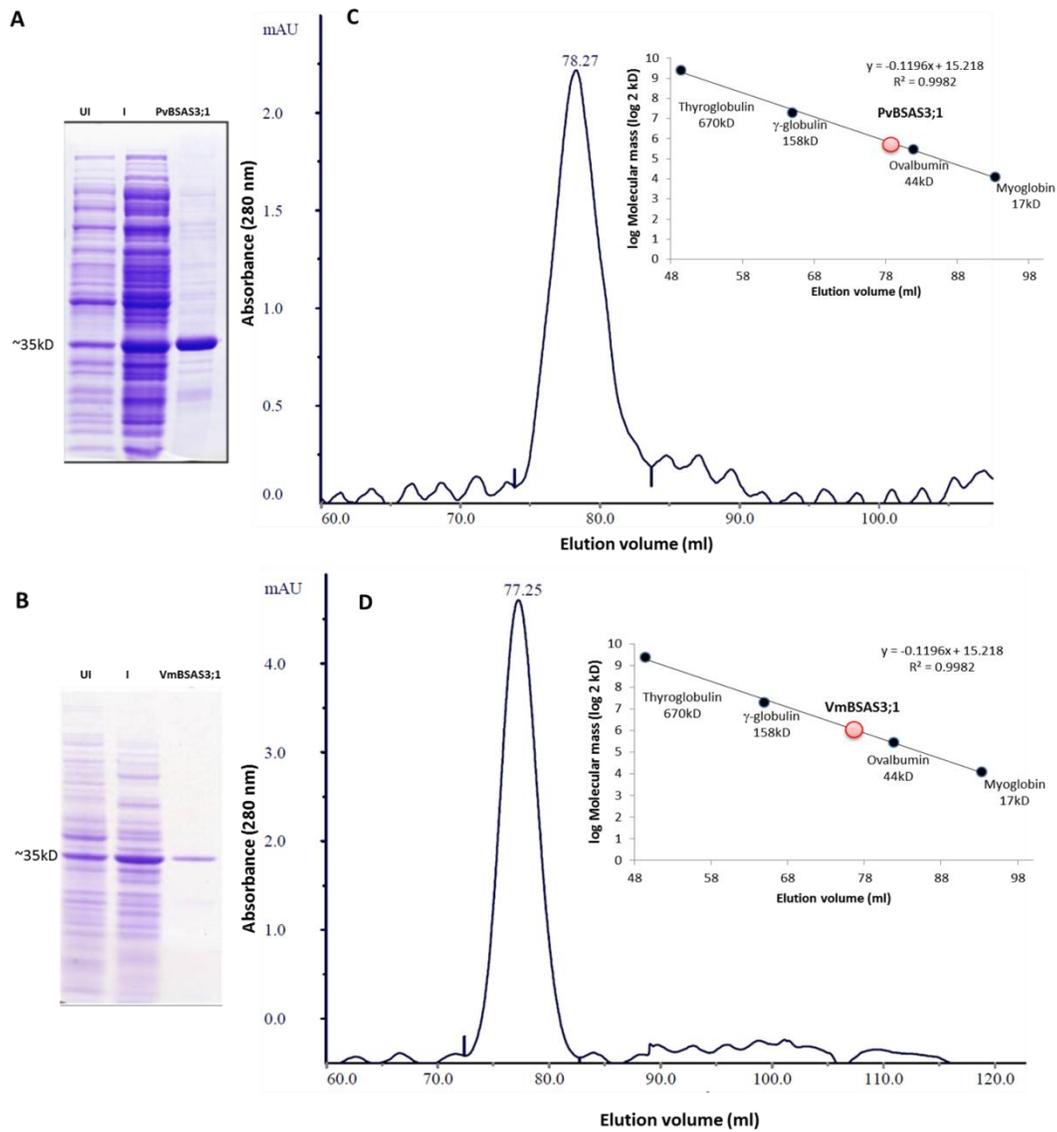


Figure 2.6: Physical and spectral properties of PvBSAS3;1 and VmBSAS3;1.

(A) and (B) SDS– PAGE analysis of uninduced (UI), induced (I) and purified PvBSAS3;1 and VmBSAS3;1. (C) and (D) Elution profile of Co^{2+} -affinity purified PvBSAS3;1 and VmBSAS3;1 in size-exclusion chromatography. Molecular weight was calculated based on a standard curve shown in the inset.

2.3.3.2 Enzymatic activity

To determine their function, His-tagged purified PvBSAS3;1 and VmBSAS3;1 were used to perform CAS enzyme assay. Both PvBSAS3;1 and VmBSAS3;1 were highly active as a CAS, using cyanide and cysteine as substrates. The specific activity of PvBSAS3;1 and VmBSAS3;1 for β -cyanoalanine production was 21.0×10^{-7} katal mg^{-1} and 19.2×10^{-7} katal mg^{-1} , respectively. These enzymes catalyzed a similar thiol exchange reaction using methanethiol and cysteine as substrates, forming *S*-methylCys. The rates of the two reactions were significantly different for both enzymes using student's t-test (Figure 2.7). In the case of PvBSAS3;1 there was significantly higher activity with potassium cyanide as well as sodium thiomethoxide than VmBSAS3;1.

After observing significantly higher activity with PvBSAS3;1 kinetic studies were performed for this enzyme. Enzyme kinetics data showed that PvBSAS3;1 has a higher affinity for cyanide than methanethiol (Table 2.3). The apparent K_m value for cyanide was lower than any other CAS reported from other crops (Table 2.4). However, the apparent K_m value for methanethiol was also in the low mM range (Table 2.3). This analysis identifies BSAS3;1 as a putative candidate enzyme for the formation of *S*-methyl-Cys in seeds of common bean.

2.3.4 Subcellular localization

According to WoLF PSORT PvBSAS3;1 was predicted to be localized in plastids rather than mitochondria. A translational fusion of full-length BSAS3;1 was created with reporter gene YFP and expressed transiently in epidermal cells of *N. benthamiana* leaf. Co-localization of PvBSAS3;1 with CFP tagged mitochondrial marker protein confirmed PvBSAS3;1 localization in mitochondria (Figure 2.8).

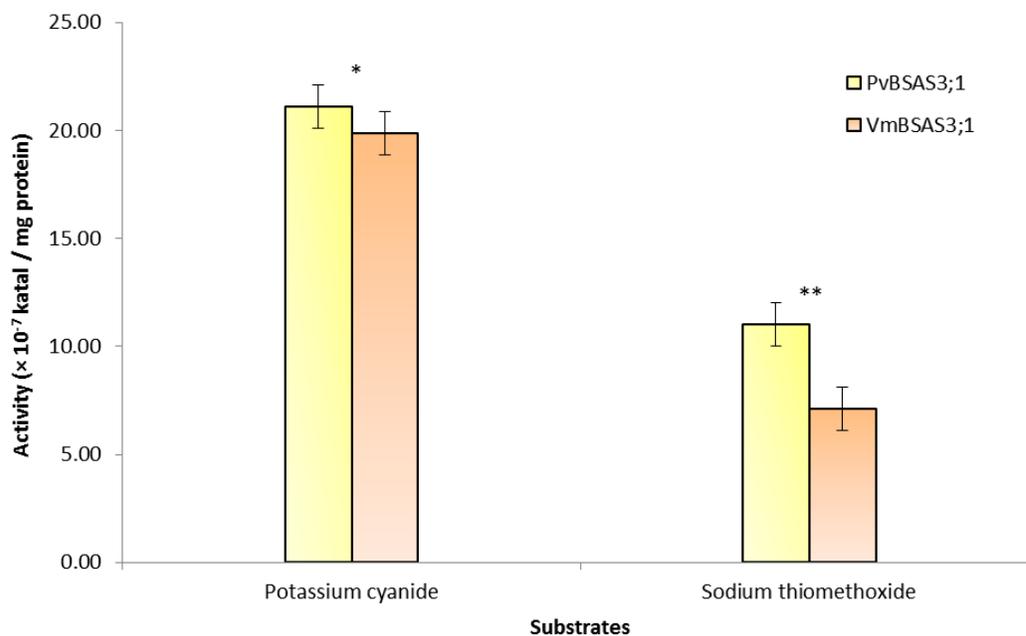


Figure 2.7: Specific activity of PvBSAS3;1 and VmBSAS3;1 with 2mM potassium cyanide and 2mM sodium thiomethoxide (methanethiol). 1mM cysteine was common substrate in both the reactions.

Substrate concentration of 1 mM cysteine and 2 mM KCN or 2 mM sodium thiomethoxide was used in the reaction. (* statistically significant at $p \leq .001$; ** $p \leq .0001$).

Table 2.3: Kinetic parameters of PvBSAS3;1 with different substrates

Substrate	V_{\max} ($\times 10^{-7}$ katal mg^{-1})	K_m (mM)	V_{\max}/K_m ($\times 10^{-7}$ katal mg^{-1})/mM
L-Cysteine + Potassium cyanide \longrightarrow β -Cyanoalanine + Hydrogen Sulfide			
Potassium cyanide	21.4 ± 0.3	0.05 ± 0.01	428.0
Cysteine	41.6 ± 2.9	0.70 ± 0.11	59.4
L-Cysteine + Sodium thiomethoxide \longrightarrow S-methylCys + Hydrogen Sulfide			
Sodium thiomethoxide	18.7 ± 1.5	1.18 ± 0.09	15.9
Cysteine	15.1 ± 0.2	0.28 ± 0.01	54.1
All values are expressed as a mean \pm SD ($n = 3$)			

Table 2.4: Kinetics of CAS reported in other plant species

Plant species	Specific activity μmoles/min/mg	K _m (mM)		Reference
		Cysteine	KCN	
Blue lupine	21.5	2.5	0.55	(Hendrickson and Conn, 1969)
Spinach	212	2.3	0.73	(Ikegami <i>et al.</i> , 1988b)
<i>Lathyrus latifolius</i>	132	16	0.51	(Ikegami <i>et al.</i> , 1988a)
<i>Vicia angustifolia</i>	NR	3.6	0.5	(Ikegami <i>et al.</i> , 1989)
Cassava	105	2.5	8.0	(Elias <i>et al.</i> , 1997)
Spinach	157	2.14	0.10	(Hatzfeld <i>et al.</i> , 2000)
Arabidopsis	62.1	2.54	0.06	(Hatzfeld <i>et al.</i> , 2000)
Potato	66.2	2.76	0.134	(Maruyama <i>et al.</i> , 2001)
Rice	13.3	0.84	0.27	(Lai <i>et al.</i> , 2009)
Soybean	NR	0.81	0.26	(Yi and Jez, 2012)
<i>P. vulgaris</i>	126	0.70	0.05	This study

*NR: Not reported

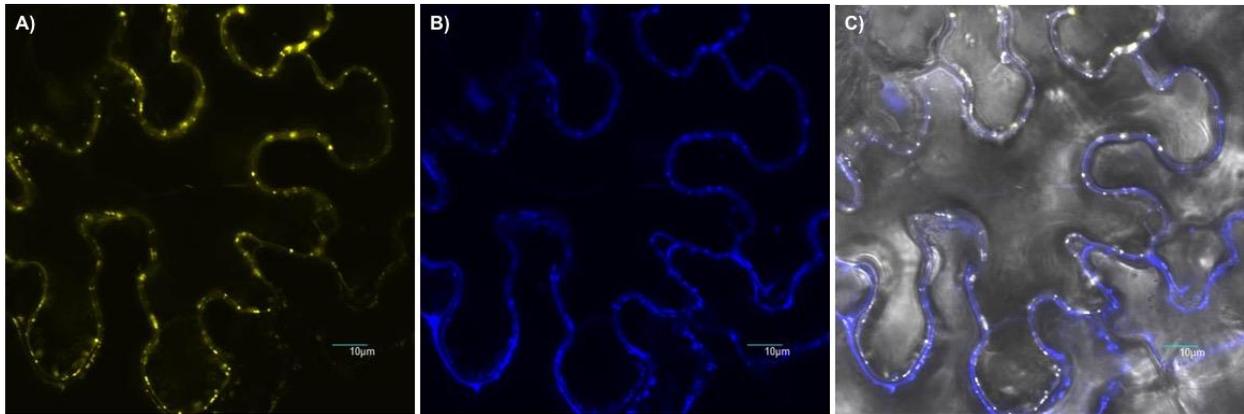


Figure 2.8: Subcellular localization of PvBSAS3;1.

Full length *PvBSAS3;1* was translationally fused upstream of the reporter gene YFP, transformed into *N. benthamiana* by *A. tumefaciens* mediated transformation and visualized in the leaf epidermal cells by confocal laser-scanning microscopy. A CFP-tagged mitochondrial marker was coexpressed with PvBSAS3;1. A) YFP tagged PvBSAS3;1, B) CFP tagged mitochondrial marker (Mt-CFP) and c) Co-localization of PvBSAS3;1 with Mt-CFP. Scale bar indicates 10 μm . YFP: Yellow fluorescent protein; CFP, cyan fluorescent protein.

2.4 Discussion

BSAS constitutes a large superfamily of PLP-dependent enzymes comprising CSase (OAS-TL) and CASase in plants. In *P.vulgaris* a genome-wide search led to identification of eight genes which encode for CS or CAS activity. These genes are located on six chromosomes and encode proteins present in different cellular compartments. Similar to *Arabidopsis*, cytosolic BSAS1;1 and plastidic BSAS2;1 are likely major players of cysteine biosynthesis in common bean while mitochondrial PvBSAS3;1 is involved in detoxification with CAS activity. PvBSAS3;1 is a relatively seed specific CAS in common bean and its expression is high during early developmental stages (Figure 2.5). Researchers have reported a role of ethylene biosynthesis during seed development (Johnson-Flanagan and Spencer, 1994; Hays *et al.*, 2000; Matilla, 2000; Matilla and Matilla-Vázquez, 2008). Ethylene biosynthesis gives rise to cyanide which should be removed due to its toxicity. High expression of PvBSAS3;1 during seed development can be explained in relation to cyanide production. PvBSAS3;1 takes part in detoxification of cyanide and production of β -cyanoalanine which enters in asparagine biosynthesis. PvBSAS3;1 is a key candidate in cysteine catabolism in seed tissue.

Structurally, there are two groups of β -CAS present in different plant species (Hasegawa *et al.*, 1995). In one group monomeric CAS of 52 kD with one PLP cofactor binding site was reported in blue lupine (Akopyan *et al.*, 1975). On the other hand, CAS reported in spinach, cocklebur, rice, *Lathyrus latifolius*, soybean and *Arabidopsis* is a homodimer with a monomeric unit of 20-35 kDa and PLP binding domain present in both subunits (Ikegami *et al.*, 1988a; Ikegami *et al.*, 1988b; Ikegami *et al.*, 1989; Maruyama *et al.*, 1998; Hatzfeld *et al.*, 2000; Maruyama *et al.*, 2000; Maruyama *et al.*, 2001; Lai *et al.*, 2009; Yi *et al.*, 2012; Yi and Jez, 2012). In my study, size-exclusion chromatography and sequence analysis of PvBSAS3;1 and VmBSAS3;1 suggested them as members of homodimer group of CASs with one PLP binding domain present in each subunit.

BSAS family members are reported to be PLP dependent enzymes that have a lysine residue at their active site to bind with PLP covalently through a Schiff base linkage. All BSAS family members reported utilize *O*-acetylserine, serine, or cysteine as the donor of the alanyl group to

PLP Schiff base. This binding of alanyl group to PLP Schiff base moiety determines catalytic promiscuity of the enzymes (Akopyan *et al.*, 1975; Percudani and Peracchi, 2003; Bonner *et al.*, 2005). Sequence analysis demonstrates conservation of the lysine residue at the active site in all common bean BSAS members, though a few substitutions were noticed in case of PvBSAS3;1 at PLP binding and SERAT interaction sites. The “true” OAS-TLs are defined by the ability to form a complex with SERAT. Lai *et al.* (2009) correlated substitutions at the PLP binding and SERAT interaction sites with the substrate specificity of β -CAS subfamily for cyanide and cysteine. According to sequence alignment (Figure 2.3) PvBSAS3;1 retain such characteristics. Our kinetic data for PvBSAS3;1 shows that similar to cyanogenic plants PvBSAS3;1 prefers cysteine and cyanide as substrates (Table 2.4). Cyanogenic plants release cyanide while other crops such as rice and spinach produce cyanide during seed germination and under stress conditions (Machingura and Ebbs, 2014). These kinetic data suggest a key role of PvBSAS3;1 in detoxifying cyanides in seed tissue during development and germination.

Based on the amino acid sequences another difference that can be noted between the β -CAS and CS isoforms is the presence of a signal peptide within their sequence for mitochondrial or chloroplast targeting, respectively (Saito *et al.*, 1994). Cyanide acts as an inhibitor in electron transport chain in the mitochondria as it binds to terminal cytochrome c oxidase (Solomonson, 1981). Localization of β -cyanoalanine formation to quench cyanide suggests the presence of BSAS3;1 in mitochondria. In our study, transient expression of PvBSAS3;1 with YFP tag in *N. banthemiana* leaves confirmed PvBSAS3;1 localization in mitochondria in agreement with previous studies. Previously conducted organellar fractionation methods and immunogold labelling studies also suggested CAS activity to be localized in mitochondria (Akopyan *et al.*, 1975; Wurtele *et al.*, 1984; Wurtele *et al.*, 1985;Lai *et al.*, 2009).

Previously, studies have predicted cysteine as a precursor of *S*-methylCys biosynthesis. In radish leaves, recovery of radioactive carbon in *S*-methylCys was observed when treated with radiolabelled cysteine or methionine (Thompson and Gering, 1966). The initial studies of cyanoalanine synthesis also demonstrated that the purified β -cyanoalanine synthase could use methanethiol, ethanethiol and 2-mercaptoethanol as substrates *in vitro* (Hendrickson and Conn, 1969; Akopyan *et al.*, 1975). In the case of blue lupine and spinach, synthesis of *S*-methylCys

was observed when cyanide was replaced by methanethiol as substrate (Hendrickson and Conn, 1969; Ikegami *et al.*, 1988b). However, the rate of biosynthesis of methylated cysteine was reported to be very low for the blue lupine enzyme (Hendrickson and Conn, 1969). In our study, PvBSAS3;1 used methanethiol efficiently to produce *S*-methylCys and the rate of biosynthesis of *S*-methylCys was approximately the same as for β -cyanoalanine formation. However, it is unlikely that the enzyme catalyzes the formation of *S*-methylcysteine *in vivo*. Based on our subcellular localization study, PvBSAS3;1 is present in mitochondria. Methionine- γ -ligase (MGL) which catalyzes biosynthesis of methanethiol is reported to be present in the cytosol (Rebeille *et al.*, 2006). So it is unlikely that BSAS3;1 and MGL can work in coordination to produce *S*-methylCys in developing seeds of common bean. Our *in vitro* kinetics data suggest that although cyanide is a preferred substrate, synthesis of *S*-methylCys may happen as a side reaction by PvBSAS3;1 if methanethiol is present in mitochondria with lower affinity for methanethiol vs. cyanide.

2.5 Conclusion

With the completed genome sequence of Andean (Schmutz *et al.*, 2014) and Mesoamerican (Vlasova *et al.*, 2016) varieties eight BSAS family members were identified to be present in the common bean genome. Phylogenetic and synteny analysis of common bean with *Arabidopsis* and soybean BSAS family members helped to predict their function and subcellular localization. Physical and biochemical characterization of PvBSAS3;1 has not only revealed the role of PvBSAS3;1 in cyanide detoxification but also suggested its possible role in *S*-methylCys biosynthesis. Due to seed-specific expression of PvBSAS3;1 and its pivotal role in cysteine metabolism further investigation is required, *in planta*, to determine whether it can influence cysteine and methionine concentration in bean varieties.

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CHAPTER THREE - PROBING SULFUR AMINO ACID METABOLISM IN SEED OF COMMON BEAN USING ISOTOPE LABELLING

3.1 Introduction

In plants, the sulfur containing amino acid cysteine plays a central role in sulfur metabolism. Apart from incorporation into proteins and synthesis of methionine, an essential amino acid, another important usage of cysteine by both plants and animals is the biosynthesis of glutathione (GSH), a tripeptide essential to maintain cellular redox homeostasis (Noctor *et al.*, 2012). Other fates of cysteine include phytochelatins, iron-sulfur clusters, vitamin cofactors, and the biosynthesis of multiple secondary metabolites (Bonner *et al.*, 2005).

Protein quality in legumes is compromised due to suboptimal levels of the sulfur amino acids, cysteine and methionine. Among all the legumes produced in the world, common bean (*Phaseolus vulgaris*) is considered as one of the best for human consumption (Broughton *et al.*, 2003). Major seed proteins present in common bean, such as the 7S globulin phaseolin and the lectin phytohaemagglutinin, have a low methionine and cysteine content (Sathe, 2002; Montoya *et al.*, 2010). In contrast, common bean accumulates the non-protein sulfur amino acid, *S*-methylcysteine (*S*-methylCys) and a related dipeptide, γ -glutamyl-*S*-methylcysteine (γ -Glu-*S*-methylCys) (Giada *et al.*, 1998; Taylor *et al.*, 2008).

S-methylCys and its oxide are considered Cinderella phytochemicals due to their medicinal and health benefits (Edmands *et al.*, 2013). The presence of *S*-methylCys and its derivatives is widely noted in the *Brassicaceae*, *Alliaceae* and *Fabaceae* families (Jones *et al.*, 2004). The characteristic aroma and flavor of *Allium* spp. is due to the *S*-alk(en)yl-cysteine sulfoxides, while members of the *Fabaceae* accumulate these non-proteinogenic *S*-amino acid derivatives to transport reduced sulfur or act as storage sink for sulfur (Zacharius *et al.*, 1959; Giada *et al.*, 1998; Taylor *et al.*, 2008). *Astragalus bisulcatus* which can accumulate

high selenium content produces a selenium analogue of *S*-methylCys to prevent non-specific incorporation of selenocysteine into proteins or conversion into selenomethionine. The seeds accumulate γ -glutamyl-methyl selenocysteine as a storage compound (Sors *et al.*, 2005; Sors *et al.*, 2009).

In the past, scattered reports have come related to the potential benefit or anti-nutritional effect of *S*-methylCys and its oxide on animal and human health. In 1976, *S*-methyl-L-cysteine sulfoxide was reported to be 'kale anemia factor' having toxic effects on ruminant (Whittle *et al.*, 1976). Many animal studies have reported anti-nutritional effects of an *S*-methylCys rich diet on animals (Benevenga *et al.*, 1976; Case and Benevanga, 1976; Padovese *et al.*, 2001). Potential benefits of *S*-methylCys and its sulfoxide include antidiabetic and antioxidant effects in humans (Akash *et al.*, 2014; Hsia and Yin, 2015). In a human dietary intervention study *S*-methyl-L-cysteine sulphoxide was identified as a biomarker of cruciferous vegetable intake (Edmands *et al.*, 2011). Recently, *S*-methylCys along with pipercolic acid were reported as biomarkers for bean rich diet in a controlled human feeding and mouse feeding study (Perera *et al.*, 2015).

In common bean, the concentration of *S*-methylCys is inversely related to that of cysteine and methionine. Three genetically related lines, SARC1, SMARC1-PN1 and SMARC1N-PN1 were developed that are deficient in major storage proteins (Osborn *et al.*, 2003). A proteomic analysis revealed that the deficiency in storage proteins is associated with increased cysteine and methionine content. In SMARC1N-PN1, cysteine and methionine was increased by 70% and 10%, respectively, at the expense of *S*-methylCys (Taylor *et al.*, 2008; Marsolais *et al.*, 2010).

Despite recent progress towards elucidating the biosynthetic pathway of cysteine and its fate in plant tissue, little is known about the biosynthesis of *S*-methylCys or γ -Glu-*S*-methylCys. Various schools of thought exist for *S*-methylCys biosynthesis in plants. In radish, the relatively high recovery of radioactivity in methyl cysteine sulfoxide after radiolabelled cysteine or methyl-labelled methionine application indicated that *S*-

methylCys is formed by direct methylation of cysteine (Thompson and Gering, 1966). *Allium* genus members, onion (*Allium cepa*) and garlic (*Allium sativum*) accumulate *S*-methylCys as a sulfoxide and biosynthesis is initiated by *S*-alkylation of GSH (Turnbull *et al.*, 1980; Lancaster *et al.*, 1989; Yoshimoto *et al.*, 2015b). Production of *S*-methylCys in *Arabidopsis* cell cultures was reported to be associated with methionine catabolism (Giovanelli *et al.*, 1980; Rebeille *et al.*, 2006). In *Astragalus* species the synthesis of *Se*-methylselenocysteine is catalyzed by *Se*-methyltransferase (SMT) by using either *S*-adenosylmethionine (SAM) or *S*-methylmethionine (SMM) as methyl sources (Sors *et al.*, 2009). Comparative transcriptome profiling of sulfur metabolism related genes in *P. vulgaris* and *Vigna mungo*, which accumulates γ -Glu-Met and not γ -Glu-*S*-methylCys, found HMTs (methyltransferases) as well as BSAS family members to be candidate genes associated with *S*-methylCys based on differential expression (Liao *et al.*, 2013).

To identify the precursor(s) of *S*-methylCys in common bean, the fate of labelled ($^{13}\text{C}_3$, ^{15}N) cysteine or serine was monitored by a combination of targeted and non-targeted high-resolution mass spectrometry (HRMS). Targeted, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to obtain product ion spectra of the unlabelled compounds which could be involved in the biosynthetic pathway of *S*-methylCys. Two possible pathways of *S*-methylCys biosynthesis were proposed and isotopologues containing $^{13}\text{C}_3$ and ^{15}N were tracked along these proposed pathways. In one pathway, methylation of cysteine was proposed as a source of *S*-methylCys biosynthesis, while in another pathway serine was proposed as a precursor of *S*-methylCys (Figure 3.1).

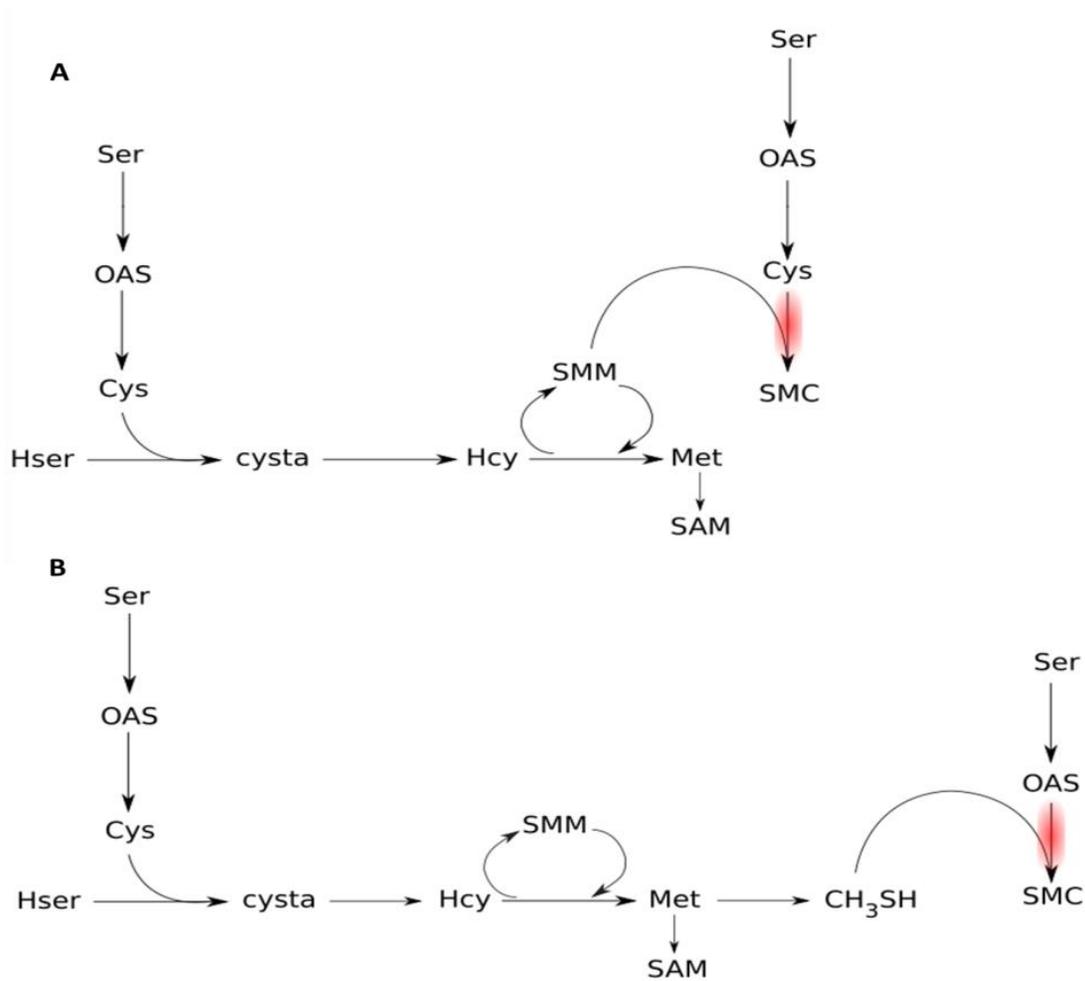


Figure 3.1: Schematic representation of two possible *S*-methylCys biosynthesis pathways.

A) Upper panel: *S*-methylCys biosynthesis takes place via cysteine methylation. SMM acts as a methyl donor. B) Lower panel: *S*-methylCys biosynthesis may take place *via* condensation of *O*-acetylserine with methanethiol. In this scheme methanethiol is produced *via* cleavage of methionine. HSer: *O*-phospho-L-homoserine, Ser: Serine, OAS: *O*-acetylserine, Cys: Cysteine, cysta: Cystathionine, Hcy: Homocysteine, SMM: *S*-methylmethionine, CH₃SH: methanethiol, SMC: *S*-methylCys.

3.2 Material and methods

3.2.1 Chemicals

¹³C and ¹⁵N isotope labelled serine and cysteine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Other components of growth media were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

3.2.2 Plant material

Common bean (*Phaseolus vulgaris*) genotype BAT93 plants were grown in growth cabinets (Environmental Growth Chambers, Chagrin Falls, OH, USA) under 16 h light (300-400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at a temperature cycling between 18 and 24 °C as described by Pandurangan et al. (2012). Seeds were germinated in vermiculite and 12-day old seedlings were transplanted to pots (17 × 20 cm) containing Promix BX soil (Premier Tech Horticulture, Québec, Canada).

3.2.3 Embryo culture

Thirteen to 15 days post fertilization, developing pods were harvested, and surface sterilized with 0.5% bleach and soap (Alconox powdered precision cleaner, Alconox, NY, USA) for 5 min. Surface sterilization was followed up with three thorough, 5 min washings, in sterile distilled water and dissection for seed collection. Every seed was weighed and the seed coat was removed. Cotyledon weight was taken before transfer to 25 mL standard line cell culture flasks (VWR, Mississauga, Ontario, Canada) containing culture media. These cell culture flasks had a filtered vent to provide oxygen for the developing seeds. Each flask contained six cotyledons from three developing seeds and 2.5 mL of filter-sterilized culture media in the flasks.

3.2.4 Culture media

Culture medium contained 8 mM MgSO₄, 10 mM KCl, 3 mM CaCl₂, 1.25 mM KH₂PO₄, 0.5 mM MnSO₄, 0.15 mM ZnSO₄·7H₂O, 0.1 mM sodium EDTA ferric salt, 0.1 mM

H₃BO₃, 27 μM glycine, 2.5 μM CuSO₄·5H₂O, 5 μM KI, 1 μM Na₂MoO₄, 0.1 mM CoCl₂·6H₂O, 4 μM nicotinic acid, 1 μM thiamine-HCl, 0.5 μM pyridoxine-HCl, 0.56 mM *myo*-inositol, 0.1 mM Na₂EDTA and 5 mM MES to buffer the final solution to pH 5.8. Sucrose and glutamine was provided at levels of 146 mM and 62.5 mM respectively (Thompson *et al.*, 1981; Obendorf *et al.*, 1983; Holowach *et al.*, 1984). This basic culture medium was supplemented by serine or cysteine based on treatment groups.

3.2.5 Radiolabelling treatments

Five groups of treatments were designed based on culture media: labelled serine supplementation, non-labelled serine supplementation, labelled cysteine supplementation, non-labelled cysteine supplementation and no supplementation. In each treatment group, three cotyledons grown in cell culture flasks were kept horizontally at room temperature under continuous light and slow shaking (50 rpm). After completion of incubation, seeds were washed three times with sterile water to remove any traces of non-absorbed amino acids on the surface. Seeds were dried and stored at -80 °C following flash freezing in liquid nitrogen for future experiments.

3.2.6 Amino acid extraction

The frozen seeds were homogenized using metal beads in 1.5 ml eppendorf tubes using TissueLyser II (Qiagen). The ground seeds were extracted in ethanol: water (70:30), which is optimal for sulfur containing γ -glutamyl dipeptides (Kasai *et al.*, 1986). For mass spectrometry analysis dried amino acids were reconstituted in a 500 μl methanol:water (50:50) solution and filtered through 1.2 μm filters (Pall Life Sciences, Mississauga, Ontario, Canada) into an amber glass HPLC vial. The samples were prepared immediately prior to MS analysis.

3.2.7 HRMS and HRMS/MS

MS data were acquired with a Thermo® Q-Exactive Orbitrap mass spectrometer coupled to an Agilent 1290 HPLC system. Two microliter of sample was injected onto an Agilent

Zorbax Eclipse Plus RRHD C18 column (2.1 × 50 mm, 1.8 μM) maintained at 35 °C. Mobile phase A (0.1 % formic acid in LC-MS grade H₂O, Thermo Scientific, Fairlawn, NJ) was maintained at 100% for 1.25 min. Mobile phase B (0.1 % formic acid in LC-MS grade acetonitrile, Thermo Scientific, Fairlawn, NJ) was increased to 50 % over 1.75 min, and 100 % over 0.5 min. Mobile phase B was held at 100 % for 1.5 min and returned to 0 % over 0.5 min. The following heated electrospray ionization (HESI) was optimized for the analysis of *S*-methylCys: spray voltage, 3.9 kV; capillary temperature, 250 °C; probe heater temperature, 450 °C; sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; and S-Lens RF level, 60 %. MS/MS was performed at 17,500 resolution, automatic gain control (AGC) target of 1e⁶, maximum injection time (IT) of 60 ms and isolation window of 1 *m/z*. A top 5 DDA method was comprised of a full MS scan at 35,000 resolution, AGC target of 3e⁶, maximum IT of 125 ms, scan range between *m/z* 70-450 and intensity threshold of 7.7e⁵. The DDA scan conditions were identical to the targeted MS/MS method. Normalized collision energy of 25 was used for both MS/MS and DDA methods. Data were analyzed and all theoretical masses were calculated with Xcalibur™ software. The proportion of labelled compound present was calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

Targeted, liquid chromatography and tandem mass spectrometry (LC-MS/MS) was used to obtain product ion spectra of the unlabelled compounds which could be involved in the biosynthetic pathway of *S*-methylCys (Table 3.1). The formula of the major product ions of these compounds were determined by accurate mass and used to map MS/MS dissociation pathways. High resolution LC-MS/MS was then used to monitor both the unlabelled and predicted isotopically labelled compounds listed in Table 3.1.

3.3 Results

3.3.1 Determination of optimal incubation conditions for amino acid uptake

In common bean, free *S*-methylCys biosynthesis takes place during early seed development such as stages III - heart stage, IV and V cotyledon stages (Walbot *et al.*, 1972). Previously,

free amino acids were profiled in common bean cultivar BAT93 seeds by HPLC (Supplementary data 3.1) (Yi *et al.*, 2012). In early developing stages, *S*-methylCys concentration was equal to 0.40 nmol per mg seed weight while accumulation of γ -Glu-*S*-methylCys began later in development from stage VI - maturation to mature seed. To track the incorporation of stable isotopes in *S*-methylCys, I chose to feed the labelled precursors to developing seeds at stage IV - cotyledon. Seeds were grown in the presence of 1 mM, 5 mM and 8 mM concentrations of labelled serine to determine the optimal concentrations for the feeding experiments. Amino acid extracts from these seeds were analyzed by LC-MS/MS to determine the uptake of labelled serine relative to endogenous levels. Supplementation of growth media with 8 mM serine for 24 h resulted in sufficient incorporation of ^{13}C and ^{15}N in serine pool as well as in downstream metabolites. This concentration was then used in a time course experiment in order to determine the optimal incubation time. Insufficient incubation time would prevent the detection of important intermediates and downstream products while a long incubation time could lead to scattering of the labelled isotopes across multiple pathways. Seeds collected after 24 and 48 h showed efficient incorporation of isotopologues in endogenous serine and cysteine pool while incorporation was decreased after 48 h, which indicated its movement into downstream products (Figure 3.2). Therefore, the optimal growth conditions of incubating seeds in culture media supplemented by 8 mM amino acids were 2 days. Using the optimized conditions, 42 seeds in 14 cell line culture flasks and 33 seeds in 11 cell line culture flasks was incubated with labelled cysteine and serine respectively. Every flask contained three seeds.

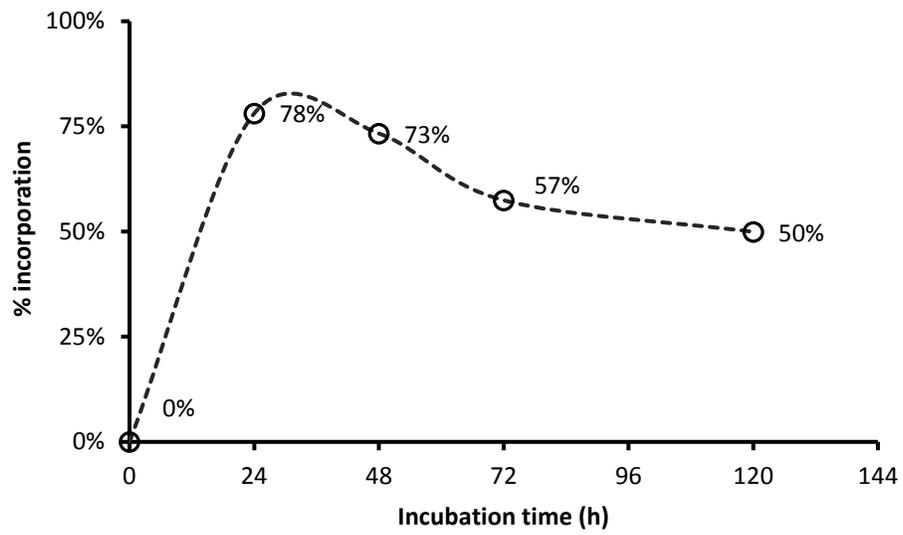


Figure 3.2: Uptake of ¹³C and ¹⁵N labelled serine over time.

Seeds were cultivated in the presence of 8 mM serine for varying time. Incorporation of ¹³C and ¹⁵N isotope in native serine is presented as % incorporation.

Following amino acid extraction, $80 \pm 4\%$ of the serine content within the $^{13}\text{C}_3^{15}\text{N}$ serine treatment was labelled. Similarly, $68 \pm 15\%$ of the cysteine content was labelled in the $^{13}\text{C}_3^{15}\text{N}$ cysteine treatment. These data suggested efficient uptake of both serine and cysteine by developing seeds from the growth media (Figure 3.3).

3.3.2 Tracking fate of labelled serine and cysteine

In order to track the path of isotopes from serine or cysteine to other metabolites and the position of isotopes in the metabolite, targeted MS/MS was performed. Accurate mass of major S-containing metabolites was used to determine the formula of product ions and to map MS/MS dissociation pathways. These dissociation pathways were used to predict the number of stable isotopes on the products ions which would be produced if $^{13}\text{C}_3$, ^{15}N cysteine or $^{13}\text{C}_3$, ^{15}N serine were incorporated. A product ion list was generated for unlabelled and labelled product ions (Table 3.1).

3.3.2.1 O-acetylserine

In the cysteine biosynthesis pathway, *O*-acetylserine is known to be the carbon backbone donor for cysteine biosynthesis. Serine gets converted to *O*-acetylserine using acetyl-CoA. Among the five carbons of *O*-acetylserine three are inherited from serine along with nitrogen. In the treatment group supplemented with labelled serine, $78 \pm 6\%$ of the *O*-acetylserine pool was labelled, while labelled cysteine treated seed showed no incorporation of stable isotopes (Figure 3.5). This is in agreement with a previous study where *O*-acetylserine was proven as a precursor of cysteine synthesis (Hell and Wirtz 2011).

3.3.2.2 S-methylCys

To increase the selectivity of detecting incorporation of a labelled amino acid into a downstream metabolite, *S*-methylCys and compounds predicted to be involved in the pathway were monitored by MS/MS. The major product ion of protonated *S*-methylCys ($\text{C}_4\text{H}_{10}\text{NO}_2\text{S}$) is $\text{C}_4\text{H}_7\text{O}_2\text{S}$ which occurs following the neutral loss of NH_3 (17.0265 Da).

Therefore, should either labelled cysteine or serine be the precursor of *S*-methylCys, the product ion formula would be $^{13}\text{C}_3\text{CH}_{10}^{15}\text{NO}_2\text{N}$, and $^{13}\text{C}_3\text{CH}_7\text{O}_2\text{S}$ (122.0262) after losing $^{15}\text{NH}_3$ (18.0236 Da) (Figure 3.4). A secondary product of *S*-methylCys is $\text{C}_3\text{H}_5\text{O}_2^+$ which arises following the neutral loss of CH_2S . This product ion does not occur in homocysteine, which is isobaric to *S*-methylCys and was used to distinguish *S*-methylCys from homocysteine (Table 3.1).

Isotopically labelled *S*-methylCys was not detected in samples treated with non-labelled serine, non-labelled cysteine or labelled cysteine. In contrast, labelled *S*-methylCys was clearly detected in seeds grown in the presence of labelled serine (Figure 3.5). The product ions observed agree with the expected positions of labelled atoms in *S*-methyl-Cys which used serine as a precursor. These findings strongly support that serine is the precursor of *S*-methylCys in the common bean variety of this study and not cysteine.

Table 3.1: Product ions tracked for labelled and unlabelled* compounds

Name	Formula	<i>m/z</i>	<i>m/z</i> *	Product ion 1	<i>m/z</i>	<i>m/z</i> *	Product ion 2	<i>m/z</i>	<i>m/z</i> *
Serine	C ₃ H ₇ NO ₃	106.0499	110.057	C ₂ H ₆ ON	60.0451	63.0481	C ₃ H ₆ O ₂ N	88.0397	92.0464
Homoserine	C ₄ H ₉ NO ₃	120.0655	124.0726	C ₃ H ₈ ON	74.0606	77.0638	C ₄ H ₈ O ₂ N	102.0552	106.0605
Cysteine	C ₃ H ₇ NO ₂ S	122.027	126.0341	C ₂ H ₆ NS	76.0222	79.0253	C ₃ H ₃ OS	86.9904	89.9999
<i>S</i> -methylCys	C ₄ H ₉ NO ₂ S	136.0427	140.0498	C ₄ H ₇ O ₂ S	119.0161	122.0262	C ₃ H ₅ O ₂	73.029	76.0385
<i>O</i> -acetylserine	C ₅ H ₉ NO ₄	148.0604	152.0675	C ₃ H ₈ O ₃ N	106.0499	110.057	C ₃ H ₆ O ₂ N	88.0396	92.0464
Methionine	C ₅ H ₁₁ NO ₂ S	150.0583	154.0654	C ₄ H ₁₀ NS	104.0531	107.0566	C ₅ H ₉ O ₂ S	133.0316	136.0418
<i>SMM</i>	C ₆ H ₁₃ NO ₂ S	164.074	168.0811	C ₆ H ₁₁ O ₂ S	147.0474	150.0575			
Cystathionine	C ₇ H ₁₄ N ₂ O ₄ S	223.0747	227.0818	C ₄ H ₈ O ₂ NS	134.0268	137.0304			
γ -Glu- <i>S</i> -methylCys	C ₉ H ₁₆ N ₂ O ₅ S	265.0853	269.0924	C ₄ H ₇ O ₂ S	119.0160	122.0262	C ₄ H ₁₀ O ₂ NS	136.0423	140.0498
GSH	C ₁₀ H ₁₇ N ₃ O ₆ S	308.0911	312.0982	C ₅ O ₃ N ₂ SH ₁₁	179.0485	183.0556			
hGSH	C ₁₁ H ₁₉ N ₃ O ₆ S	322.1067	326.1138	C ₆ H ₁₃ O ₃ N ₂ S	193.0637	197.0712			
<i>S</i> -methylhGSH	C ₁₂ H ₂₁ N ₃ O ₆ S	336.1224	340.1295	C ₇ H ₁₅ O ₃ N ₂ S	207.0792	211.0867			

SAM	$C_{15}H_{22}N_6O_5S$	399.1445	403.1516	$C_9H_{16}O_7N$	250.0928	250.0928	$C_{10}H_{20}O_7NS$	298.0962	298.0961
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SMM- *S*-methylmethionine; γ -Glu-*S*-methylCys: γ -glutamyl-*S*-methylcysteine; GSH: Glutathione; hGSH: Homoglutathione;

S-methylhGSH: *S*-methylhomoglutathione; SAM: *S*-Adenosylmethionine

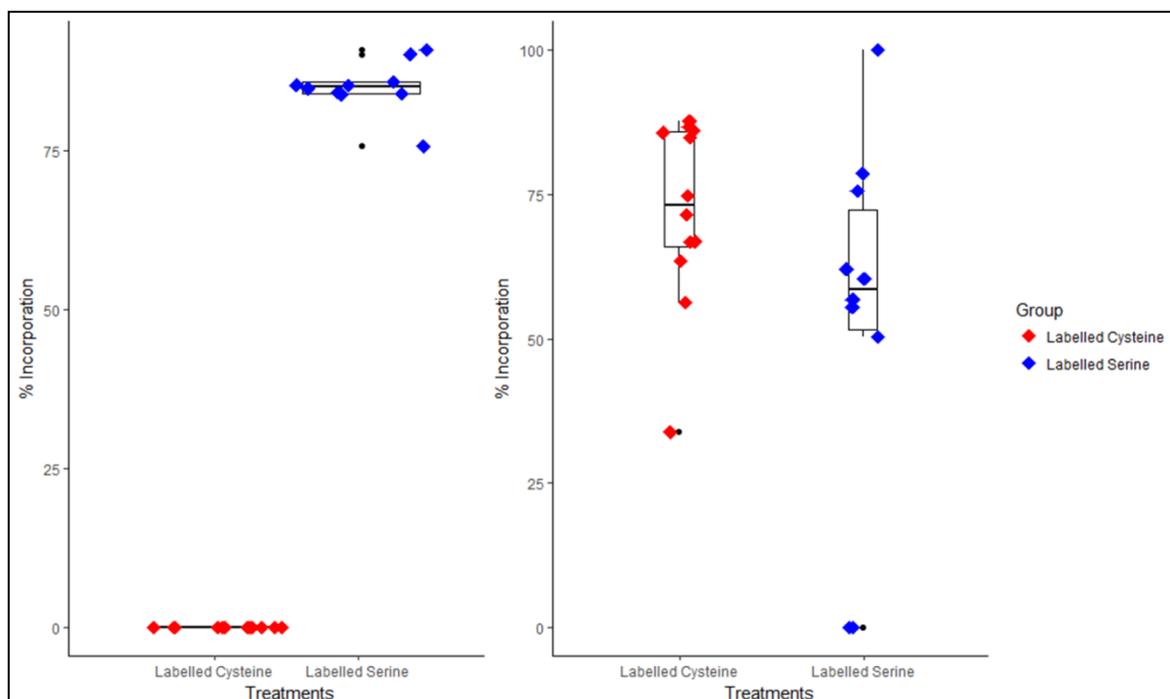


Figure 3.3: Percentage of isotopically labelled serine and cysteine compounds.

Left: percentage incorporation of $^{13}\text{C}_3$ $^{15}\text{N}_1$ in serine **Right:** percentage incorporation of ^{13}C and $^{15}\text{N}_1$ in cysteine in labelled cysteine or labelled serine supplementation to the basic culture media. Each dot represents a single LC-MS measurement of a three seed sample. The percentage incorporation of labelled compound in serine or cysteine were calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

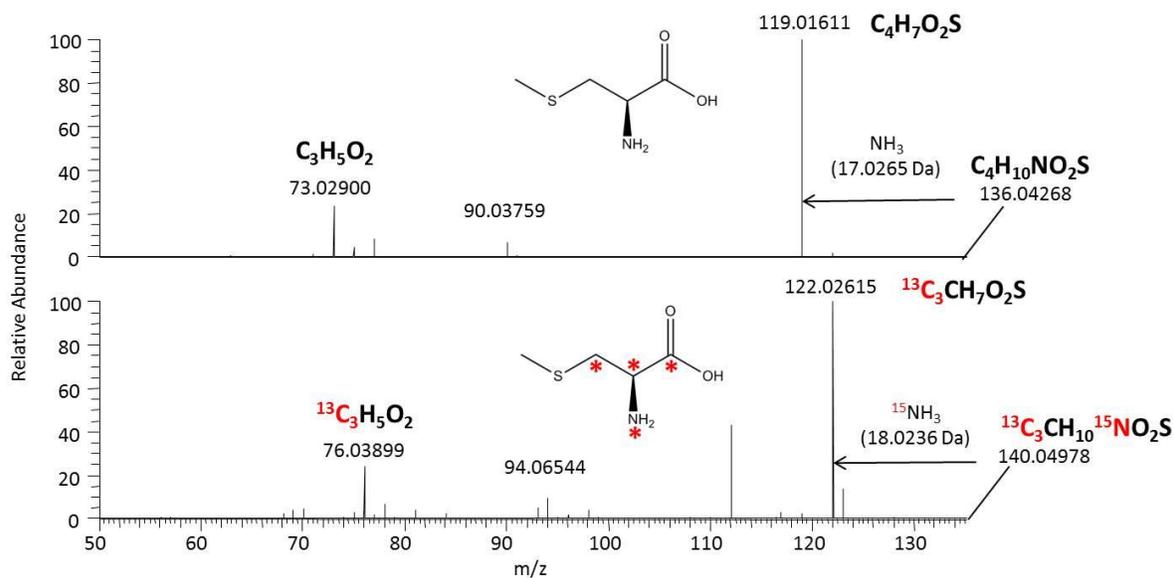


Figure 3.4: Representative dissociation of protonated unlabelled *S*-methylCys and labelled *S*-methylCys

Upper panel: Product ions of unlabelled *S*-methylCys. **Lower panel:** Product ions of labelled *S*-methylCys. The percentage incorporation of labelled compound in *O*-acetylserine or *S*-methylCys was calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

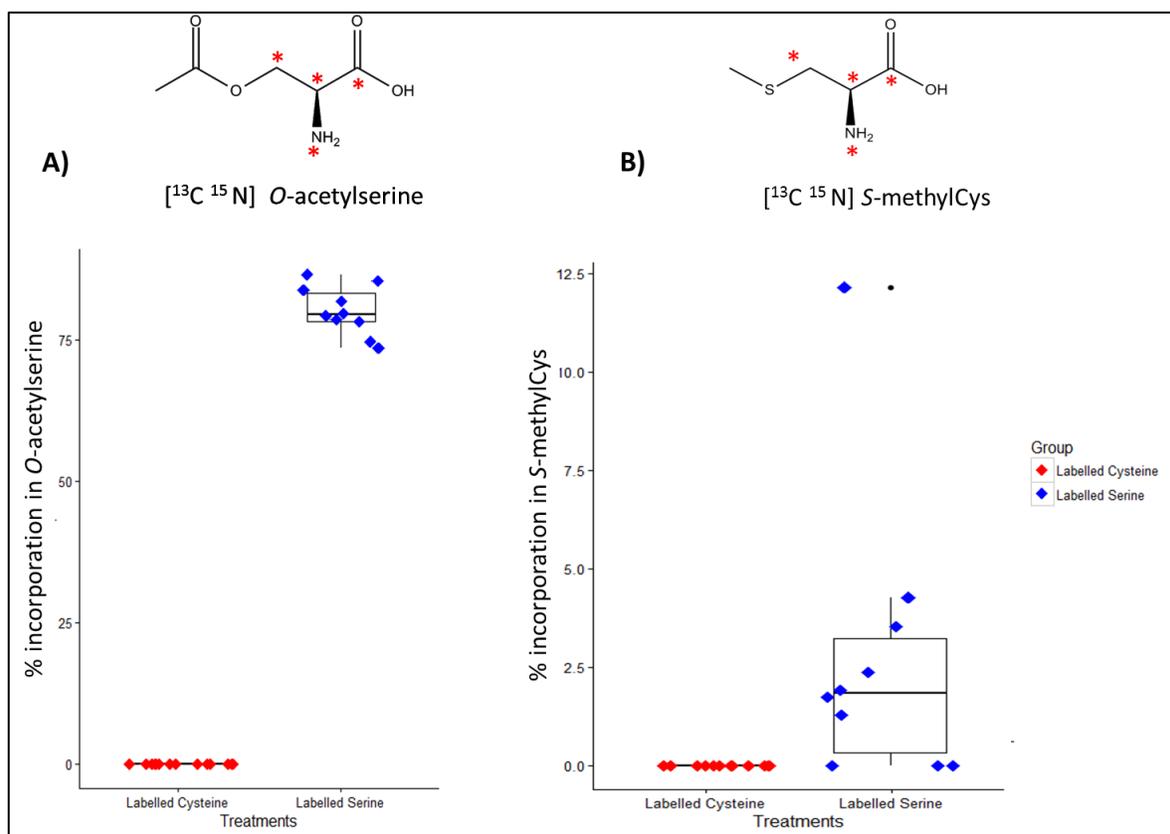


Figure 3.5: Percentage of isotopically labelled compounds in *O*-acetylserine and *S*-methylCys in labelled cysteine or labelled serine supplementation to the basic culture media.

Each dot represents a single LC-MS measurement of a three seed sample. **A)** Percentage incorporation of $^{13}\text{C}_3$ $^{15}\text{N}_1$ in *O*-acetylserine. **B)** Percentage incorporation of ^{13}C and ^{15}N in *S*-methylCys. The percentage incorporation of labelled compound in *O*-acetylserine or *S*-methylCys was calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

3.3.2.3 γ -Glu-*S*-methylCys

The dipeptide γ -Glu-*S*-methylCys, which accumulates in maturing common bean seed was also monitored by LC-MS/MS in order to identify its biosynthetic precursors. γ -Glu-*S*-methylCys ($C_9H_{15}N_2O_5S$), has m/z of 265.0853 and upon collision induced dissociation, two major product ions are produced including a *S*-methylCys fragment ($C_4H_{10}O_2NS$) and deaminated *S*-methylCys ($C_4H_7O_2S$) (Figure 3.6). Should labelled serine or cysteine be incorporated into γ -Glu-*S*-methylCys, the precursor ion would have a $^{13}C_3C_6H_{16}^{15}NNO_5S$, formula and the product ions would be $^{13}C_3CH_{10}^{15}NO_2S$ and $^{13}C_3CH_7^1O_2S$ respectively (Figure 3.6).

Interestingly, the percentage of labelled compound incorporation observed in either labeled cysteine or serine treatments was similar in both the treatments which suggest more than one pathway for γ -Glu-*S*-methylCys biosynthesis (Figure 3.7).

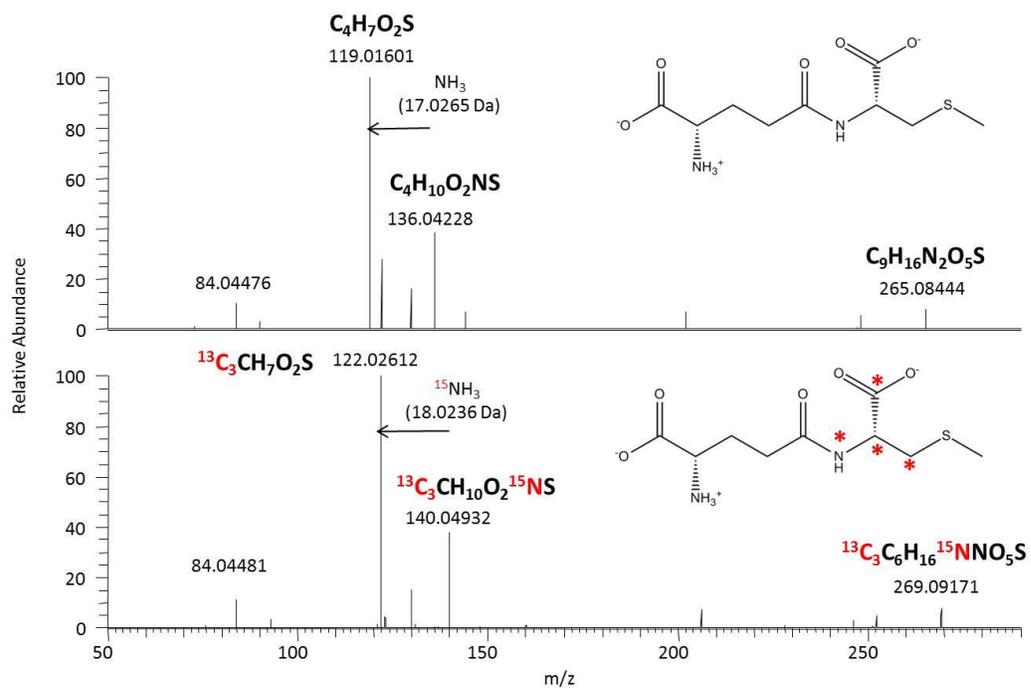


Figure 3.6: Product ion spectra of protonated unlabelled γ -Glu-S-methylCys and labelled γ -Glu-S-methylCys.

Upper panel: Product ions of unlabelled γ -Glu-S-methylCys. **Lower panel:** Product ions of labelled γ -Glu-S-methylCys.

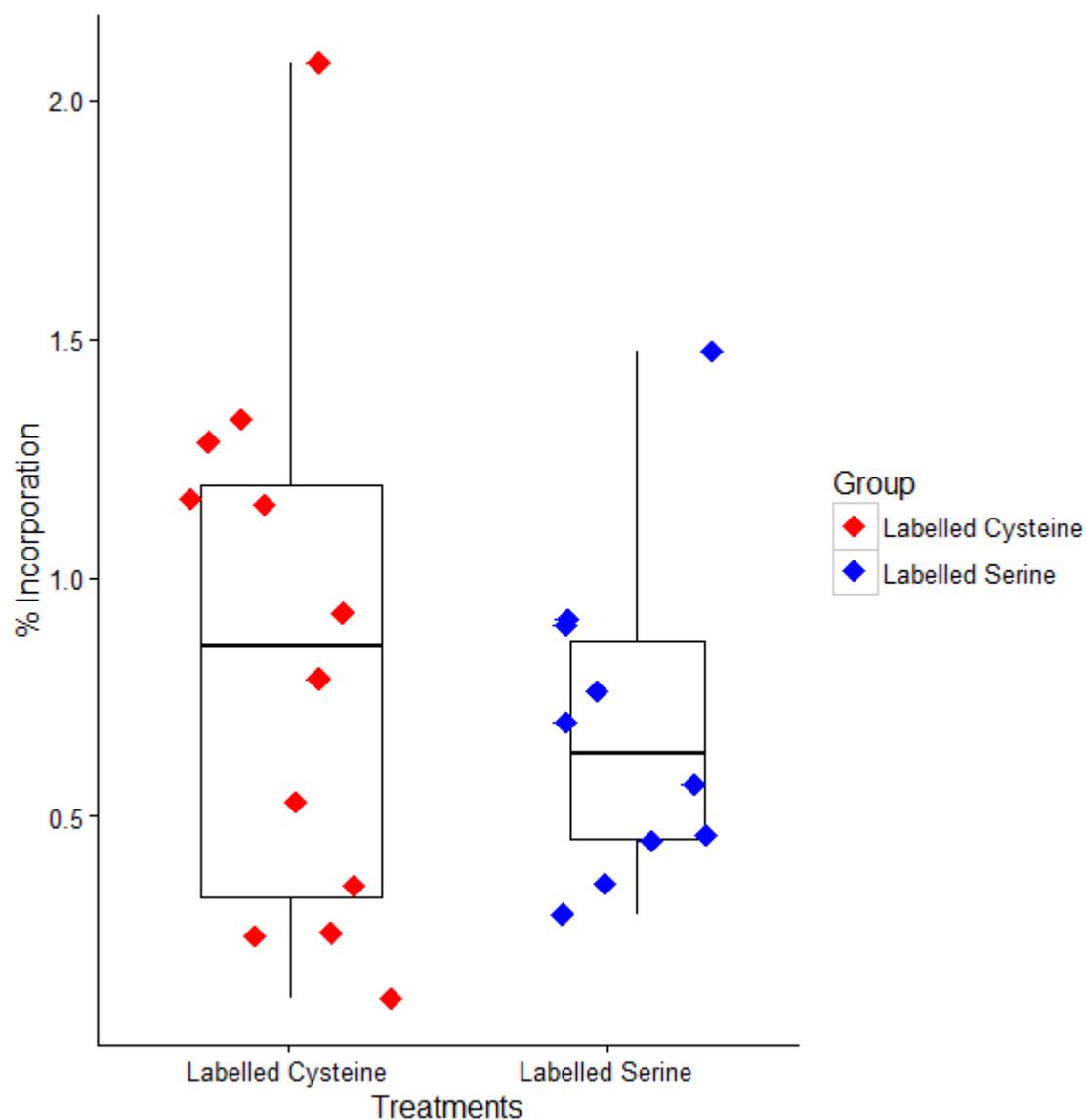


Figure 3.7: Percentage of isotopically labelled compounds γ -Glu-S-methylCys in labelled cysteine or labelled serine supplementation to the basic culture media.

Each dot represents a single LC-MS measurement of a three seed sample. The percentage incorporation of $^{13}\text{C}_3$ and $^{15}\text{N}_1$ in γ -Glu-S-methylCys pool was calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

Several studies have proposed *S*-alk(en)yl-L-cysteine sulfoxides are formed in *Allium* family members via GSH and its conjugates (Suzuki *et al.*, 1962; Turnbull *et al.*, 1980). In the proposed pathway, GSH is *S*-alk(en)ylated at the cysteine residue, followed by the removal of a glycyl group to form a biosynthetic intermediate, γ -glutamyl-*S*-alk(en)yl-L-cysteine. Based on this pathway, we investigated the incorporation of stable isotopes derived from either cysteine or serine into other sulfur containing peptides and their conjugates. Beside methionine biosynthesis, an important role of cysteine is the formation of glutathione and homoglutathione (Galant *et al.*, 2011). γ -Glutamyl cysteine (γ -Glu-Cys), the precursor of glutathione is synthesized from cysteine by γ -Glu-Cys ligase (GCL). Seeds were grown in media with labelled serine or cysteine containing ^{13}C and ^{15}N labelled γ -Glu-Cys. The percent incorporation of ^{13}C and ^{15}N was higher ($69 \pm 7\%$) in labelled serine treated seeds compared to labelled cysteine treatment ($13 \pm 4\%$) (Figure 3.8).

In legumes, GSH accumulates in the form of hGSH, which is present in addition to or in place of GSH (Loscos *et al.*, 2008; Yi *et al.*, 2010). hGSH is synthesized from γ -Glu-Cys and based on this in our experiment $62 \pm 4\%$ incorporation of labelled serine compared to $10 \pm 2\%$ of labeled cysteine follows a similar pattern as in γ -Glu-Cys (Figure 3.8).

Previously, Kasai *et al.* reported the presence of another glutathione conjugate *S*-methylhomoglutathione (*S*-methyl-hGSH) in *Vigna radiata* seeds (Kasai *et al.*, 1986). Following this, the presence of *S*-methyl-hGSH in *P. vulgaris* seeds was also reported (Liao *et al.*, 2013). In accordance with our hypothesis, incorporation of ^{13}C and ^{15}N in *S*-methyl-hGSH follows a similar pattern as γ -Glu-Cys and hGSH did in both labelled cysteine and serine treatments (Figure 3.9).

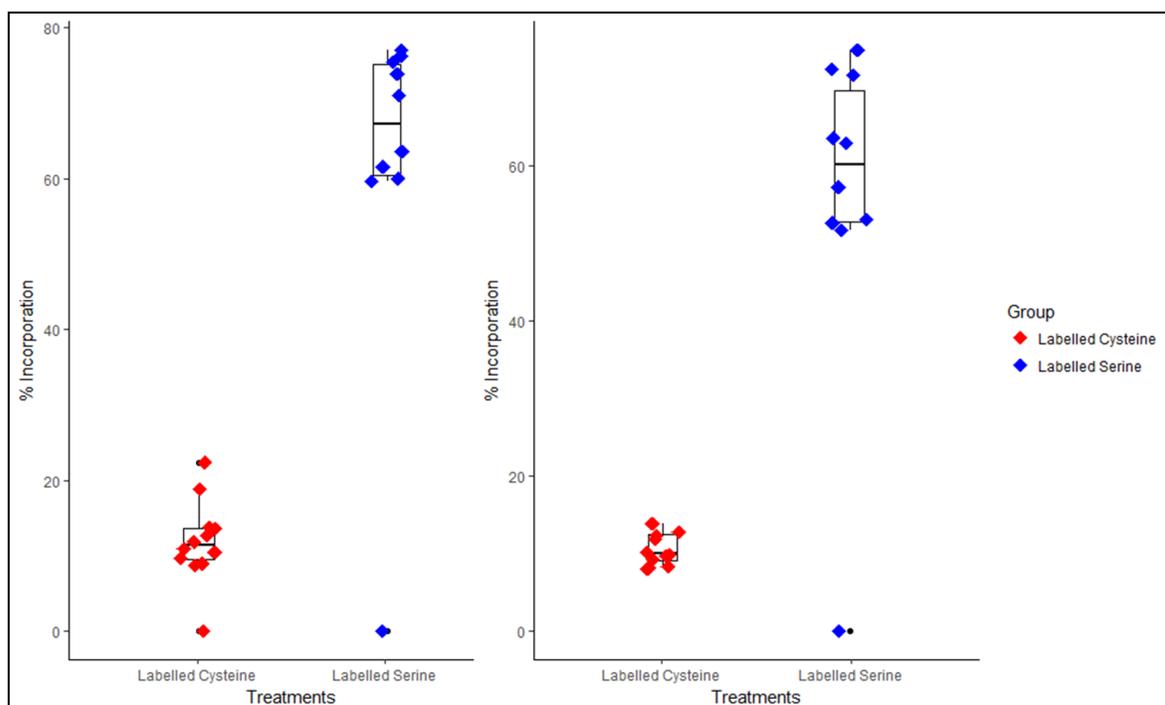


Figure 3.8: Percentage of isotopically labelled compounds γ -Glu-Cys and *h*GSH in developing seeds following incubation with labelled cysteine or labelled serine supplementation to the basic culture media.

Each dot represents a single LC-MS measurement of a three-seed sample. Left: percentage incorporation of $^{13}\text{C}_3$ $^{15}\text{N}_1$ in γ -Glu-Cys. Right: percentage incorporation of ^{13}C and $^{15}\text{N}_1$ in *h*GSH. The percentage incorporation of labelled compound in γ -Glu-Cys or *h*GSH were calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

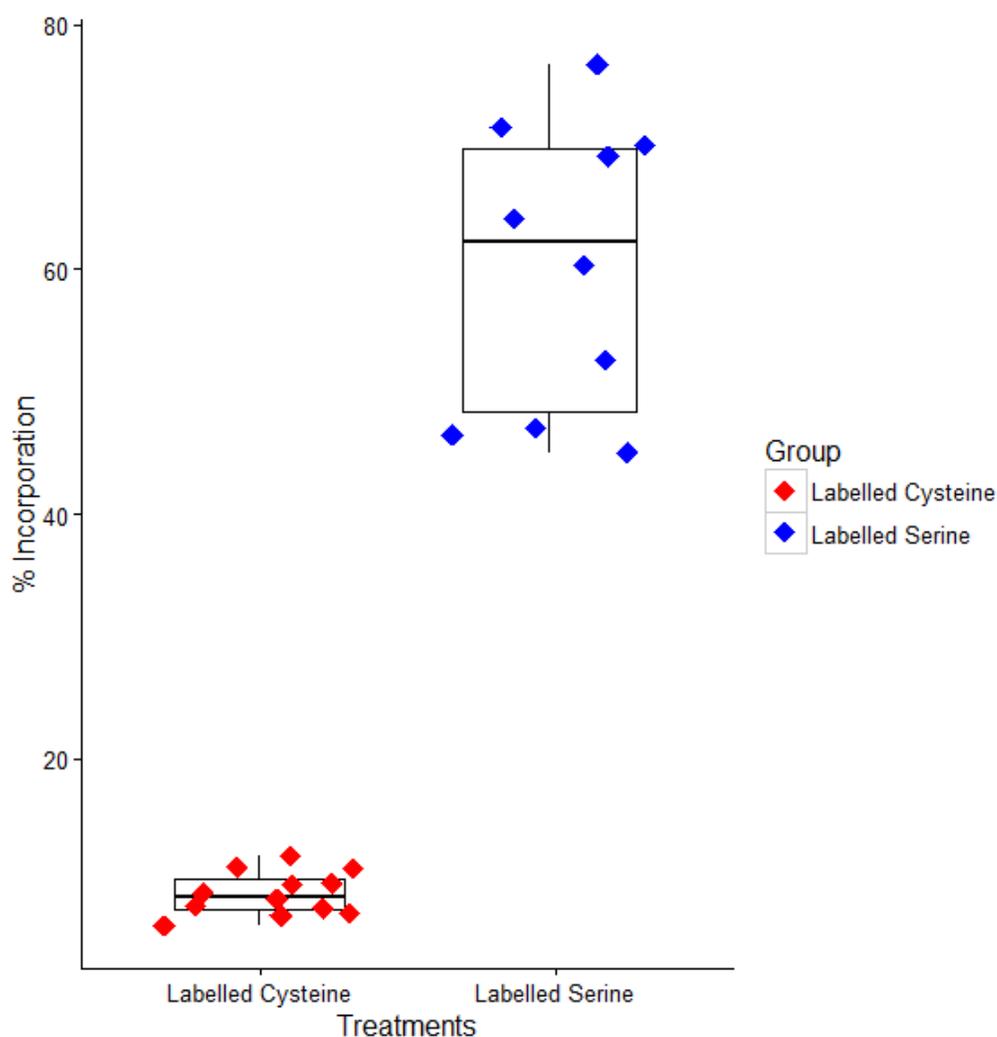


Figure 3.9: Percentage of isotopically labelled compound *S*-methyl-hGSH in developing seeds following incubation with labelled cysteine or labelled serine supplementation to the basic culture media.

Each dot represents a single LC-MS measurement of a three-seed sample. The percentage incorporation of labelled compound in *S*-methyl-hGSH were calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity

3.4 Discussion

In the past, several strategies have been developed to understand sulfur amino acid metabolism in legumes. In this study, isotope labelling was combined with high resolution liquid chromatography-tandem mass spectrometry to decipher *S*-methylCys biosynthesis in common bean. Single stage HRMS is commonly used in biosynthetic studies to monitor stable isotope incorporation, however, in the present study, using high resolution MS/MS greatly reduced the level of background interference and furthermore, provided evidence to the location of the labelled isotopes within the larger molecule itself. In addition to targeted LC-MS/MS, a non-targeted data-dependent acquisition (LC-DDA) method was also used to provide a dataset which could be mined retrospectively for other compounds which were not initially predicted to be involved in biosynthetic pathways. Use of these techniques not only helped me to overcome limitations with sulfur containing metabolite detection but also to understand complex sulfur metabolism in common bean seeds.

To simplify our understanding achieved from this study and combine these lessons with previous knowledge of sulfur metabolism in other plants, a scheme explaining sulfur amino acid metabolism in common bean seeds is proposed and presented in Figure 3.10.

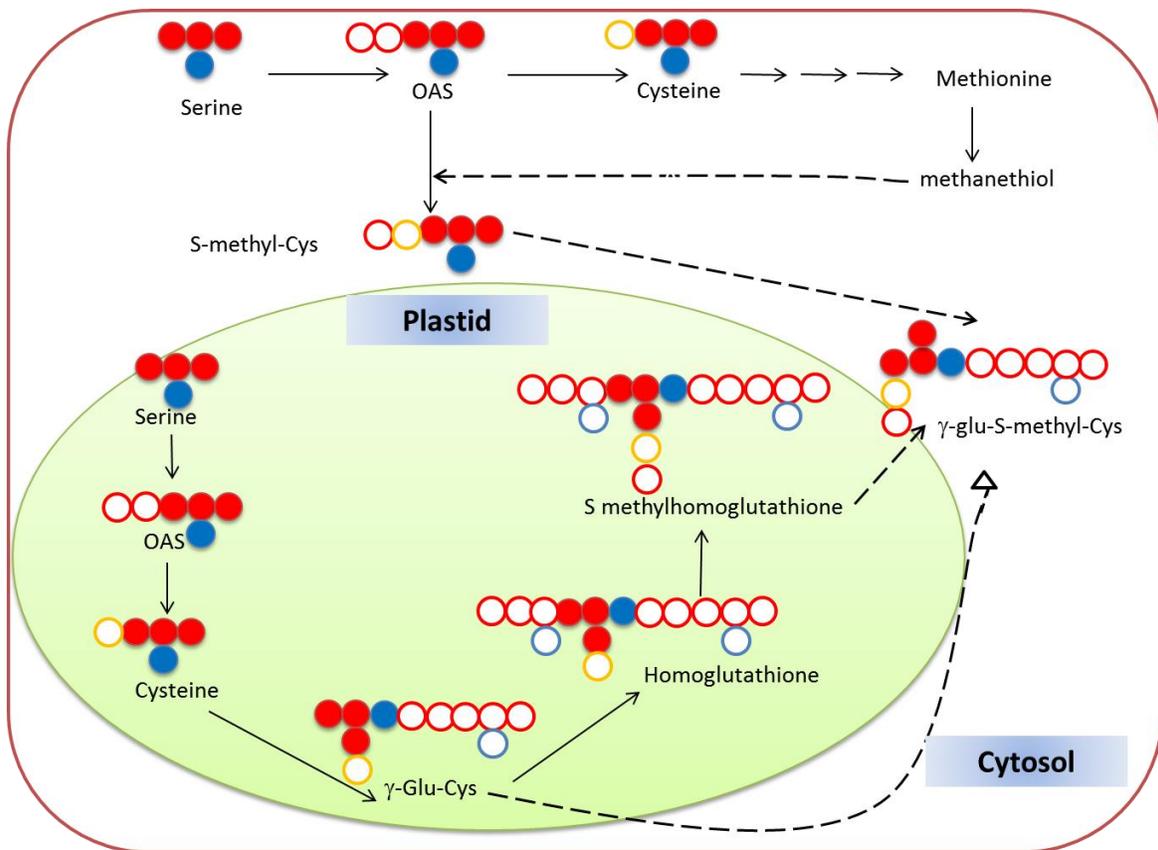


Figure 3.10: Proposed pathway of sulfur amino acid metabolism in common bean developing seeds.

Red color is assigned to carbon, blue to nitrogen and yellow to sulfur atoms of the metabolite. Hollow circle represents unlabelled carbon, nitrogen or sulfur while solid circle represents ^{13}C or ^{15}N incorporation in metabolite. Oxygen and hydrogen atoms present in metabolites are not shown in this figure. The reactions which were not confirmed in this study are represented with a broken arrow. Reactions involving multiple steps are shown with multiple arrows.

Sulfur metabolism is a complex system which is governed by subcellular compartmentation. While plastids are the only site for sulfate reduction, cysteine and methionine biosynthesis takes place in the cytosol, plastids and mitochondria. Differential accumulation of ^{13}C and ^{15}N from cysteine or serine to *S*-amino acids and derivatives suggest that metabolic pathways for *S*-methylCys and γ -Glu-methylCys biosynthesis are compartmentalized to cytosol and plastids, respectively, in common bean seeds. In our pathway, we propose the entry of exogenous amino acids into the cytosol and plastids. In cytosol and plastids serine takes part in biosynthesis of cysteine due to the presence of *bona fide* SERAT and BSAS via *O*-acetylserine formation. In legumes one fate of cysteine is hGSH. hGSH biosynthesis is reported to be governed by a two-step process catalyzed by glutamate–cysteine ligase (GCL) and homoglutathione synthetase (hGS). A major portion of GCL activity to synthesize γ -Glu-Cys takes place in plastids and homoglutathione production is regulated by GCL (Hell and Bergmann, 1990; Hicks *et al.*, 2007; Galant *et al.*, 2011). According to isotope tracking data shown in this study, a similar amount of incorporation of isotopes in γ -Glu-Cys and hGSH in common bean seeds suggests that biosynthesis of both compounds occurs in plastids. Another metabolite which follows similar isotope incorporation pattern as γ -Glu-Cys and hGSH is *S*-methylhGSH which suggests *S*-methylhGSH biosynthesis occurs in plastids. In garlic and onion *S*-alk(en)yl-L-cysteine sulfoxides and γ -glutamyl peptides are reported to be stored in the cytosol (Lancaster *et al.*, 1989; Yoshimoto *et al.*, 2015a). Following isotopes in our feeding experiment also points towards cytosolic biosynthesis of *S*-methylCys and γ -Glu-*S*-methylCys in common bean. Based on relative incorporation of isotopes in a native pool of metabolites, the plastidic pathway is more prominent than the cytosolic pathway in common bean. This may be due to preferred movement of exogenous serine or cysteine inside plastids than in the cytosol.

Our feeding experiment with labelled serine and cysteine supports serine as the precursor of *S*-methylCys in common bean seeds. Recently in the anaerobic protozoan parasite *Entamoeba histolytica* cysteine deprivation led to an increase in *S*-methylCys concentration

(Husain *et al.*, 2010). In this study using stable isotope-labelled L-serine and L-methionine, *O*-acetylserine and methanethiol was reported to be precursor of *S*-methylCys. In common bean seeds *S*-methylCys biosynthesis is also taking place from *O*-acetylserine. It is possible that during seed development SMM is transported from vegetative parts of the plant to the developing seeds where it gets converted to methionine *via* the SMM cycle (Lee *et al.*, 2008). Excessive methionine in developing bean seeds may induce the release of methanethiol. This is similar to pumpkin leaves where an L-methionine-inducible enzymatic system is present, which is capable of converting L-methionine into methanethiol (Schmidt *et al.*, 1985). The available methanethiol could be condensed with *O*-acetylserine to form *S*-methylCys. Most likely the enzyme involved in biosynthesis of *S*-methylCys is an OAS-TL which can condense *O*-acetylserine with methanethiol to produce *S*-methylCys. Previously, in *Spinacia oleracea* an OAS-TL was reported to have the capacity to synthesize *S*-methylCys in presence of *O*-acetylserine and methanethiol (Ikegami *et al.*, 1988b). Unfortunately, we did not observe any incorporation of isotopes in methionine and its derivatives but our future plan is to use labelled methionine in an experiment to confirm the condensation reaction of *O*-acetylserine and methanethiol for *S*-methylCys biosynthesis.

Incorporation of labelled ^{13}C and ^{15}N into γ -Glu-*S*-metCys in both the treatments suggests the presence of more than one pathway for γ -Glu-*S*-metCys synthesis in different cellular compartments. γ -Glutamyl-*S*-methylCys biosynthesis may be taking place *via* two different pathways, one of them involving thiomethylation and the other one involving methylation of homogluthathione. One pathway might take place in the cytosol in which methylation of γ -Glu-Cys may form γ -Glu-*S*-metCys in the presence of methyltransferases. Another one involving methylation might takes place in plastids where homogluthathione synthesizes *S*-methylhGSH. Alanine is then cleaved off from *S*-methylhGSH catalyzed by carboxypeptidase and released in the cytosol. Similar biosynthetic pathways were also proposed by Lancaster *et al.* (1989) after doing pulse chase experiments using radiolabelled $^{35}\text{SO}_4^{2-}$ in leaves of *Allium cepa* (onion), *A. sativum* (garlic) and *A. siculum*. Based on pulse

chase experiment results, it was predicted that glutathione, and γ -glutamyl peptides act as intermediates in the biosynthetic pathway of alk(en)ylcysteine sulphoxide (Lancaster *et al.*, 1989). Two biosynthetic pathways were proposed for the biosynthesis of alk(en)ylcysteine sulphoxide in garlic. One proceeds from alkylation of glutathione through γ -glutamyl peptides to yield *S*-alkyl cysteine sulphoxides while the alternative was direct thioalkylation of serine followed by oxidation to the sulphoxide (Granroth, 1970; Lancaster *et al.*, 1989; Lawson, 1996). Recently biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides via alkylation of glutathione was reported to be the major pathway in garlic (Yoshimoto *et al.*, 2015b). During the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides, *S*-alk(en)ylation of glutathione is followed by removal of glycine that leads to γ -glutamyl-*S*-alk(en)yl-L-cysteine synthesis. Later γ -glutamyl transpeptidases (GGTs) catalyze removal of γ -glutamyl moiety to synthesize *S*-allyl-L-cysteine in garlic (Lancaster *et al.*, 1989; Lancaster and Shaw, 1994; Leustek *et al.*, 2000; Yoshimoto *et al.*, 2015a; Yoshimoto *et al.*, 2015b). Based on high recovery of isotope in *S*-methylhGSH and learning from garlic studies this may be the most promising pathway to synthesize γ -Glu-*S*-methylCys.

Another less likely case may involve synthesis of dipeptide by γ -Glu-Cys synthetase from *S*-methylCys. The mammalian enzyme has substantial *in vitro* activity with *S*-methylCys as substrate and this could also take place in common bean seeds (Rathbun, 1967b; Sekura and Meister, 1977). In the future, designing a pulse chase experiment with metabolic flux analysis (MFA) to track the flux of sulfur in the sulfur metabolic pathway will be an effective solution to confirm the proposed pathways.

3.5 Conclusion

A major outcome from this research is finding a precursor for *S*-methylCys. Previously, several schools of thoughts have indicated that serine, cysteine or glutathione can act as precursors for *S*-methylCys varying from plant to plant. This study supports serine as the precursor for *S*-methylCys in common bean seeds. Furthermore, the results of the isotope labelling experiments provided evidence for *S*-amino acid metabolism producing *S*

metabolites such as γ -Glu-Cys, hGSH, γ -glu-*S*-methylCys and *S*-methylhGSH which may act as storage sinks for sulfur in seeds. Isotope tracking confirms *S*-methylhGSH as the main intermediate in γ -glu-*S*-methylCys biosynthesis. Another fate of sulfur can be in the form of phytochelatins which needs to be validated with another isotope labelling experiment. Based on the finding in this study my next approach will be to identify the genes and enzymes responsible for *S*-methylCys biosynthesis from *O*-acetylserine.

3.6 Cited Literature

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3.7 Supplementary data

Supplementary Data 3.1: Previously published free amino acid profiles in developing seeds of BAT93

Day after fertilization/seed weight (mg)/developmental stage	9/8 III-heart	13/16 IV-cotyledon	15/36 IV-cotyledon	18/98 V-cotyledon	22/180 VI-maturation	27/288 VII-maturation	33/489 VIII-maturation	163 Mature/
Asp	1.2 ± 0.3	1.2 ± 0.1	0.82 ± 0.05	0.55 ± 0.02	0.62 ± 0.04	0.79 ± 0.13	1.16 ± 0.13	0.71 ± 0.07
Glu	3.0 ± 0.6	3.0 ± 0.2	3.1 ± 0.2	4.0 ± 0.1	1.6 ± 0.1	1.7 ± 0.3	0.91 ± 0.08	0.68 ± 0.07
Asn	3.9 ± 0.6	3.4 ± 1.2	2.3 ± 0.1	2.3 ± 0.1	0.34 ± 0.02	0.12 ± 0.01	0.32 ± 0.04	n. d.
Ser	1.3 ± 0.2	1.2 ± 0.1	0.79 ± 0.03	0.53 ± 0.02	0.21 ± 0.01	0.21 ± 0.03	0.18 ± 0.02	0.058 ± 0.017
Gln	21 ± 5	15 ± 4	13 ± 1	7.7 ± 0.2	0.49 ± 0.03	0.21 ± 0.03	0.13 ± 0.02	n. d.
Gly	0.32 ± 0.05	0.24 ± 0.03	0.19 ± 0.01	0.17 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.057 ± 0.01	0.12 ± 0.02
His	0.80 ± 0.22	0.88 ± 0.03	0.64 ± 0.03	0.60 ± 0.02	0.43 ± 0.02	0.083 ± 0.016	0.30 ± 0.04	0.95 ± 0.08
γ-Glu-S-methylCys	0.050 ± 0.01	0.15 ± 0.06	0.84 ± 0.05	2.4 ± 0.1	2.3 ± 0.1	1.8 ± 0.4	3.2 ± 0.5	9.4 ± 1.0
Arg	3.4 ± 0.8	3.6 ± 0.6	4.2 ± 0.3	6.0 ± 0.1	0.87 ± 0.05	0.24 ± 0.04	0.57 ± 0.08	4.0 ± 0.4
Thr	0.72 ± 0.14	0.75 ± 0.05	0.62 ± 0.04	0.51 ± 0.03	0.30 ± 0.02	0.19 ± 0.02	0.27 ± 0.04	0.12 ± 0.01

Ala	5.8 ± 1.9	3.5 ± 1.2	1.3 ± 0.1	1.0 ± 0.1	0.33 ± 0.01	0.20 ± 0.01	0.37 ± 0.05	0.50 ± 0.06
Pro	0.34 ± 0.04	0.31 ± 0.07	0.23 ± 0.01	0.17 ± 0.01	0.080 ± 0.004	0.066 ± 0.005	0.20 ± 0.01	0.24 ± 0.11
γ-Glu-Leu	0.090 ± 0.015	0.092 ± 0.009	0.092 ± 0.004	0.31 ± 0.02	0.67 ± 0.02	0.73 ± 0.10	0.74 ± 0.08	1.8 ± 0.2
Tyr	0.041 ± 0.01	0.077 ± 0.04	0.069 ± 0.01	0.067 ± 0.02	0.048 ± 0.002	0.035 ± 0.01	0.027 ± 0.003	0.051 ± 0.01
S-methylCys	0.41 ± 0.15	0.40 ± 0.05	0.41 ± 0.02	0.21 ± 0.02	0.095 ± 0.001	0.067 ± 0.02	0.081 ± 0.01	0.16 ± 0.02
Val	0.55 ± 0.24	0.84 ± 0.04	0.62 ± 0.03	0.56 ± 0.02	0.23 ± 0.01	0.17 ± 0.02	0.29 ± 0.04	0.20 ± 0.02
Met	1.8 ± 0.3	1.6 ± 0.3	1.6 ± 0.1	0.87 ± 0.04	0.13 ± 0.01	0.10 ± 0.02	0.040 ± 0.01	0.044 ± 0.005
Ile	0.16 ± 0.02	0.41 ± 0.35	0.18 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.11 ± 0.01	0.25 ± 0.03	0.14 ± 0.01
Leu	0.28 ± 0.05	0.44 ± 0.01	1.4 ± 0.1	1.2 ± 0.03	0.13 ± 0.01	0.083 ± 0.009	0.15 ± 0.03	0.12
Phe	0.10 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.086 ± 0.038	0.052 ± 0.014	0.22 ± 0.11	0.73 ± 0.02
Lys	0.062 ± 0.017	0.093 ± 0.016	0.17 ± 0.01	0.30 ± 0.02	0.046 ± 0.001	0.030 ± 0.002	0.018 ± 0.004	0.40 ± 0.03

Values presented as nmol per mg seed weight; average ± standard deviation; n. d.: not determined

Supplementary Data 3.2: Summary of % incorporation of ¹³C and ¹⁵N in metabolites in all the samples

Experimental group: LC: Labelled cysteine treatment; LS: Labelled serine treatment; NLC: non-labelled cysteine treatment; NLS: non-labelled serine treatment, NT: no supplementation

	Serine	Cysteine	<i>O</i> -acetylserine	<i>S</i> -methylCys	γ -Glu- <i>S</i> -metCys	γ -Glu-Cys	hGSH	<i>S</i> -methylhGSH
Non-labelled								
Fragments	88.0397	76.0222	106.0499	119.0160	119.0160	251.0696	193.0637	207.0792
Labelled								
Fragments	92.0464	79.0253	110.0570	122.0262	122.0262	255.0767	197.0712	211.0867
LC1	0.00	86.71	0.00	0.00	1.33	12.73	10.18	9.65
LC2	0.00	66.83	0.00	0.00	1.15	9.65	9.23	7.72
LC3	0.00	66.81	0.00	0.00	0.12	8.66	7.97	7.14
LC4	0.00	74.74	0.00	0.00	0.53	10.47	9.85	9.03
LC5	0.00	71.50	0.00	0.00	0.26	10.92	8.11	6.36
LC6	0.00	33.81	0.00	0.00	2.08	11.81	9.68	7.89
LC7	0.00	63.43	0.00	0.00	0.25	8.96	8.34	7.31
LC8	0.00	85.73	0.00	0.00	1.17	22.37	12.29	11.10
LC9	0.00	56.23	0.00	0.00	0.93	13.62	12.80	9.78
LC10	0.00	84.89	0.00	0.00	1.28	0.00	13.80	12.06
LC11	0.00	87.69	0.00	0.00	0.36	13.81	13.88	8.45

LC12	0.00	86.13	0.00	0.00	0.79	18.81	11.86	11.03
LS1	90.94	50.37	86.52	4.27	0.70	59.63	51.67	45.04
LS2	90.29	60.39	85.32	1.28	0.36	73.89	62.93	64.16
LS3	85.33	75.58	81.73	1.92	0.57	63.60	57.32	60.41
LS4	84.04	55.50	78.13	2.37	0.76	61.61	53.05	46.50
LS5	84.28	56.78	78.54	3.52	0.91	60.05	52.71	52.56
LS6	83.97	62.13	83.78	0.00	0.30	75.52	71.76	69.23
LS7	75.76	0.00	73.53	12.16	1.47	0.00	0.00	47.00
LS8	84.91	100.00	74.65	1.74	0.45	71.00	63.64	70.14
LS9	85.44	78.59	79.17	0.00	0.90	76.29	72.52	71.62
LS10	85.98	0.00	79.57	0.00	0.46	77.04	74.93	76.84
NLC1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLC2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLC3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLC4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLC5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLS1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLS2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLS3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLS4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

NLS5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NLS6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NT1	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NT2	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
NT3	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06
NT4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NT5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NT6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NT7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

CHAPTER FOUR - EVIDENCE THAT BSAS4;1 UTILIZES A METHYL GROUP FROM METHIONINE FOR S-METHYLCYSTEINE BIOSYNTHESIS IN COMMON BEAN (*Phaseolus vulgaris*)

4.1 Introduction

Methionine is a sulfur amino acid and is the most limiting essential amino acid in legumes along with cysteine (Galili and Amir, 2013). Cysteine is the first organic compound in which inorganic sulfur is assimilated in the plant system. The fate of cysteine lies mainly in protein, methionine or glutathione biosynthesis (Hell and Wirtz, 2011). Methionine biosynthesis from cysteine leads to the formation of *S*-adenosylmethionine (SAM), a universal methyl donor. SAM not only acts as primary methyl group donor for various cellular processes but also plays a major regulatory role in the biosynthesis of biotin, polyamines, phytohormones, ethylene, chlorophyll and cell wall polymers (Roje, 2006). Another known metabolic fate of methionine is *S*-methylmethionine (SMM) which is unique to higher plants (Bourgis *et al.*, 1999). SMM biosynthesis results from SAM-dependent methylation of methionine, generating *S*-adenosylhomocysteine, which cycles back to synthesize methionine through the SMM cycle (Ranocha *et al.*, 2001). Catabolism of methionine leads to the formation of volatile molecules such as methanethiol, dimethyl disulfide, or dimethyl sulfide (Schmidt *et al.*, 1985; Boerjan *et al.*, 1994; Hacham *et al.*, 2002; Goyer *et al.*, 2007) (Figure 4.1). Several reports of these volatiles are published in various crops but till today the physiological significance of these volatiles is yet unknown.

Methionine- γ -lyase (MGL; EC 4.4.1.11), a pyridoxal 5'-phosphate (PLP) dependent enzyme is reported to be involved in the production of these volatiles (Christen and Mehta, 2001). Taxonomically MGL is present in a vast majority of genera and characterized in microbes, protozoa and plants (Inoue *et al.*, 1995; Dias and Weimer, 1998; Manukhov *et al.*, 2005; Goyer *et al.*, 2007; Sato and Nozaki, 2009). It catalyzes the conversion of methionine to α -ketobutyrate, methanethiol and ammonia. Further, α -ketobutyrate enters

the isoleucine biosynthetic pathway, while the fate of methanethiol is yet to be discovered (Joshi and Jander, 2009). Production of methanethiol is a consequence of the high accumulation of free methionine in the plant tissue. An MGL knockout mutant accumulated high amount of SMM and methionine in leaves, flowers and seeds of *Arabidopsis* (Saini *et al.*, 1995; Goyer *et al.*, 2007; Joshi and Jander, 2009). However, release of methanethiol seemed not be the case with *Arabidopsis* treated with methionine, suggesting entry of methanethiol in metabolism (Goyer *et al.*, 2007).

In various legume crops *S*-amino acids methionine and cysteine are reported in suboptimal levels (Galili *et al.*, 2005). In contrast to this common bean (*P. vulgaris*) seeds accumulate high levels of a non proteinacious *S*-amino acid *S*-methylCys which has an inverse relationship with methionine and cysteine content (Taylor *et al.*, 2008; Yin *et al.*, 2011). *Arabidopsis* cell culture lines which were grown in methionine rich media have shown production of *S*-methylCys and isoleucine. Production of *S*-methylCys in these cells was predicted to be linked with MGL driven γ -cleavage of methionine releasing methanethiol (Rebeille *et al.*, 2006).

In Chapter 3, isotopic tracking of labelled serine and cysteine revealed *O*-acetylserine as a precursor of *S*-methylCys biosynthesis in developing seeds of common bean. To synthesize *S*-methylCys from *O*-acetylserine one possibility is a condensation reaction between *O*-acetylserine and methanethiol. In 1988, Ikegami *et al.* reported the presence of a cysteine synthase (OAS-TL) in *Spinacia oleracea* that could synthesize *S*-methylCys using *O*-acetylserine and methanethiol as substrates (Ikegami *et al.*, 1988b). The common bean BSAS family has eight members that have either cysteine synthase or cysteine synthase like activity. Structural and functional analysis of OAS-TL from different plants suggests a two-step reaction mechanism. In the first half reaction, *O*-acetylserine reacts with the PLP cofactor to yield a reactive α -aminoacrylate intermediate. In the second half reaction, this intermediate interacts with a sulfur containing moiety to synthesize cysteine (Warrilow and Hawkesford, 2002; Bonner *et al.*, 2005). The present study provides lines of evidence for the role of a cysteine synthase in *S*-methylCys biosynthesis. Isotope labeling method was

used to track the source of the methyl group in *S*-methylCys and to characterize seed specific cysteine synthase in common bean that is a candidate enzyme for *S*-methylCys biosynthesis.

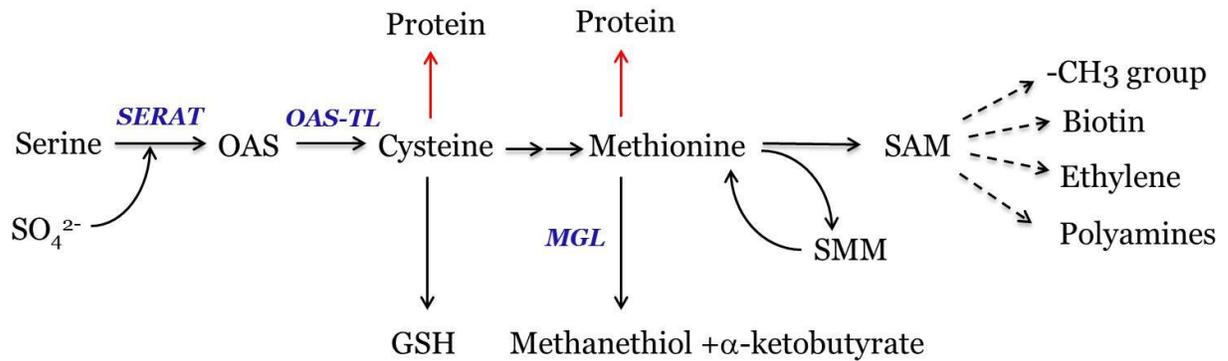


Figure 4.1: Schematic representation of cysteine and methionine fate in plants.

OAS: *O*-acetylserine, SAM: *S*-adenosylmethionine, SMM: *S*-methylmethionine, GSH: Glutathione. Key enzymes for these reactions are SERAT: Serine acetyltransferase; OAS-TL: *O*-acetylserine (thiol) lyase and MGL: Methionine- γ -lyase. Multiple arrows represent more than one step in that conversion.

4.2 Materials and methods

4.2.1 Chemicals

¹³C, ¹⁵N isotope labelled methionine, serine and cysteine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Other components of growth media were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

4.2.2 Plant materials

Common bean (*Phaseolus vulgaris*) genotype BAT93 plants were grown in growth cabinets (Environmental Growth Chambers, Chagrin Falls, OH, USA) under 16 h light (300-400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at a temperature cycling between 18 and 24 °C as described by Pandurangan *et al.* (2012). Seeds were planted and fertilized as described in section 3.2.2.

4.2.3 Isotope labelling and embryo culture

Isotopic labelling and embryo culture was performed in 25 mL standard line cell culture flasks (VWR, Mississauga, Ontario, Canada) containing culture media following the methods described in section 3.2.3.

4.2.4 Culture media

Seeds were grown in a basic culture media with some modifications. (Thompson *et al.*, 1981; Obendorf *et al.*, 1983; Holowach *et al.*, 1984). Composition of basic media is described in section 3.2.4. This basic culture media was supplemented by 8 mM methionine, serine or cysteine based on treatment groups.

4.2.5 Treatments

Seven groups of treatments were designed based on culture media: labelled methionine/serine/cysteine supplementation, non-labelled methionine/serine/cysteine supplementation and no supplementation. In each treatment group, three cotyledons grown

in cell culture flasks were kept horizontally at room temperature under continuous light and slow shaking (50 rpm). After completion of 48 h incubation, seeds were washed three times with sterile water to remove any traces of non-absorbed amino acids on the surface. Seeds were dried and stored at -80 °C following flash freezing in liquid nitrogen for future experiments.

4.2.6 Amino acid extraction

From the frozen seeds amino acids were extracted in ethanol: water (70:30) (Kasai *et al.*, 1986) and processed for mass spectrometry analysis following the method described in section 3.2.6.

4.2.7 HRMS and HRMS/MS

MS data were acquired with a Thermo® Q-Exactive Orbitrap mass spectrometer coupled to an Agilent 1290 HPLC system. Method described in section 3.2.7 for MS/MS and DDA was used to acquire data. Xcalibur™ software was used to calculate all theoretical masses and to analyze data. The proportion of labelled compound present was calculated by dividing the intensity of the labelled signal over the sum of labelled and unlabelled intensity.

4.2.8 Expression profile

Transcript expression profile of BSAS family members in different tissues was recorded according to the gene atlas (O'Rourke *et al.* 2014). A heat map was generated in R using the heatmap.2 function from the gplots and RColorBrewer CRAN library. Expression values are represented as RPKM (reads per kilobase of transcript per million mapped reads).

4.2.9 Cloning BSAS4;1 for recombinant protein expression

A full length β -substituted alanine synthase (BSAS) gene was amplified from common bean (*P. vulgaris*) line BAT93. RNA was extracted from developing seeds as mentioned by Wang and Vodkin (1994) and quantified by spectrophotometry with a NanoDrop 1000

(ThermoScientific, Wilmington, DE, USA). Quality of RNA was evaluated from A_{260/280} ratio and by 1% agarose gel electrophoresis. One microgram of total RNA treated with amplification-grade DNase I (Life Technologies, Burlington, ON, Canada) was used for cDNA synthesis using qScript™ cDNA SuperMix (Quanta Biosciences).

A 936 bp long BSAS4;1 was amplified by polymerase chain reaction (PCR) using *Pfx50* DNA polymerase (Life Technologies, Burlington, ON), and the primers designed based on the accession number Phvul.008G061100 in *P. vulgaris v2.1* available in Phytozome. The primer sequences for *PvBSAS4;1* (Fw: 5'-ATTACTGGATCCGAGCCAAAGTGTGCGATTAAGAAG-3' and Rvs: 5'-AGTAATAAGCTTTTAGTCAAATGTCATTTGTTTCAGCTTCT-3') contained the vector-specific sequences and restriction sites (underlined). The amplification conditions were 94 °C for 30 s; 30 cycles of 58.5 °C for 30 s, 68 °C for 60 s. The PCR product was cloned into the pSC-B-amp/kan vector as per the manufacturer's protocol (StrataClone Blunt PCR Cloning Kit, Agilent Technologies) and transformed in *Escherichia coli*. Positive recombinant clones selected on ampicillin plates were confirmed by restriction digests (*Bam*HI and *Hind*III) and DNA sequencing using the M13 sequencing primers and checked against the BAT93 genome. Confirmed plasmid DNA was digested with *Bam*HI and *Hind*III and subcloned in the bacterial expression vectors pQE30 (Qiagen, Toronto, ON) by restriction enzyme-mediated cloning using *Bam*HI and *Hind*III restriction endonucleases and T4 DNA ligase following manufacturers' guidelines. The expression constructs were transformed in *E. coli* XL10-Gold (Agilent Technologies, Mississauga, ON).

4.2.10 Protein purification

XL10-Gold cells were grown in NZY media with ampicillin (100 µg/ml) as antibiotic at 37 °C. At OD of 0.6, cells were induced by 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) and allowed to grow at room temperature for 14-16 h. Cells were harvested by centrifugation (6,000 × g, 30 min at 4 °C) and suspended in lysis buffer (50 mM sodium

phosphate, pH 8.0, 500 mM NaCl and 20 mM imidazole, pH 7.4). Suspended cells were treated with 1 mg/mL lysozyme for 30 min, lysed with French press and supernatant was collected after centrifugation at $18,000 \times g$ for 45 minutes. After centrifugation, the supernatant was purified by immobilized metal affinity chromatography on a 5 mL HisTrap Ni²⁺-Sepharose column using an ÄKTApurifier system (GE Healthcare). The column was washed with five column volumes of wash buffer (50 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) and eluted with a linear gradient varying from 0 to 100% of elution buffer (50 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Purified protein was desalted in 100 mM MOPSO, pH 7.5 with PD10 column using gravity method and later concentrated using Amicon Ultra-15 Ultracel 30 K filter unit (Millipore, Billerica, MA). Purified protein was stored at -80 °C in 20% glycerol (v/v) after flash freezing in liquid nitrogen. Protein quantification and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed as previously described.

4.2.11 Size exclusion chromatography

Size exclusion chromatography was performed on ÄKTApurifier system (GE Healthcare) using HiLoad Superdex 200 prep grade column with FPLC buffer (20 mM Tris buffer, pH 7.5, 150 mM sodium chloride). Molecular weight of eluted protein was calculated based on a standard curve generated with Gel Filtration Standard (Bio-Rad). The protein quantification was performed using the Bio-Rad Protein Assay solution (Mississauga, ON), and bovine serum albumin (BSA) as standard. Purified protein was analyzed by SDS-PAGE on 12% gel, and the protein bands were visualized by Coomassie staining as described (Laemmli, 1970).

4.2.12 Absorption spectroscopy of PvBSAS4;1 protein

Absorption spectrum of purified recombinant protein was determined using a DU 600 spectrophotometer (Beckman Coulter, Mississauga, ON). The typical wavelength scan was between 250 and 600 nm and used 1.5 mg/ml of purified protein in 25 mM HEPES buffer with 100 mM sodium chloride (pH 7.5)

4.2.13 Ligand binding assay

Binding of cysteine and *O*-acetylserine to PvBSAS4;1 was monitored by using PowerWave XS plate reader equipped with Gen5.5 software (BioTeK Instruments, Winooski, VT). Change in PLP binding signal was collected from 350 to 600 nm wavelength spectra using 400 µg protein (in 25 mM HEPES, pH 7.5, 100 mM NaCl) and varying concentration of *O*-acetylserine (0 - 1.5 mM) and cysteine (0 - 1.5 mM).

4.2.14 Enzyme assays

Cysteine synthase activity of PvBSAS4;1 was measured by measuring cysteine biosynthesis using acid ninhydrin assay as described previously by Gaitonde (1967). To prepare acid ninhydrin reagent 250 mg of ninhydrin was dissolved in a mixture of 6 mL acetic acid and 4 mL conc. HCl. The mixture required 30 min continuous mixing at room temperature in order to dissolve ninhydrin completely (Gaitonde, 1967). A 250 µL reaction containing 100 mM MOPSO (pH 7.0), 8 mM *O*-acetylserine, and 0.75 mM Na₂S was initiated by adding 80 ng of enzyme. After incubating at 25 °C for 15 min, the reaction was stopped by adding 50 µL of 40 mM HCl. Two hundred fifty µL of the reaction was mixed with an equal volume of freshly prepared acid-ninhydrin reagent. The mixture was heated (95 °C) for 5 min and then cooled on ice for 5 min and 500 µL of 100% cold ethanol was added. Production of cysteine was determined colorimetrically at 546 nm using PowerWave XS plate reader. A standard curve of cysteine was developed from 10 µM -100 µM. Varied concentration of *O*-acetylserine (0-8 mM), and Na₂S (0-0.75 mM) was used to determine steady-state kinetic parameters. For *S*-methylCys biosynthesis assay, the cysteine synthase enzyme assay was used using 2 mM methanethiol and 10 mM *O*-acetylserine. Production of *S*-methylCys was detected by mass spectrometry using previously described method (section 3.2.7). In *S*-methylCys assay 10 µM labelled methionine was used for isotopic dilution to reduce the matrix effects.

To determine the cyanoalanine synthase (CAS) activity of PvBSAS4;1, a reaction mixture of 500 μ L containing 50 mM Tris HCl (pH 9.0) buffer, 1 mM cysteine, 2 mM KCN and 50 ng of protein was incubated at 25 °C for 10 min. The reaction was stopped by adding 50 μ L of 30 mM FeCl₃ in 1.2 N HCl and 50 μ L of 20 mM N, N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) in 7.2 N HCl. Production of methylene blue due to the reaction of one molecule of produced H₂S with two molecules of DMPD was colorimetrically determined at 670 nm using a PowerWave XS plate reader with Gen5.5 software (BioTeK Instruments, Winooski, VT). CAS activity was expressed as the amount (moles) of H₂S produced by 1 mg protein in 1 sec (katal mg⁻¹). To determine the kinetic parameters of PvBSAS4;1, various concentrations of substrates such as cysteine (0-2 mM) and KCN (0-1 mM) were used in the described enzyme assay reaction. Various concentrations of Na₂S were used to generate a standard curve to determine released H₂S in the reaction. Steady state kinetic parameters K_m and V_{max} were determined by nonlinear fitting of initial velocity versus substrate concentration to the Michaelis-Menten equation.

4.3 Results

4.3.1 Methyl donor for *S*-methylCys biosynthesis

In our previous study, I discovered that serine acts as a precursor for *S*-methylCys. To determine the role of methionine in *S*-methylCys, common bean seeds were incubated with labelled methionine (¹³C₅H₁₀¹⁵NO₂S). After 48 h of incubation seeds treated with labelled methionine had a 98 ± 4% methionine pool labelled as ¹³C₅¹⁵N methionine, which suggests efficient uptake of methionine from media by developing seeds.

Methionine catabolism by MGL leads to methanethiol, α -ketobutyrate and ammonia. In common bean if MGL is active and methionine is taking part in isoleucine biosynthesis as reported in *Arabidopsis*, then ¹⁵N should be lost as ¹⁵NH₃ and methanethiol should inherit one ¹³C from methionine. Isoleucine biosynthesis from methionine should lead to the inclusion of four isotopic labelled carbons from methionine and the expected *m/z* is 136.1153. Our labelled methionine treated common bean seeds showed significantly more

m/z 136.1153 ions than any other amino acid treated seeds (Supplementary Data 4.1). These data suggest the presence of active MGL in developing seed of common bean which hydrolyzes methionine into α -ketobutyrate and methanethiol.

If methionine is the source of methanethiol for *S*-methylCys biosynthesis, then in labelled methionine treated seeds one carbon of *S*-methylCys ($m/z=136.0427$) will be labelled and parent ion will become 137.0422. Comparing labelled methionine treatment with no supplementation treatment $78.4 \pm 0.6\%$ of *S*-methylCys was labelled on one carbon in labelled methionine treated seeds. When I compared, labelled methionine treated samples with labelled serine or cysteine treated samples, I detected the presence of one carbon labelled *S*-methylCys ($^{13}\text{CC}_3\text{H}_{10}\text{NO}_2\text{S} = 137.0422$) only in labelled methionine treated samples not in the other treatments (Figure 4.2).

To confirm the position of ^{13}C labelled carbon incorporated from methionine to *S*-methylCys MS/MS was performed. The major primary product ion of protonated *S*-methylCys ($\text{C}_4\text{H}_{10}\text{NO}_2\text{S}$) after neutral loss of NH_3 (17.0265 Da) is $\text{C}_4\text{H}_7\text{O}_2\text{S}$ (119.01644 m/z). If the methyl group was donated by labelled methionine to *S*-methylCys, the product ion formula would be $^{13}\text{CC}_3\text{H}_7\text{O}_2\text{S}$ (120.0198 m/z) after losing NH_3 (17.0264 Da), which is what I observed (Figure 4.3). A secondary product of *S*-methylCys is $\text{C}_3\text{H}_5\text{O}_2^+$ (73.029 Da) which arises following the neutral loss of CH_2S . In case of one carbon labelled *S*-methylCys, the labelled carbon is lost as the neutral loss of $^{13}\text{CH}_2\text{S}$ and product ion remains $\text{C}_3\text{H}_5\text{O}_2$ (73.029 Da) and that is what I observed (Figure 4.3). These findings strongly suggest methionine as a methyl donor for *S*-methylCys biosynthesis.

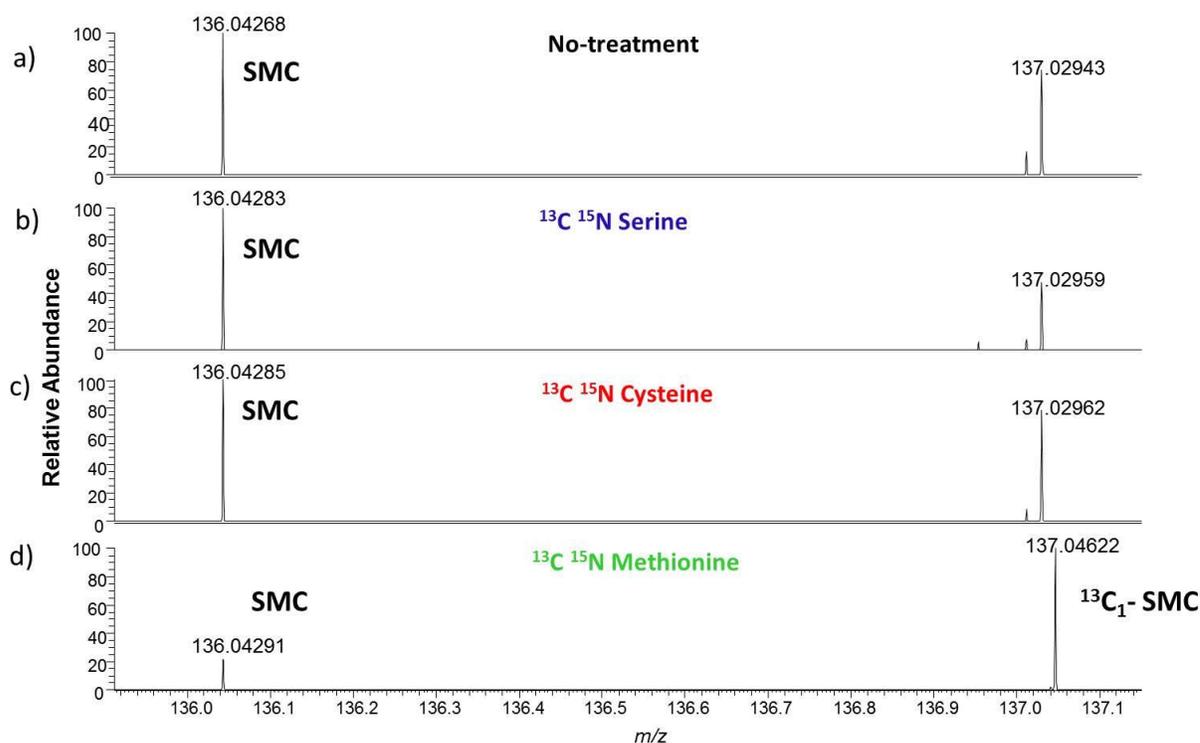


Figure 4.2: Comparison of isotopic pattern of *S*-methylCys in various treatments.

a) no amino acid supplementation; **b)** seeds treated with $^{13}\text{C}_3$ and ^{15}N serine; **c)** seeds treated with $^{13}\text{C}_3$ and ^{15}N cysteine; **d)** seeds treated with $^{13}\text{C}_4$ and ^{15}N methionine. One carbon labelled *S*-methylCys ($m/z = 137.04603$) is not detectable in seeds treated with labelled serine or cysteine but in case of labelled methionine treated samples it is present prominently. An unknown peak ($m/z=137.02962$) was detected no treatment, serine and cysteine treated seeds which is not one carbon labelled *S*-methylCys ($m/z = 137.04603$).

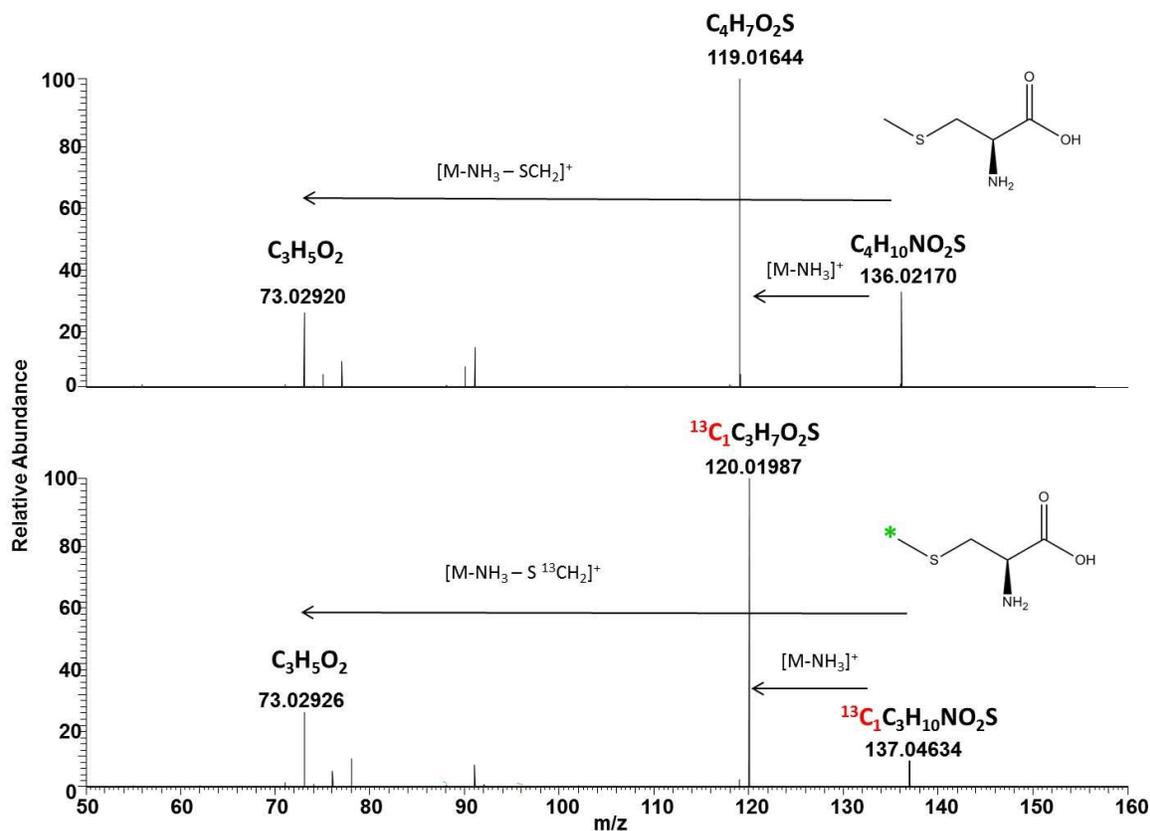


Figure 4.3: Representative dissociation of protonated unlabelled *S*-methylCys and labelled *S*-methylCys in unlabelled methionine treatment and labelled methionine treated seeds.

Upper panel: product ions of unlabelled *S*-methylCys in non-labelled methionine treatment. **Lower panel:** product ions of one carbon labelled *S*-methylCys in labelled methionine treatment. The asterisk represents the position of the labelled carbon in *S*-methylCys after labelled methionine treatment.

To determine whether methionine plays any role in γ -Glu-*S*-methylCys biosynthesis, another non-proteinaceous *S*-amino acid in common bean, I followed ^{13}C and ^{15}N labelling of methionine. Interestingly, γ -Glu-*S*-methylCys also inherits one ^{13}C from methionine, which is not the case in any other treatment (Figure 4.4). Upon collision induced dissociation of γ -Glu-*S*-methylCys ($\text{C}_9\text{H}_{15}\text{N}_2\text{O}_5\text{S}$) two major product ions are produced, including a *S*-methylCys fragment ($\text{C}_4\text{H}_{10}\text{O}_2\text{NS}$) and deaminated *S*-methylCys ($\text{C}_4\text{H}_7\text{O}_2\text{S}$). In case of ^{13}C labelled γ -Glu-*S*-methylCys ($\text{C}_9\text{H}_{15}\text{N}_2\text{O}_5\text{S}$) these product ions changed to a labelled *S*-methylCys fragment ($^{13}\text{CC}_3\text{H}_{10}\text{O}_2\text{NS}$) and labelled deaminated *S*-methylCys ($^{13}\text{CC}_3\text{H}_7\text{O}_2\text{S}$) which have m/z 137.04609 and 120.01972 respectively. The product ion of m/z 73.029 Da confirms incorporation in the *S*-methyl group of *S*-methylCys (Figure 4.5). Around 17% of γ -Glu-*S*-methylCys had one carbon labelled. Movement of ^{13}C from methionine to γ -Glu-*S*-methylCys determines the role of methionine in γ -Glu-*S*-methylCys biosynthesis either via *S*-methylCys or SAM. During the search of downstream metabolite of methionine biosynthesis, I could track isotope label incorporation into SMM, SAM and *S*-methylhomoglutathione (Supplementary Data 4.2 and 4.3).

Based on this isotope tracking experiment I conclude that methionine catabolism leads to methanethiol synthesis which enters the *S*-metabolism pathway and gets incorporated into *S*-methylCys and γ -Glu-*S*-methylCys. Now the question is: what are the key enzymes involved in *S*-methylCys biosynthesis?

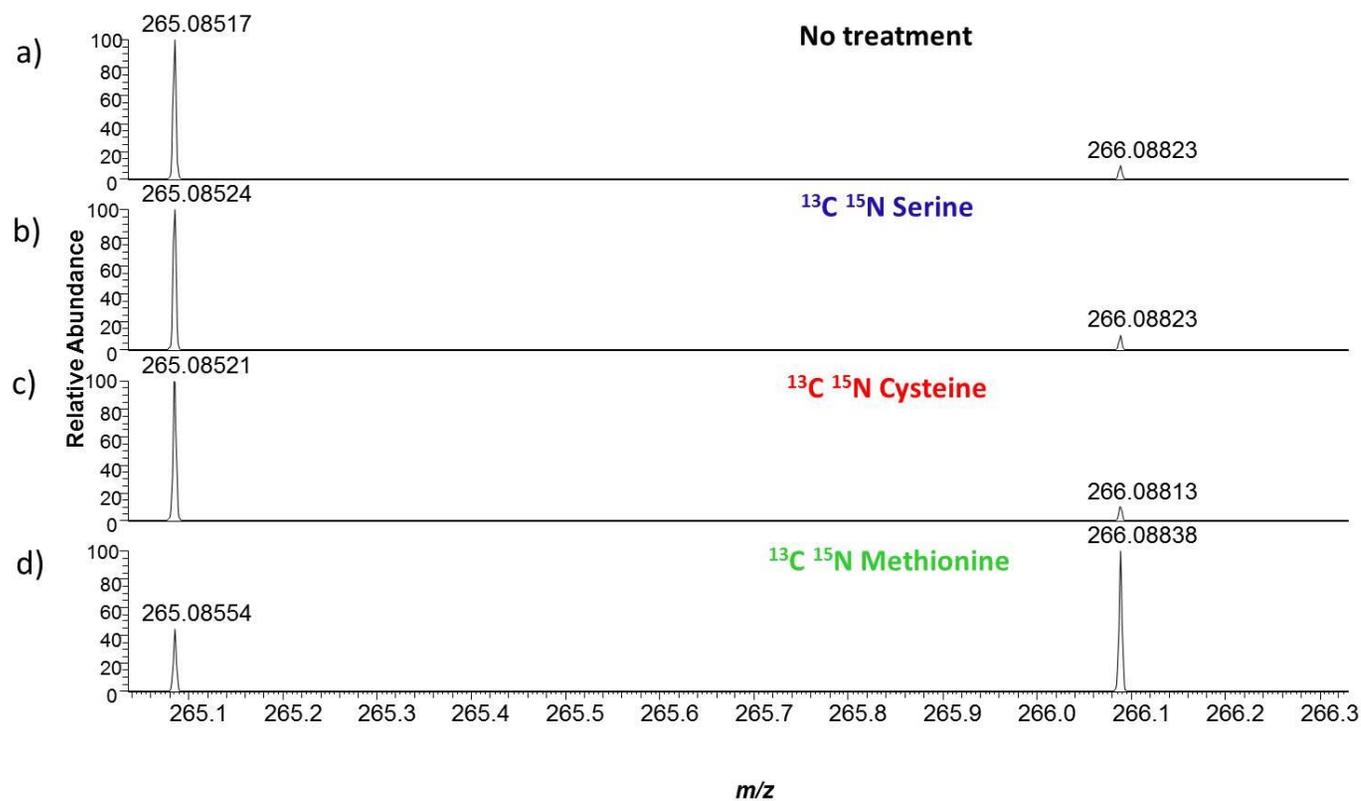


Figure 4.4: Comparison of isotopic pattern of γ -Glu-S-methylCys in various treatments.

a) no amino acid supplementation; **b)** seeds treated with $^{13}\text{C}_3$ and ^{15}N serine; **c)** seeds treated with $^{13}\text{C}_3$ and ^{15}N cysteine; **d)** seeds treated with $^{13}\text{C}_4$ and ^{15}N methionine. One carbon labelled γ -Glu-S-methylCys ($m/z=137.04603$) is not detectable in seeds treated with labelled serine or cysteine but in case of labelled methionine treated samples it is present prominently.

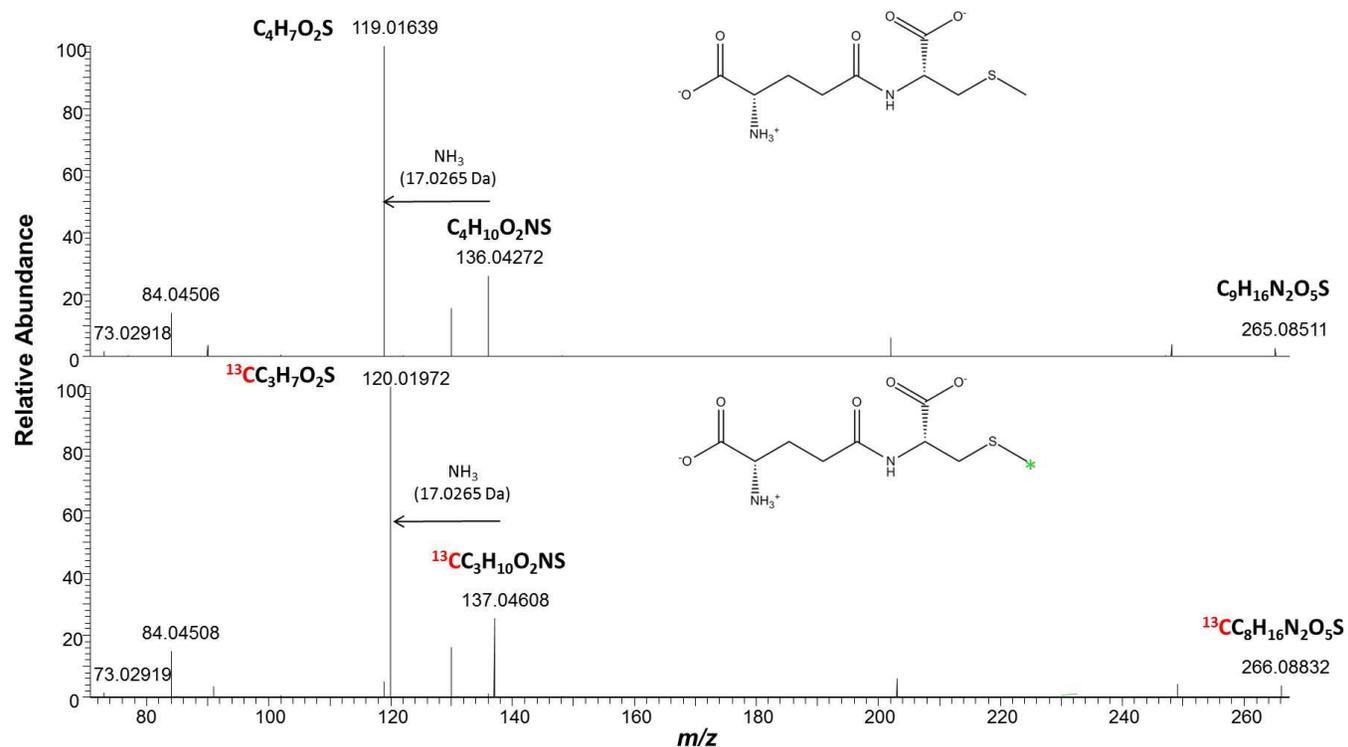


Figure 4.5: Representative dissociation of protonated unlabelled γ -Glu-S-methylCys and labelled γ -Glu-S-methylCys in unlabeled methionine treatment and labelled methionine treated seeds.

Upper panel: Product ions of unlabelled γ -Glu-S-methylCys in non-labelled methionine treatment. **Lower panel:** Product ions of one carbon labelled γ -Glu-S-methylCys labelled methionine treatment. The asterisk represents position of labelled carbon in γ -Glu-S-methylCys after labelled methionine treatment.

4.3.2 Expression profile of PvBSAS family members

In order to determine the enzyme that can catalyze *S*-methylCys biosynthesis using *O*-acetylserine and methanethiol I looked at eight BSAS family members in the *P. vulgaris* genome. Comparing the expression profile of eight BSAS family members in various tissues reveals variation in expression (O'Rourke *et al.*, 2014). Out of all BSAS members PvBSAS4;1 is highly expressed in early seed development (Figure 4.6). PvBSAS3;1 is another member that is highly expressed in seeds as described in detail in Chapter 2 of this thesis. . PvBSAS3;1 prefers cysteine as a substrate while our isotopic labelling suggests *O*-acetylserine as precursor of *S*-methylCys. Based on the expression profile, PvBSAS4;1 is predicted to be a cysteine synthase that may use *O*-acetylserine and methanethiol as substrates to produce *S*-methylCys.

4.3.3 Sequence analysis of BSAS4;1 for cysteine synthase activity

To check whether PvBSAS4;1 has all the structural characteristics of a cysteine synthase, the PvBSAS4;1 amino acid sequence was compared with the known cysteine synthase AtBSAS1;1, and with AtBSAS4;1 and GmBSAS4;1. AtBSAS1;1 acts as a control sequence because a lot of studies are available on its crystal structure and cysteine synthase activity.

Sequence alignment among different species suggests that the sequence is conserved across the species. Based on *Arabidopsis* cysteine synthase (AtOAS-TL) crystal structures, for cysteine synthase activity Lys should be present at the active site (Bonner *et al.*, 2005). In the PvBSAS4;1 active site it is present as Lys⁴⁸. In AtOAS-TL, Asn⁷⁷ and Ser²⁶⁹ residues form hydrogen bonds with oxygen and nitrogen of the PLP pyridine ring. In PvBSAS4;1 these residues are conserved. The presence of Thr⁷⁴ and Ser⁷⁵ is suggested to be important in stabilizing the transition state of the second half-reaction of cysteine synthesis (Bonner *et al.*, 2005). To incorporate sulfur in a carbon backbone Thr⁷⁴, Ser⁷⁵ and Gln¹⁴⁷ play major roles (Tai and Cook, 2001; Rabeh *et al.*, 2005) and all of these residues are conserved in

PvBSAS4;1 (Figure 4.7). Based on our expression data and sequence alignment we can annotate PvBSAS4;1 as cysteine synthase.

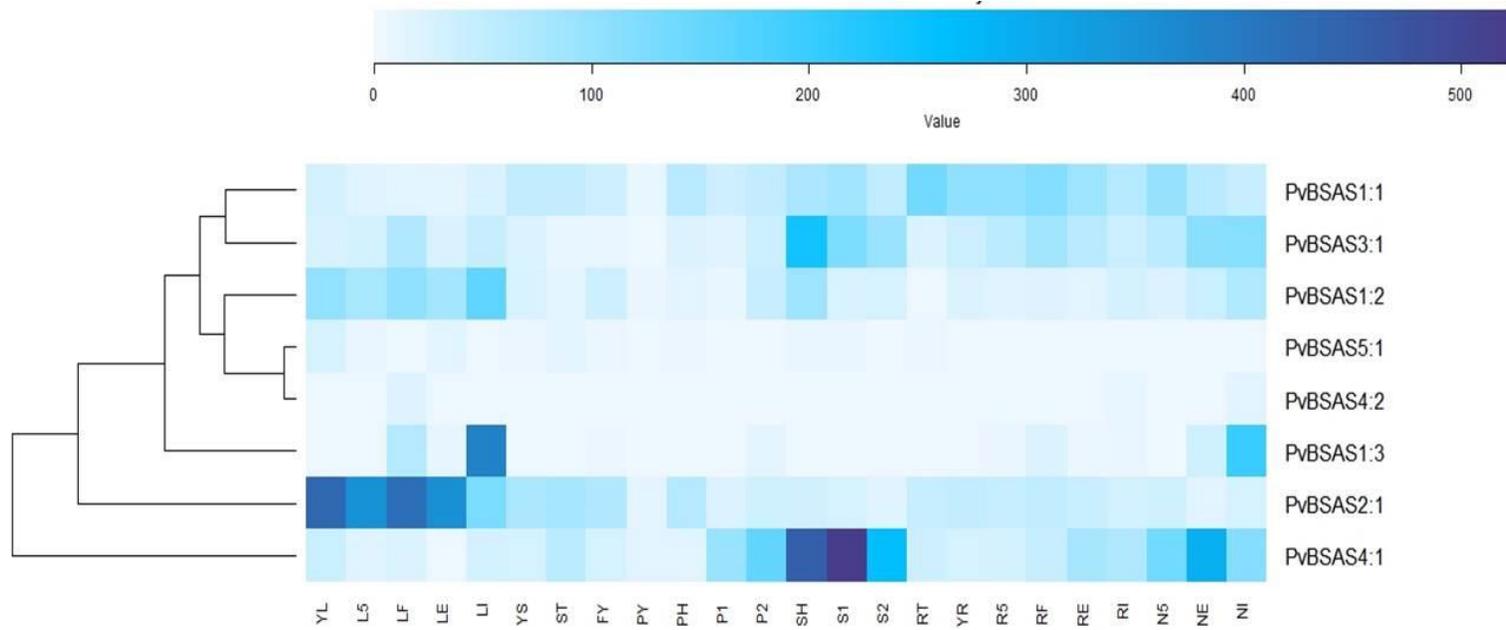


Figure 4.6: Heatmap of expression profile of the *PvBSAS* gene family in various plant tissues.

Values are presented in RPKM. Descriptions of relevant tissues are as follows: YL- Fully expanded 2nd trifoliolate leaf tissue; L5- Leaf tissue collected 5 days after rhizobium inoculation; LF- Leaf tissue from fertilized plants collected at the same time of LE and LI; LE- Leaf tissue collected 21 days after rhizobium inoculation; LI- Leaf tissue collected 21 days after plants were inoculated with ineffective rhizobium; YS- All stem internodes above the cotyledon collected at the 2nd trifoliolate stage; ST- Shoot tip, including the apical meristem, collected at the 2nd trifoliolate stage; FY- Young flowers, collected prior to floral emergence; PY- pods containing globular stage embryos (1-4 days after fertilization); PH- pods containing heart stage seeds; P1- pods associated with stage 1 seeds (pods only); P2- pods associated with stage 2 seeds (pods only); SH- heart stage seeds (ca. 7 mg); S1- stage 1 seeds (ca. 50 mg); S2- stage 2 seeds (ca. 150 mg); RT- Root tips; YR- Whole roots, including root tips; R5- Whole roots separated from 5 day old pre-fixing nodules; RF- Whole roots from fertilized plants collected at the same time as RE and RI; RE- Whole roots separated from fix+ nodules collected 21 days after inoculation; RI- Whole roots separated from fix- nodules collected 21 days after inoculation; N5- Pre-fixing (effective) nodules collected 5 days after inoculation; NE- Effectively fixing nodules collected 21 days after inoculation; NI- Ineffectively fixing nodules collected 21 days after inoculation.

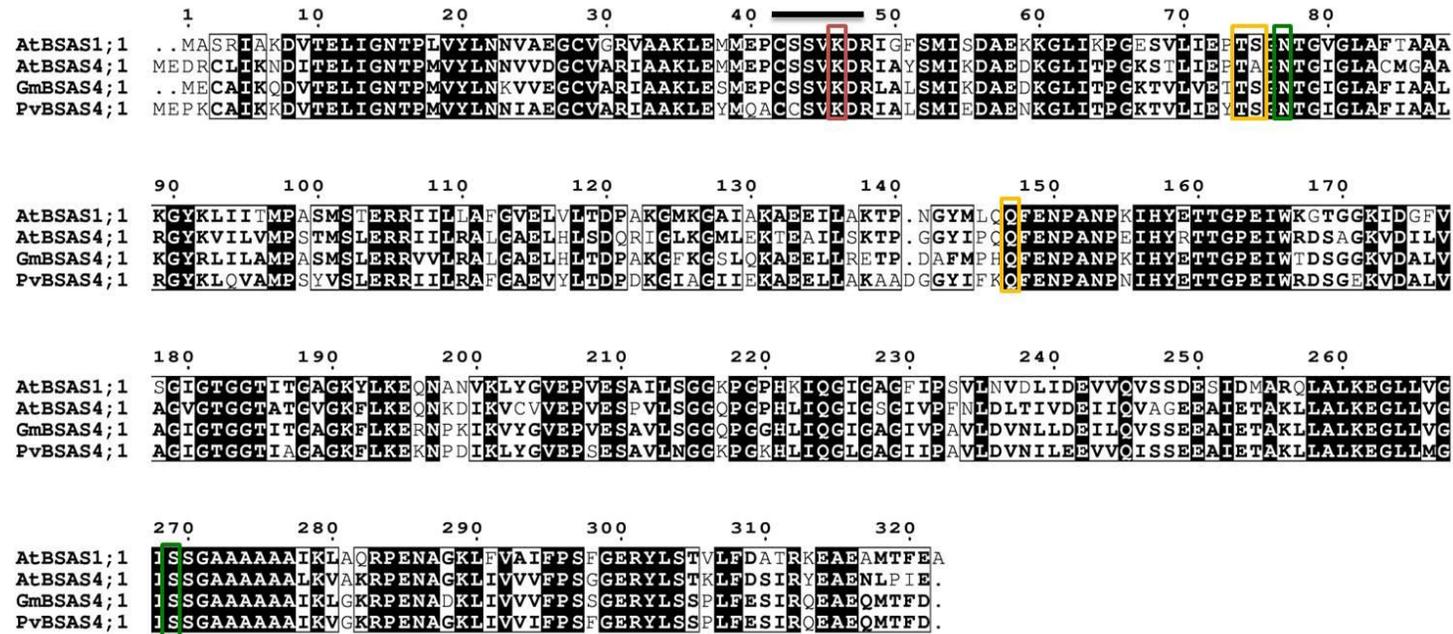


Figure 4.7: Multiple sequence alignment of deduced amino acid sequences of the BSAS4;1 gene from *Arabidopsis*, soybean and common bean.

Black shade indicates conserved sequence. Residues in the PLP binding site are highlighted by black solid line. The active site contains Lys which is highlighted in red. Thr⁷⁴, Ser⁷⁵ Gln¹⁴⁷ residues highlighted by yellow boxes are important for sulfur incorporation. Asn⁷⁷ and Ser²⁶⁹ residues making hydrogen bonds with oxygen and nitrogen of the PLP pyridine ring are highlighted in green.

4.3.4 Physical and spectral properties of PvBSAS4;1

To determine whether PvBSAS4;1 is a functional BSAS, a full-length coding sequence was cloned and expressed in *E. coli* as a His-tagged recombinant protein. The predicted molecular mass of the 324-amino acid residue protein is 37 kDa. Comparison of elution profile of purified PvBSAS4;1 with known molecular weight proteins using size exclusion chromatography suggested PvBSAS4;1 as homodimer with molecular weight 74 kDa (Figure 4.9 A, B).

The spectrum of nickel affinity-purified recombinant PvBSAS4;1 between 250 and 600 nm showed a peak at 412 nm which is characteristic of bound PLP (Figure 4.8 C). This characteristic peak also points out why purified protein is yellowish green color, suggesting that PLP was bound to the enzyme.

4.3.5 Functional analysis of PvBSAS4;1

4.3.5.1 Ligand binding assay

To determine whether PvBSAS4;1 has CS or CAS activity, 400 µg protein was incubated with 0-1.5 mM *O*-acetylserine and 0-1.5 mM cysteine. Absorption spectroscopy of ligand binding assay showed shift of maximum absorbance of PLP from 412 nm to 470 nm in *O*-acetylserine treatment which is associated with formation of an α -aminoacrylate intermediate (Bonner *et al.*, 2005). In the case of the cysteine assay no such shift was observed using any concentration of cysteine. These experiments demonstrate that PvBSAS4;1 prefers *O*-acetylserine over cysteine to form the reaction intermediate α -aminoacrylate (Figure 4.9) which is the intermediate for the first half reaction of cysteine synthase. Together sequence analysis and substrate binding analysis characterize PvBSAS4;1 as a cysteine synthase in the PvBSAS family.

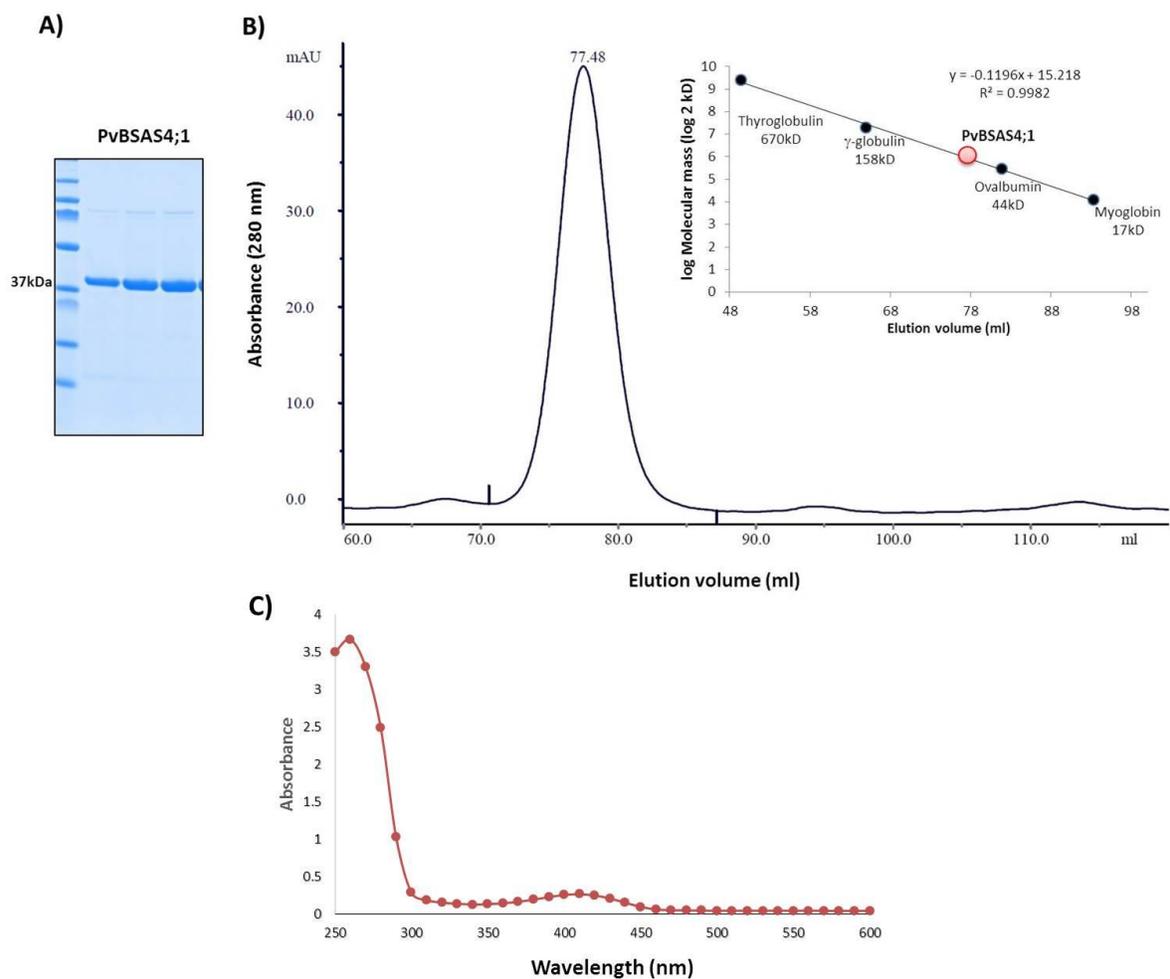


Figure 4.8: Physical and spectral properties of PvBSAS4;1.

A) SDS- PAGE analysis of purified denatured PvBSAS3;1 **B)** Elution profile of Ni²⁺-affinity purified PvBSAS3;1 in size-exclusion chromatography. Molecular weight was calculated based on a standard curve shown in the inset. **C)** Absorption spectrum of PvBSAS4;1.

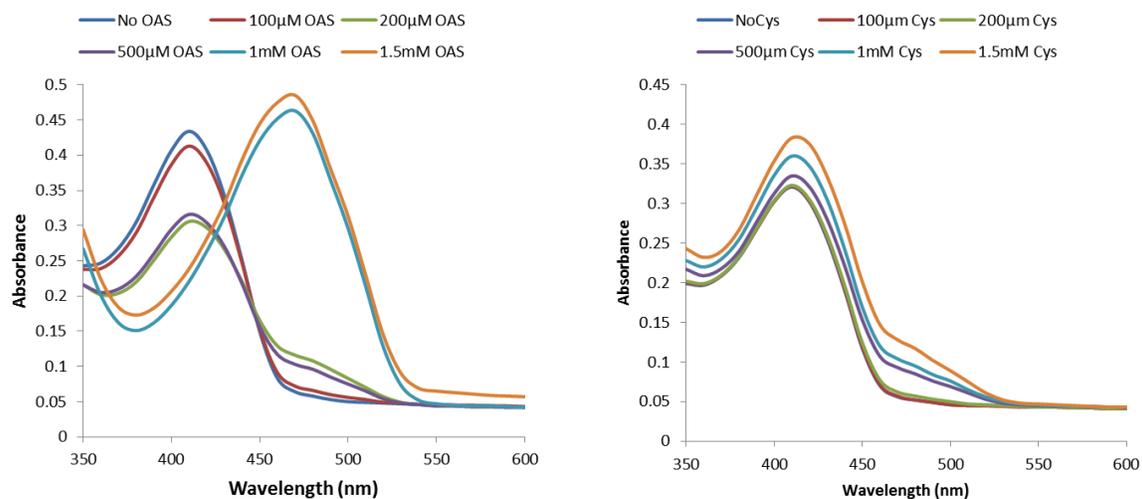


Figure 4.9: Ligand binding assay of PvBSAS4;1.

A) Change in absorption spectrum of PLP from 412 nm to 470 nm after addition of *O*-acetylserine (OAS; 0-1.5 mM). **B)** No change in absorption spectrum of PLP from 412 nm after addition of cysteine (0- 1.5 mM).

4.3.5.2 Enzyme assay

To measure the cysteine synthase activity of PvBSAS4;1, the His-tagged purified protein was used to perform enzyme assays (Table 4.1). In the presence of PvBSAS4;1, *O*-acetylserine and Na₂S, cysteine was produced, which was detected by a colorimetric detection method using the ninhydrin acid assay. β-cyanoalanine activity of BSAS4;1 was also detected but was very low compared to cysteine synthase activity (not presented). Production of cysteine suggests that PvBSAS4;1 belongs to the cysteine synthase subfamily of BSASs. Enzyme kinetics data also showed that PvBSAS4;1 has a high cysteine biosynthesis activity. The K_m values for Na₂S and *O*-acetylserine were 0.13 mM and 1.91 mM respectively, which is comparable to other cysteine synthases reported in other crops (Hatzfeld *et al.*, 2000; Yamaguchi *et al.*, 2000).

Along with the Cys synthase activity assays, methanethiol and *O*-acetylserine were used as substrates in another enzyme assay to determine the role of PvBSAS4;1 in *S*-methylCys biosynthesis. LC-MS/MS analysis of reaction mixtures revealed production of *S*-methylCys by PvBSAS4;1. The rate of the reaction was similar to the cysteine synthase reaction. Enzyme kinetics data also indicated low K_m values for *O*-acetylserine and methanethiol. K_m values for methanethiol and *O*-acetylserine were 1.78 mM and 0.73 mM, respectively, which identify PvBSAS4;1 as potential candidate for *S*-methylCys biosynthesis (Table 4.1).

Table 4.1: Kinetic parameters of PvBSAS4;1 with different substrates

Substrate	V_{\max} ($\times 10^{-7}$ katal mg^{-1})	K_m (mM)	V_{\max}/K_m ($\times 10^{-7}$ katal mg^{-1})/mM
<i>O</i> -acetylserine + Na ₂ S \longrightarrow Cysteine + Sodium acetate			
<i>O</i> -acetylserine	4.47 \pm 0.16	1.91 \pm 0.13	2.35
Na ₂ S	4.16 \pm 0.30	0.13 \pm 0.02	33.44
<i>O</i> -acetylserine + Sodium thiomethoxide \longrightarrow <i>S</i> -methylCys + Sodium acetate			
<i>O</i> -acetylserine	2.41 \pm 0.02	0.73 \pm 0.15	3.40
Sodium thiomethoxide	2.86 \pm 0.78	1.78 \pm 0.83	1.64
All values are expressed as a mean \pm SD ($n = 3$)			

4.4 Discussion

Our study establishes that methionine acts as methyl donor for *S*-methylCys biosynthesis. This is the first report on the role of cysteine synthase in the biosynthesis of *S*-methylCys in common bean developing seeds. The results of isotope labeling experiments conducted in this study present a mode of methionine catabolism and *S*-methylCys biosynthesis in common bean (Figure 4.10). Previously, emission of methanethiol was reported for plants such as wheat (*Triticum aestivum*), and grassland and saltmarsh species (Rennenberg, 1991). Production of methanethiol was also reported in some crops that were exposed to excess sulfur. In a feeding experiment of leaf discs of pumpkin with ³⁵S methionine, more than 80% of the methanethiol emitted was derived from the labelled methionine. In the same experiment methanethiol production also took place when leaf discs were treated with *S*-methylCys but with a slower rate (Schmidt *et al.*, 1985). In these studies, production of methanethiol was associated with methionine accumulation in tissues. In tobacco plants, suppression of *S*-adenosyl-methionine synthetase (SAM-S) which is involved in SAM production from methionine, led to methanethiol production due to over-accumulation of methionine (Boerjan *et al.*, 1994). All these studies reported methanethiol production from free methionine but did not explain the physiological or metabolic roles of methanethiol in plants. Our isotope labelling results demonstrate a role for methanethiol in common bean *S* amino acid metabolism, specifically, its role in biosynthesis of nonproteinaceous *S*-amino acids, *S*-methylCys and γ -Glu-*S*-methylCys. A seed specific cysteine synthase PvBSAS4;1 can use methanethiol as substrate and synthesize *S*-methylCys from *O*-acetylserine.

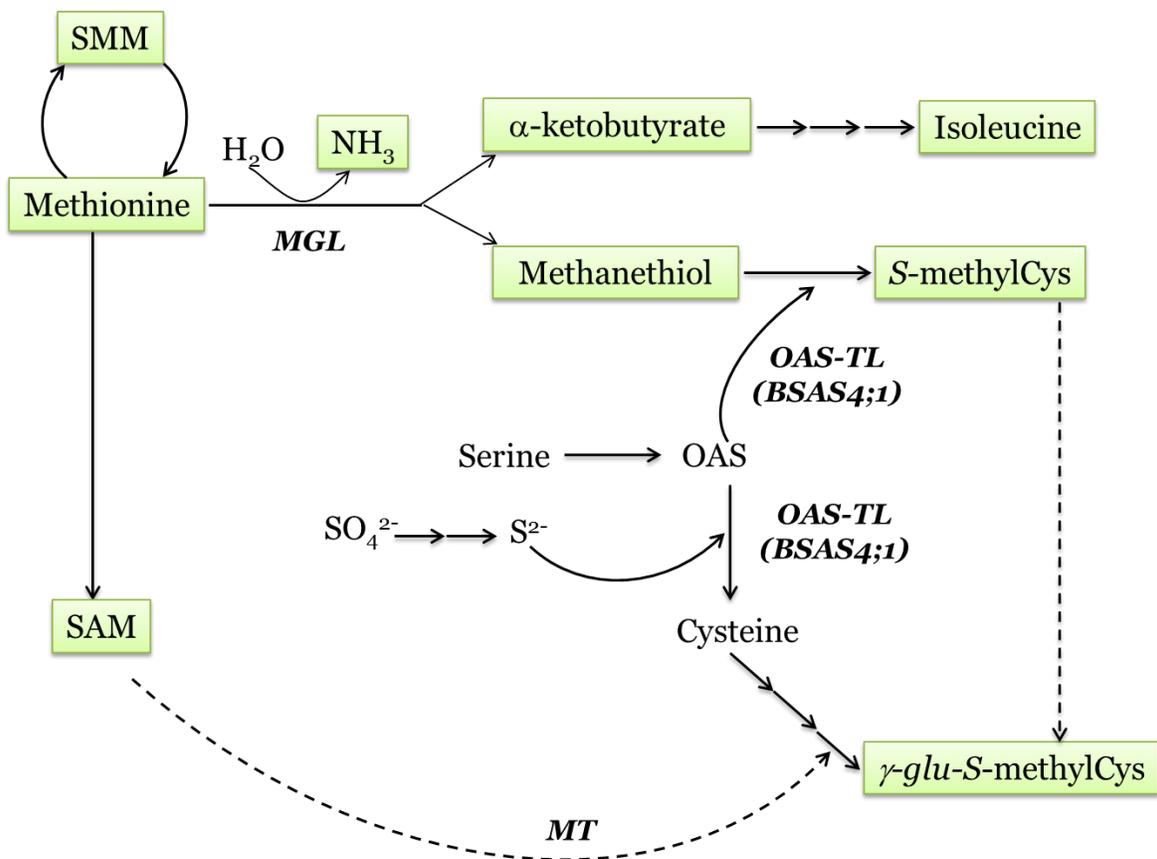


Figure 4.10: Isotopic movement in methionine pathway in common bean seeds.

Green boxes represent metabolites which acquired label from labelled methionine. Multiple arrows represent more than one step involved in metabolite synthesis. *Methanethiol and α -ketobutyrate could not be detected. MGL: Methionine- γ -lyase; OAS-TL: *O*-acetylserine (thiol) lyase (BSAS4;1); MT: methyltransferase; SAM: *S*-adenosylmethionine.

In *Arabidopsis*, BSAS4;1 was reported as a cysteine synthase but with low cysteine synthase activity (Hatzfeld *et al.*, 2000). In the present study, PvBSAS4;1 which shares 70% sequence identity with AtBSAS4;1 has been shown to be more active than AtBSAS4;1. In case of *Arabidopsis* the cysteine synthase activity of AtBSAS4;1 was reported as 0.2 $\mu\text{mol Cys mg}^{-1} \text{ protein min}^{-1}$ (Hatzfeld *et al.*, 2000). In my study PvBSAS4;1 activity was equal to 18 $\mu\text{mol cysteine mg}^{-1} \text{ protein min}^{-1}$ which is 90-fold higher than the AtBSAS4;1. Based on our sequence analysis, ligand binding assay, enzyme assay and the previously well studied cysteine synthase mechanism of an *Arabidopsis* BSAS family member (Bonner *et al.*, 2005), a reaction mechanism for *S*-methylCys biosynthesis can be proposed as shown in Figure 4.11. As a PLP enzyme, BSAS4;1 exists in the in the form of enzyme with an internal Schiff base, as the aldehyde group of PLP forms a linkage with the lysine residue on the enzyme (A). The α amino group of *O*-acetylserine replaces the bound Lys molecule and forms an unstable external Schiff base with the PLP (B). Formation of external Schiff base allows Lys⁴⁷ to act as a general base in the α, β -elimination of acetate from *O*-acetylserine, similar to other reported cysteine synthase reactions (Burkhard *et al.*, 1999; Tai and Cook, 2001; Bonner *et al.*, 2005). This elimination leads to formation of an α -aminoacrylate intermediate (C) which is a common first half reaction intermediate in all enzymatic reactions of BSAS family members (Yi *et al.*, 2012). In the second half reaction, the α -aminoacrylate is attacked by a nucleophile (e.g., CH₃S) and forms an external aldimine with a methylated sulfur group attached to it (D). Subsequent protonation by Lys helps to release *S*-methylCys as a product and regenerate BSAS4;1 for further reactions (Figure 4.8). In the near future, studying crystal structures of PvBSAS4;1 will shed more light on the mechanism of *S*-methylCys biosynthesis.

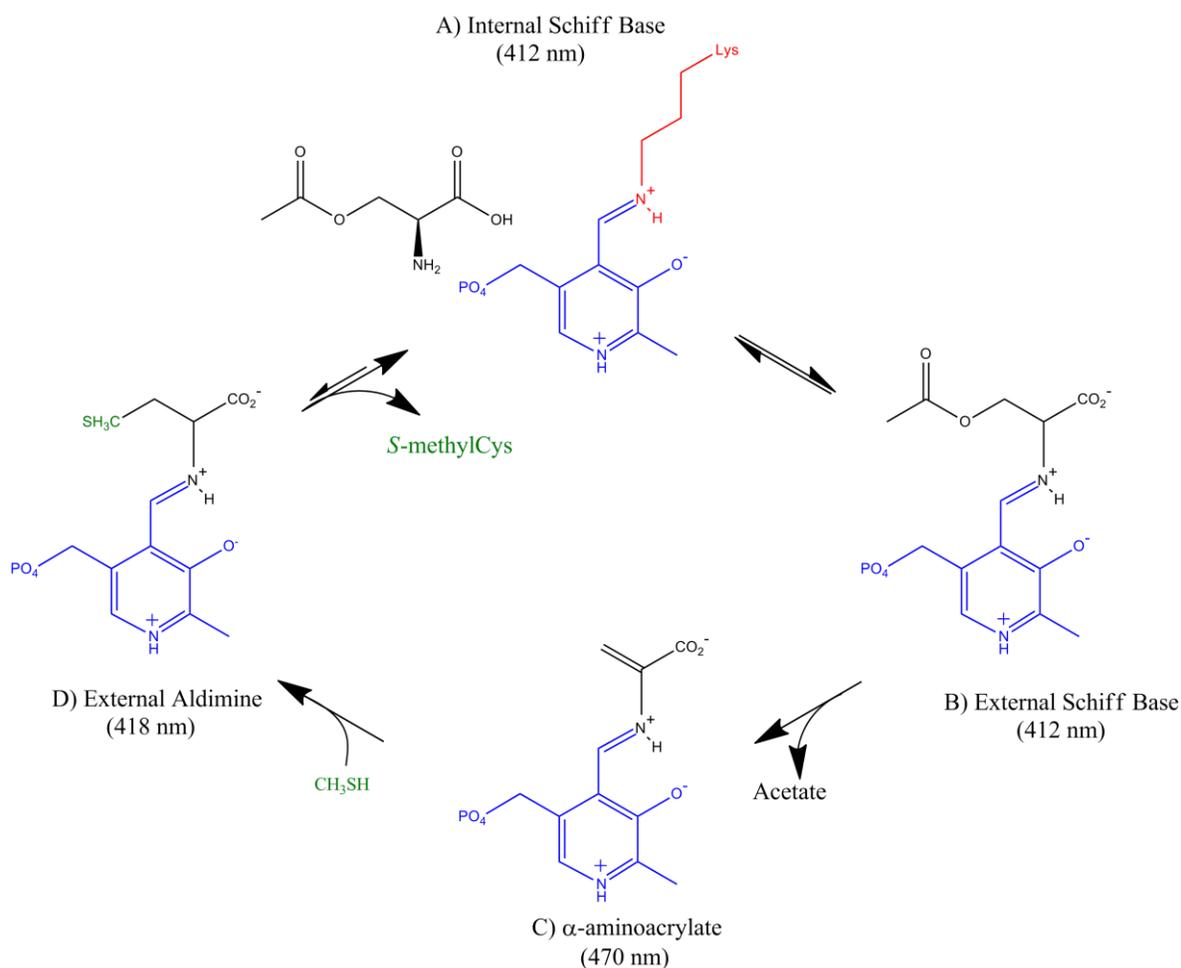


Figure 4.11: Proposed mechanism of *S*-methylCys in common bean catalyzed by PvBSAS4;1.

Four steps of mechanism involve **A)** initial Schiff base form of PLP ($A_{\text{max}} = 412 \text{ nm}$), **B)** external Schiff base form of PLP with *O*-acetylserine attached to it ($A_{\text{max}} = 412 \text{ nm}$), **C)** α -aminoacrylate an intermediate after the first half reaction ($A_{\text{max}} = 470 \text{ nm}$), and nucleophilic attack (CH_3SH) in the second half reaction leading to the **D)** external aldimine ($A_{\text{max}} = 418 \text{ nm}$), and release of *S*-methylCys.

Methionine catabolism takes place in the cytosol. MGL is reported to be cytosolic in nature, where γ -cleavage of methionine produces methanethiol, ammonia and α -ketobutyrate (Rebeille *et al.*, 2006). PvBSAS4;1 predicted to be in cytosol can trap this methanethiol and produce *S*-methylCys. Previous studies could not predict the fate of *S*-methylCys but in my isotope tracking experiment one carbon label was observed in *S*-methylCys and subsequently incorporated in γ -Glu-*S*-methylCys, which may be acting as a storage sink for sulfur. γ -Glu-Cys ligase (γ -GCL) may be the one candidate enzyme which is involved in biosynthesis of γ -Glu-*S*-methylCys from *S*-methylCys. Mammalian γ -GCL has shown activity with *S*-methylCys *in vitro* (Rathbun, 1967a). Among two γ -GCL present in the *P. vulgaris* genome (Phvul.002G289200, Phvul.002G157600), Phvul.002G157600 is highly seed specific and predicted to be present in cytosol. Co-localization of metabolites, *S*-methylcysteine and γ -Glu-*S*-methylCys with γ -GCL enzymes in cytosol supports the hypothesis on the role of γ -GCL in γ -Glu-*S*-methylCys biosynthesis. Another candidate enzyme γ -glutamyl transpeptidase (E.C. 2.3.2.2; GGT) was reported to catalyze transpeptidation of methionine in the presence of γ -glutamyl-*p*-nitroanilide (GGPNA) in onion (Shaw *et al.*, 2005). In the case of common bean, GGTs may be transferring the glutamyl group from hGSH to *S*-methylCys and synthesize γ -Glu-*S*-methylCys.

BSAS4;1 is a cysteine synthase predominantly expressed in seeds and one of the MGLs (Phvul.001G082000) is also expressed in the early developing stages of the seed. Expression data and subcellular localization of BSAS4;1 and MGL suggest an inverse relationship, I suggest an inverse relationship between methionine and *S*-methylCys levels. Combining our tissue expression analysis, isotope labelling with enzyme kinetics results suggests a co-ordinated effort among serine acetyltransferases, OAS-TL, and MGL for inverse relationship. Based on my results, in developing seeds SMM gets accumulated as phloem transport takes place from vegetative tissue to developing seeds. SMM is converted to methionine by a seed specific homocysteine methyl transferase (HMT3). While a large part of this methionine is incorporated into protein or SAM, excessive methionine serves as a substrate for methanethiol production by MGL. A condensation reaction takes place

between OAS and methanethiol generating *S*-methylCys takes place through the function of BSAS4;1 in the cytosol.

In the past, several strategies have been used to improve essential amino content in legumes. One of the strategies involves targeting methionine biosynthesis and catabolism related genes. Overexpression of cystathionine- γ -synthase (CGS), the first committed enzyme for methionine biosynthesis, resulted in 6-15 fold increase in methionine content of various crops such as alfalfa (Avraham *et al.*, 2005), potato (Di *et al.*, 2003), *Arabidopsis* (Kim *et al.*, 2002), and tobacco (Hacham *et al.*, 2008). Similarly, catabolism related genes, such as SAM synthetase, were silenced in search of high methionine content (Boerjan *et al.*, 1994;Goto *et al.*, 2002). In these studies methionine content improved, up to 430-fold, but one limitation of this approach was an abnormal phenotype due to the role of SAM in essential metabolic reactions. In this study, PvBSAS4;1 was identified as a potential candidate for *S*-methylCys synthesis that shares an inverse relationship with methionine content. Since the BSAS family has other members that take part in cysteine synthesis, future targeting of PvBSAS4;1 along with MGL could lead to enhance methionine content in common bean seed with minimal perturbation to other metabolites.

4.5 Conclusion

The main objectives of this study were to identify the *S*-methyl group donor in the biosynthesis of *S*-methylCys and the enzyme responsible for its synthesis in developing seeds of common bean. With the help of an isotope tracking method, the methyl group of *S*-methylCys has been shown to originate from methionine. This is a first report of the role of methionine catabolism in *S*-methylCys and γ -Glu-*S*-methylCys biosynthesis in common bean. In this study, Cysteine synthase BSAS4;1 has been identified to be involved in *S*-methylCys synthesis in common bean. Our observation that BSAS4;1 and MGL are putative candidates for *S*-methylCys biosynthesis in common bean seed suggests the potential to develop common bean lines with silenced BSAS4;1 and MGL expression for higher cysteine and methionine containing bean varieties.

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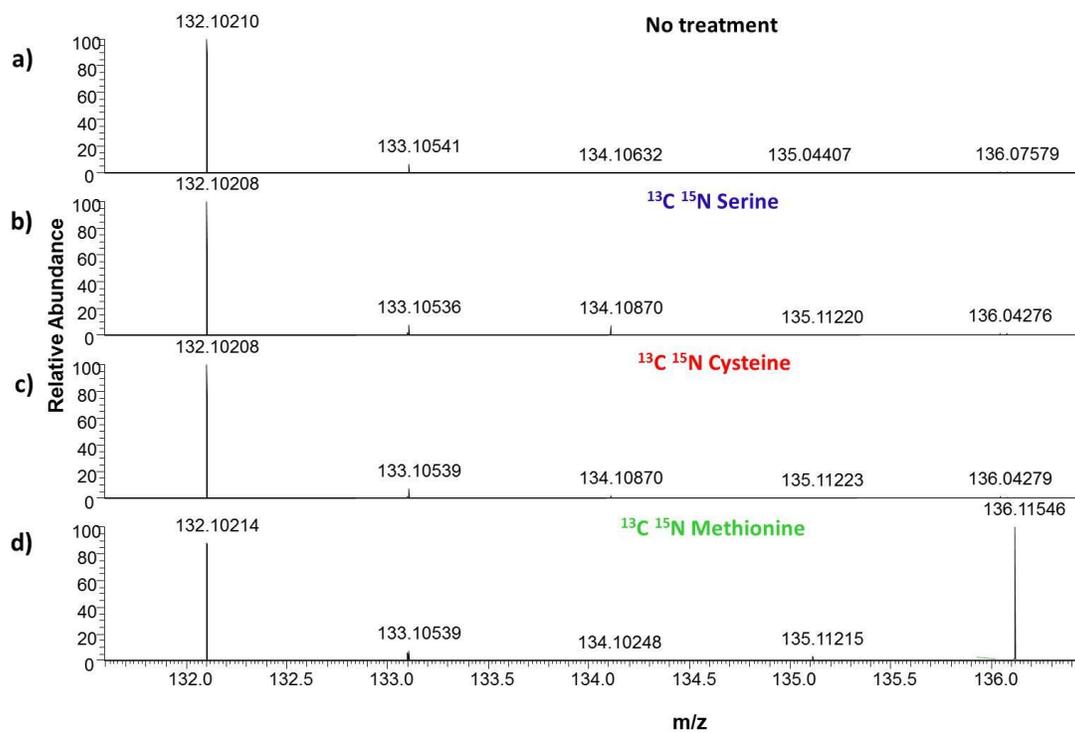
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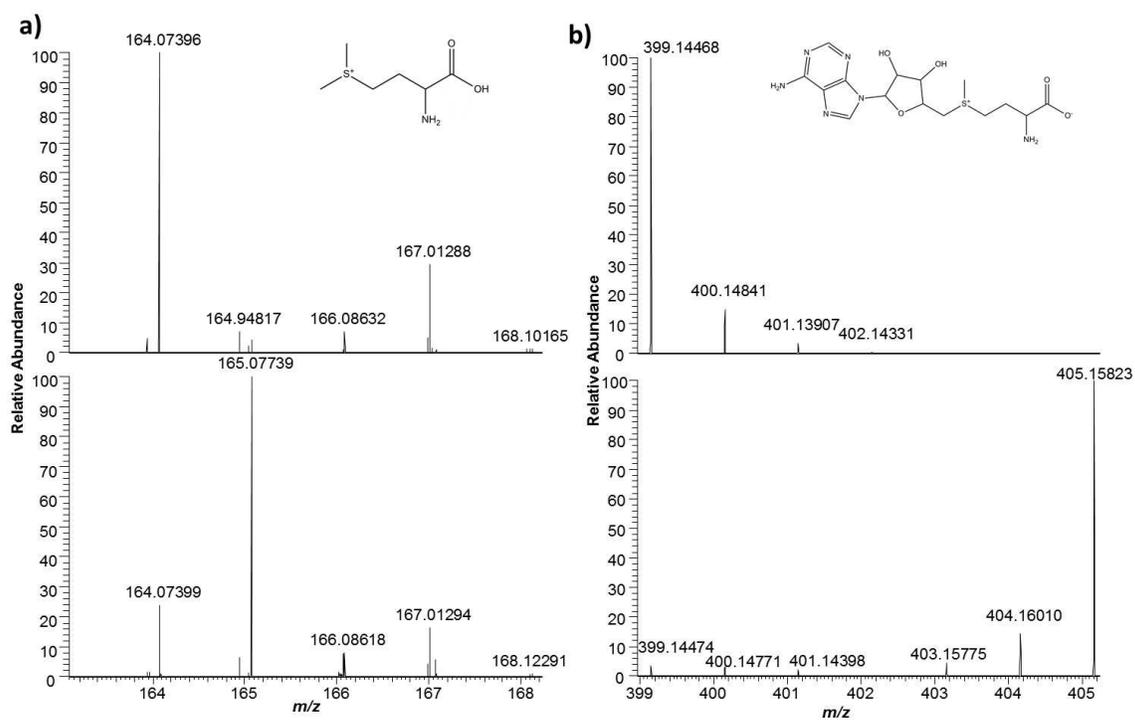
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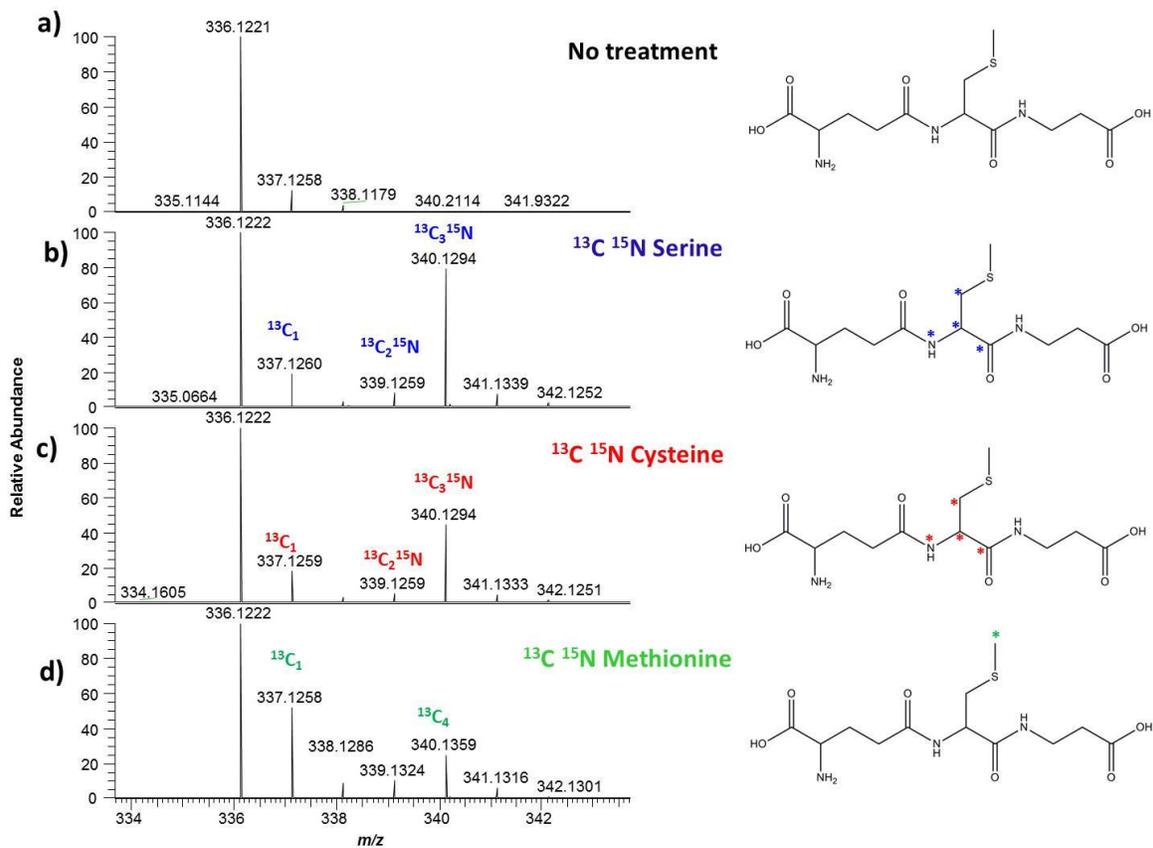
4.7 Supplementary data



Supplementary Data 4.1: Comparison of isotopic pattern of isoleucine in various treatments.

a) no amino acid supplementation; **b)** seeds treated with $^{13}\text{C}_3$ and ^{15}N serine; **c)** seeds treated with $^{13}\text{C}_3$ and ^{15}N cysteine; **d)** seeds treated with $^{13}\text{C}_4$ and ^{15}N methionine. Four carbon labelled isoleucine ($m/z=136.11546$) is not detectable in seeds treated with labelled serine or cysteine but in case of labelled methionine treated samples it is present prominently.





Supplementary Data 4.3: Comparison of isotopic pattern of *S*-methylhomoglutathione in various treatments.

a) no amino acid supplementation; **b)** seeds treated with $^{13}\text{C}_3$ and ^{15}N serine; **c)** seeds treated with $^{13}\text{C}_3$ and ^{15}N cysteine; **d)** seeds treated with $^{13}\text{C}_4$ and ^{15}N methionine.

CHAPTER FIVE - GENERAL DISCUSSION

5.1 Exploring sulfur metabolome in common bean: A complex affair

The sulfur metabolic network does not exist in isolation. It is in tight connection with carbon and nitrogen metabolism and affects nitrogen and carbon ratio in a plant system (Kopriva *et al.*, 2002). Sulfur deprivation, for example, has profound consequences on carbon and nitrogen assimilation that ultimately affect the growth, quality and yield of crops (Maruyama-Nakashita *et al.*, 2003; Hirai *et al.*, 2004; Nikiforova *et al.*, 2004; Brychkova *et al.*, 2015; Khan *et al.*, 2016). Sulfur metabolism is important not only for plant growth or yield, it also affects quality of primary and secondary metabolites (Møldrup *et al.*, 2011). Understanding sulfur metabolism has become very important to improve our knowledge of plant metabolism.

Sulfur metabolism and its regulation differ among members of plant genera (Davidian and Kopriva, 2010; Romero *et al.*, 2014; Tavares *et al.*, 2015). In common bean, the sulfur metabolome shares another level of complexity due to the presence of *S*-amino acid derivatives *S*-methylCys, γ -Glu-*S*-methylCys and *S*-methylhomoglutathione. Prior to this research these metabolites were reported in common bean and other crops, but their biosynthesis was not discussed. This research explores a possible pathway involved in the biosynthesis of *S*-amino acid derivatives and metabolites in common bean seeds (Figure 5.1).

Due to the complexity of sulfur metabolism, enzymes involved are present in more than one isoform. In the case of *Arabidopsis*, five isoforms of SERAT and eight isoforms of BSAS have been reported, some of which are redundant, but play a significant role in sulfur metabolism (Watanabe *et al.*, 2008a; Watanabe *et al.*, 2008b). In common bean, six isoforms of SERAT and eight BSAS isoforms (Appendix A and B) are reported, which suggests the complex molecular basis of the sulfur metabolome. Plastids are the only site for reduction of sulfur from sulfate to sulfide. However, *O*-acetylserine biosynthesis takes place in mitochondria, plastids and cytosol (Lunn *et al.*, 1990; Hell *et al.*, 2002). Studies

using *Arabidopsis thaliana* T-DNA knockout mutants of OAS-TL demonstrated that cysteine and its derivatives could be exchanged between compartments, and that compartmental regulation plays a role in maintaining this transport (Heeg *et al.*, 2008; Watanabe *et al.*, 2008a; Watanabe *et al.*, 2008b).

In this thesis, evidence was presented that *S*-methylCys biosynthesis takes place in the cytosol where serine acts a precursor. The observation was based on isotopic tracking of ¹³C and ¹⁵N labelled serine and cysteine. As shown in Figures 3.8 and 3.9, seeds treated with isotopic serine had higher ¹³C and ¹⁵N incorporation than labelled cysteine treated seeds which points to a role of spatial compartmentation. The compounds that acquired the highest amount of ¹³C and ¹⁵N included γ -Glutamyl cysteine (γ -Glu-Cys), homoglutathione (hGSH) and *S*-methylhomoglutathione (*S*-methylhGSH). In *Arabidopsis*, major portion of γ -Glu-Cys is reported to be synthesized in plastids while hGSH biosynthesis takes place both in plastids and cytosol (Galant *et al.*, 2011; Koffler *et al.*, 2011). However, the high uniform incorporation of isotopic label in γ -Glu-Cys and hGSH in common bean seeds suggests the same subcellular localization for this group of metabolites. Inheritance of high percentage of isotopes in labelled serine treated seeds than labelled cysteine treated seeds also suggests that uptake of serine in plastids may be favored over cysteine. The results shown in this thesis provide evidence for biosynthesis of *S*-methyl-hGSH, a methylated tripeptide in developing seeds of common bean, which was previously reported in *Vigna radiata* (Kasai *et al.*, 1986). BaHigh incorporation of isotopes in *S*-methyl-hGSH suggests a major role of this methylated tripeptide in movement of sulfur flux in developing seeds. In future *S*-methyl-hGSH could be considered as a possible precursor for γ -Glu-*S*- in presence of a carboxypeptidase (Figure 5.1). Another possible fate of *S*-amino acid derivatives could be *S*-methylated phytochelatins which needs to be validated. Phytochelatin biosynthesis requires presence of heavy metals (Zenk, 1996; Cobbett, 2000;Oven *et al.*, 2001). In case of AtPCS1 (Phytochelatin synthase) *S*-alkylglutathiones acts as a substrates for the synthesis of *S*-alkyl-phytochelatins in the complete absence of added heavy metal (Vatamaniuk *et al.*, 2000). This suggests that *S*-methylated

phytochelatins may be acting as a sink for *S*-methylCys, γ -Glu-*S*-methylCys and *S*-methyl-hGSH in common bean seeds. Presence of isotopes in *S*-methylated phytochelatins in an isotope tracking experiment could provide evidence for phytochelatins as a reservoir for sulfur accumulation.

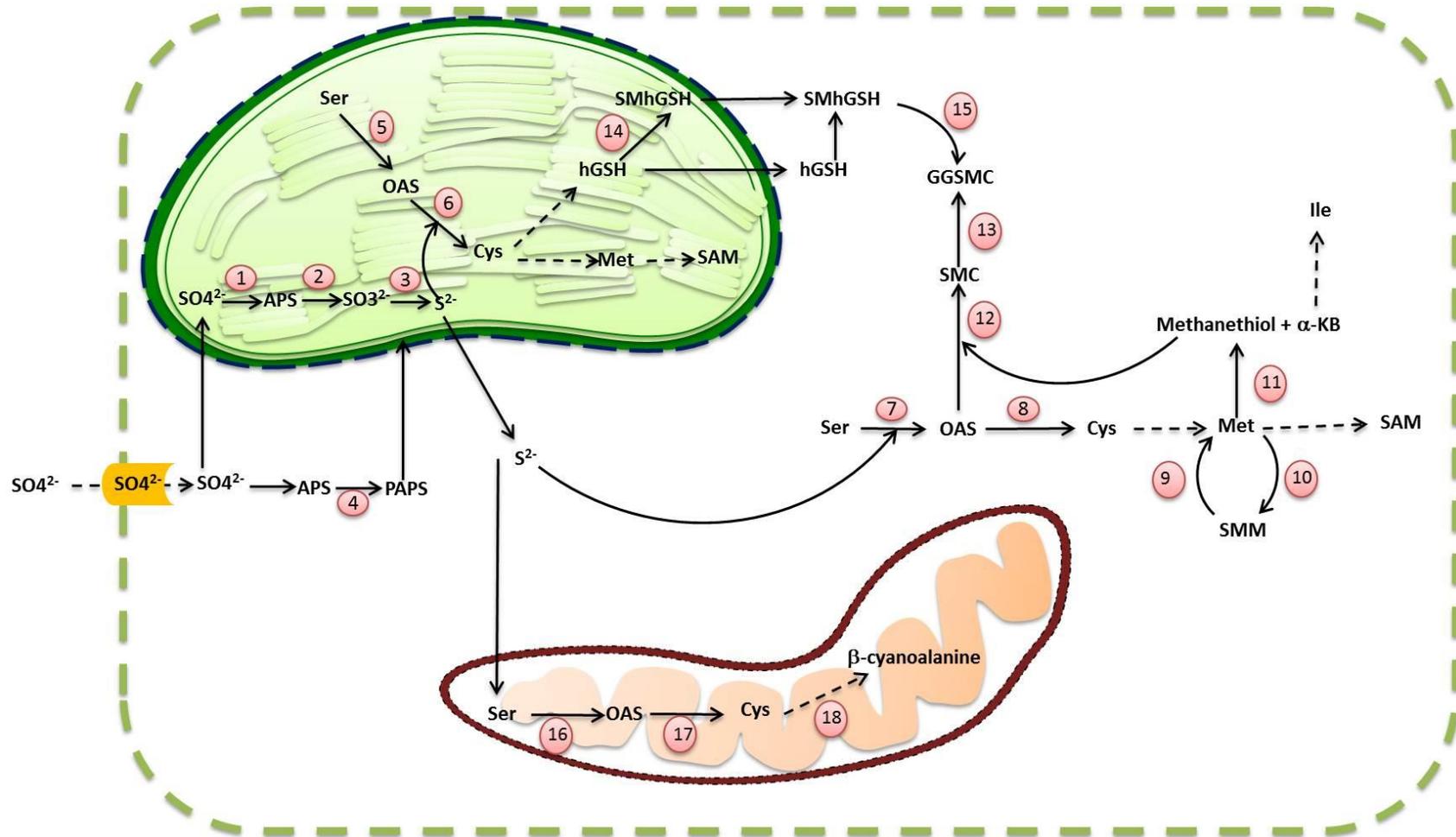


Figure 5.1: A model of sulfur amino acid metabolism in *P. vulgaris* seeds.

In common bean seeds sulfur metabolism takes place in three different compartments. 1: ATP sulfurylase 2: APS reductase; 3: Sulfite reductase; 4: APS kinase 5: Serine acetyltransferase (SERAT2;1); 6: *O*-acetylserine (thiol) lyase (BSAS2;1); 7: Serine acetyltransferase (SERAT1;1); 8: *O*-acetylserine (thiol) lyase (BSAS1;1 and BSAS4;1); 9: Homocysteine methyltransferase (HMT3); 10: Methylmethionine transferase (MMT); 11: Methionine- γ -lyase (MGL) 12: *O*-acetylserine (thiol) lyase (BSAS4;1) 13; γ -Glutamyl-cysteine ligase (GCL) 14: Methyltransferase (MT); 15: Carboxypeptidase 16: Serine acetyltransferase (SERAT2;2) 17: *O*-acetylserine (thiol) lyase (BSAS2;2) 18: β -Cyanoalanine synthase (BSAS3;1). Ser: serine; OAS: *O*-acetylserine; Cys: cysteine; Met: Methionine; SAM: *S*-adenosylmethionine; SMM: *S*-methylmethionine; SMC: *S*-methylCys; GGSMC: γ -Glu-*S*-methylCys; hGSH: Homoglutathione ; SMhGSH: *S*-methylhomoglutathione; α -KB: α -ketobutyrate. Multiple arrow represents more than one steps.

5.2 *S*-methylCys biosynthesis: Cytosolic *O*-acetyl serine and methionine work together

The natural occurrence of *S*-methylCys was first reported in 1955 as sulfoxide in cabbage leaves (Synge and Wood, 1956). In common bean, the first report of *S*-methylCys goes back to 1956 (Thompson *et al.*, 1956). Due to its aromatic and medicinal properties, this metabolite was extensively studied in the *Alliaceae*, but not in *Phaseolus*. Reports that the amount of *S*-methylCys shares an inverse relationship with *S*-amino acid in 2008 have widened our view of sulfur metabolism due to its relationship with protein quality of legumes. To date no such effort was made to decipher *S*-methylCys biosynthesis in common bean. This study is the first to focus on identifying the precursor for *S*-methylCys biosynthesis. In the case of garlic, glutathione has been reported as the precursor for *S*-allyl-cysteine biosynthesis (Yoshimoto *et al.*, 2015a), while cysteine methylation was the reaction producing *S*-methylCys in leaves of radish (Thompson and Gering, 1966). Rebeille *et al* (2006) showed that methionine treated *Arabidopsis* cells produce *S*-methylCys, following methionine catabolism. In my study, I provided several pieces of evidences using isotopic tracking, confocal microscopy and enzyme kinetics for the role of *O*-acetyl serine and methionine in *S*-methylCys biosynthesis.

5.3 Cysteine biosynthesis is through highway while *S*-methylCys follows byway

In common bean, isoforms of SERAT and BSAS are present to perform cysteine biosynthesis in the cytosol, plastids and mitochondria (Figure 5.1). Cysteine, the first organic form of sulfur is quickly used up to synthesize other metabolites. Our isotope tracking and enzymatic assay results demonstrate cysteine biosynthesis as a primary reaction in a developing cell and *S*-methylCys biosynthesis as a side reaction. BSAS4;1 is a seed specific cysteine synthase whose primary role is cysteine biosynthesis in the cytosol. During the seed developmental stages when excess methionine is catabolized and methanethiol produced in the cytosol, BSAS4;1 may help in the biosynthesis of *S*-methylCys.

5.4 Amino acid catabolism: needs much attention

Amino acid biosynthesis in plants has been investigated for the past several decades, while catabolism of these amino acids has not had much attention. My study points out the importance of methionine catabolism in the biosynthesis of *S*-methylCys, which is predicted in *Arabidopsis* cells grown in methionine rich media (Rebeille *et al.*, 2006). Methanethiol is produced from methionine catabolism and is volatile in nature. During cysteine deprivation, the anaerobic protozoan parasite *Entamoeba histolytica* produces *S*-methylCys using over accumulated *O*-acetylserine and methanethiol (Husain *et al.*, 2010). My study identifies a similar mechanism of *S*-methylCys biosynthesis in common bean seeds. Seeds grown under high methionine condition had high incorporation of ¹³C in *S*-methylCys compared to other amino acid treated seeds (Figure 4.2). Due to excessive methionine accumulation in developing seeds, methionine catabolism plays a major role in the biosynthesis of *S*-methylCys, which acts as a reservoir to store catabolized sulfur. Under sulfur starvation this sulfur reservoir may play a major role, and can be targeted to improve methionine content in common bean seeds.

5.5 Isotopic tracking combined with HRMS- An effective tool to explore the sulfur metabolome

This study reflects that isotopic tracking combined with HRMS has considerable untapped potential to elucidate unknown biochemical pathways. In the sulfur metabolome, large proportions of metabolites are still unassigned, and need re-evaluation (Glaser *et al.*, 2014). In addition to targeted LC-MS/MS, samples were also analyzed with a non-targeted LC-DDA method to provide a dataset which will be helpful to assign identity to unknown metabolites. In the future, use of HRMS with combination of ¹³C, ¹⁵N with ³⁴S isotopic ions to track sulfur movement in a pathway will widen our horizon regarding sulfur metabolism in common bean. One limitation of using ³⁴S in isotopic tracking experiments is that it will go everywhere in the plant cell, and this may turn out to be a chaotic system to decipher the pathway.

5.6 Interdependence of amino acid metabolism

My analysis reveals how different branches of amino acid metabolism work in close relation. This study highlights the interdependency of serine, cysteine, methionine and isoleucine pathways in common bean seed. In *Arabidopsis* the interaction among threonine, methionine and isoleucine metabolism was attributed to abiotic stress (Joshi *et al.*, 2010). In common bean, the interaction among these pathways plays a major role during seed development. SMM travels from vegetative tissue to seeds via phloem (Lee *et al.*, 2008). The SMM cycle plays an important function in methionine biosynthesis in seeds (Cohen *et al.*, 2017b). Due to high methionine content, seeds produce methanethiol and α -ketobutyrate. α -ketobutyrate makes an entry to isoleucine biosynthesis, while methanethiol interacts with serine and cysteine metabolism to synthesize *S*-methylCys (Figure 5.1). This study asked for a new prospect to improve protein quality. Due to interdependence, we should be very careful in engineering any amino acid metabolism. The best strategy would be manipulating genes which create minimum perturbation in another pathway. One such pathway can be *S*-methylCys biosynthesis. *BSAS4;1*, the enzyme product of which is suggested to be responsible for *S*-methylCys synthesis, could be silenced without disturbing other biosynthetic pathways, as other BSAS isoforms present in the cytosol could act in its absence for cysteine biosynthesis, as reported in *Arabidopsis* (Watanabe *et al.*, 2008a).

5.7 *S*-amino acid derivatives: a goldmine of nonprotenacious sulfur

Sulfur amino acid derivatives are reported in *Brassicaceae* and *Alliaceae*, where they account for taste and aromatic characteristics. In the case of common bean, no such characteristics are attributed to them and they may act as a sink for excessive sulfur. *S*-methylCys and γ -Glu-*S*-methylCys account for 2-3% of the total amino acids, but do not contribute to protein biosynthesis. In *Arabidopsis*, vacuoles are predicted to be the storage compartment for *S*-methylCys (Rebeille *et al.*, 2006). In the case of common bean one third of total *S*-methylCys was recovered as free *S*-methylCys, mostly as γ -Glu-*S*-methylCys, and the rest was unaccounted (Taylor *et al.*, 2008). In this study incorporation

of isotopic labels from serine to hGSH and *S*-methylhomoglutathione suggests additional sulfur reservoirs in common bean seeds, such as *S*-methylated phytochelatins. Seed storage deficiency in common bean is associated with an alteration of the protein deposition pathway (Taylor *et al.*, 2008). If we rechannelled sulfur from these reservoirs to storage proteins, then we would be able to improve protein quality in common bean.

5.8 Tracking sulfur flux

Lack of knowledge of metabolic flux for a metabolite remains the bottleneck for any metabolic engineering strategy. Metabolic flux analysis has the potential not only to increase the chances of success in metabolic engineering but can also lead to the discovery of novel metabolic routes (Libourel and Shachar-Hill, 2008; Schwender, 2008). In my thesis, I identified a pathway for *S*-methylCys biosynthesis and the enzymes involved in it. In the future, calculating metabolic flux of this *S*-metabolic pathway will help us to develop an effective metabolic engineering strategy to develop high cysteine and methionine containing common bean varieties.

5.9 Metabolic engineering of common bean with high *S* metabolites

In the past, several strategies have been used to improve methionine and cysteine content in legumes, including transgenic lines. In common bean, production of transgenic lines is a daunting task. Due to low efficiency of the current stable transformation methods, characterization of candidate genes in seeds is extremely challenging (Gepts *et al.*, 2008). Recently a particle bombardment method was used to produce stable transformants of common bean for high folate content (Ramírez Rivera *et al.*, 2016). In the near future, the development of effective transformation systems will allow us to silence *S*-methylCys pathway genes and achieve high cysteine and methionine containing bean varieties. Development of TILLING (Targeted Induced Local Lesions IN Genomes) mutagenized lines can be a good alternative to genetic transformed line.

According to the Canadian Food Inspection Agency, the label “Good source of protein” can be used for beans and other legumes. In the future silencing of *S*-methylCys pathway genes or development of TILLING lines with high cysteine and methionine levels may change bean from a good source to an “excellent source of protein”. Hence my findings provide a helping hand to overcome food insecurity in the growing world.

5.10 Cited Literature

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APPENDICES

Appendix A: BSAS family members and their corresponding accession numbers in, *P. vulgaris Arabidopsis thaliana* and *Glycine max* genome.

Protein name	Accession number	Species
PvBSAS1;1	Phvul.002G045200	<i>Phaseolus vulgaris</i>
PvBSAS1;2	Phvul.006G099100	<i>Phaseolus vulgaris</i>
PvBSAS1;3	Phvul.007G057600	<i>Phaseolus vulgaris</i>
PvBSAS2;1	Phvul.003G060200	<i>Phaseolus vulgaris</i>
PvBSAS3;1	Phvul.008G061100	<i>Phaseolus vulgaris</i>
PvBSAS4;1	Phvul.007G185200	<i>Phaseolus vulgaris</i>
PvBSAS4;2	Phvul.007G185100	<i>Phaseolus vulgaris</i>
PvBSAS5;1	Phvul.001G107500	<i>Phaseolus vulgaris</i>
AtBSAS1;1	At4g14880	<i>Arabidopsis</i>
AtBSAS2;1	At2g43750	<i>Arabidopsis</i>
AtBSAS2;2	At3g59760	<i>Arabidopsis</i>
AtBSAS3;1	At3g61440	<i>Arabidopsis</i>
AtBSAS4;1	At5g28020	<i>Arabidopsis</i>
AtBSAS4;2	At3g04940	<i>Arabidopsis</i>
AtBSAS4;3	At5g28030	<i>Arabidopsis</i>
AtBSAS5;1	At3g03630	<i>Arabidopsis</i>
GmBSAS1;1	Glyma.11G005800	<i>Glycine max</i>
GmBSAS1;2	Glyma.19G242300	<i>Glycine max</i>
GmBSAS1;3	Glyma.03G244800	<i>Glycine max</i>
GmBSAS1;5	Glyma.10G247900	<i>Glycine max</i>
GmBSAS2;1	Glyma.02G138400	<i>Glycine max</i>
GmBSAS2;2	Glyma.07G206800	<i>Glycine max</i>
GmBSAS3;1	Glyma.09G258000	<i>Glycine max</i>
GmBSAS3;2	Glyma.18G234600	<i>Glycine max</i>

GmBSAS4;1	Glyma.15G262500	<i>Glycine max</i>
GmBSAS4;2	Glyma.10G159800	<i>Glycine max</i>
GmBSAS4;3	Glyma.20G228900	<i>Glycine max</i>
GmBSAS4;4	Glyma.10G159700	<i>Glycine max</i>
GmBSAS4;5	Glyma.20G229000	<i>Glycine max</i>
GmBSAS5;1	Glyma.03G006700	<i>Glycine max</i>

Appendix B: SERAT family members and their corresponding accession numbers in *P.vulgaris* genome.

Protein name	Accession number	Species
PvSERAT1;1	Phvul.001G170600	<i>Phaseolus vulgaris</i>
PvSERAT1;2	Phvul.010G110600	<i>Phaseolus vulgaris</i>
PvSERAT2;1	Phvul.006G055200	<i>Phaseolus vulgaris</i>
PvSERAT2;2	Phvul.008G277800	<i>Phaseolus vulgaris</i>
PvSERAT3;1	Phvul.003G269000	<i>Phaseolus vulgaris</i>
PvSERAT3;2	Phvul.002G114700	<i>Phaseolus vulgaris</i>

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Education

2013-present Ph.D. candidate (Cell and molecular biology)

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Honours and awards

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2016 *Dr. Rene R. Roth Memorial Award*,
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2016 *Graduate Travel Award*,
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2013 *Pioneer Award*,
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Work experience

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Referred publications

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Shivashakar, S., **Joshi, J.**, and Sumathi, M. (2013). The role of seed viability in the development of corky tissue in sapota (*Manilkara achras*) fruit in India. *Journal of Horticultural Science & Biotechnology*. 88 (5) 671–677.

Note: This paper resulted from my MSc thesis. Under the supervision of Dr. S. Shivashankar, I designed and performed the experiments, conducted statistical analysis and wrote the first draft of the manuscript. Policy at the Indian Institute of Horticulture Research required that heads of laboratories are listed as first authors.

Bellur, P., Lakkanna, S., **Joshi, J.**, Cornelius, J., Tripodi, F. and Boddupalli, S. (2013). Food Biofortification: Breeding and biotechnology approaches to improve nutrients in vegetables and oil quality in soybean. In *Present Knowledge in Nutrition*, Tenth Edition (J. W. Erdman, I. A. Macdonald and S. H. Zeisel eds.), Wiley-Blackwell, Oxford, UK.

Conference presentations

Joshi, J. Renaud, J., and Marsolais, F. (2017). Deciphering sulfur amino acid metabolism in developing seed of common bean- An approach towards an excellent source of protein. Plant Metabolic Engineering (Gordon Research Seminar), Waterville Valley, NH, U.S.A. (Invited speaker)

Joshi, J. Renaud, J., and Marsolais, F. (2017). BSAS4;1- Key candidate in developing common bean as an excellent source of protein. Plants from Sea to Sky 2017, University of British Columbia, Vancouver, Canada.

Joshi, J. Renaud, J., and Marsolais, F. (2016). Unveiling sulfur amino acid metabolism in common bean seeds (*Phaseolus vulgaris*). Plant Biotech 2016, Queen's University, Kingston, Canada. (Best oral presentation award)

Joshi, J., Pandurangan, S., and Marsolais, F. (2014). Analysis of candidate enzymes for the conversion of cysteine into S-methyl-cysteine in seed of common bean (*Phaseolus vulgaris*). 5th Annual Biology Graduate Research Forum, University of Western Ontario, London, Canada.

Poster presentations

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Joshi, J., Pandurangan, S., and Marsolais, F. (2014). Analysis of candidate enzymes for the biosynthesis of S-methyl-cysteine in seed of common bean (*Phaseolus vulgaris*). 6th International Food Legumes Research Conference and 7th International Conference on Legume Genetics and Genomics, Saskatoon, Canada.

Joshi, J., and Shivashankar, S. (2010). Mechanism of corky tissue formation, an internal breakdown disorder in Sapodilla [*Manilkara achras* (Mill) Fosberg] fruits. 28th International Horticulture Congress, Lisbon, Portugal.

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