Application for Authorization of Food Derived from Imidazolinone-tolerant Soybean BPS-CV127-9 under Standard 1.5.2. – Food Produced using Gene Technology in the Food Standards Code

BASF Plant Science Company GmbH

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Abbreviations

ADF	acid detergent fibre
ahas	acetohydroxyacid synthase gene
ahasl	acetohydroxyacid synthase large subunit gene
AHAS	acetohydroxyacid synthase enzyme
AHASL	acetohydroxyacid synthase large subunit
AHASS	acetohydroxyacid synthase small subunit
ai	active ingredient
ANOVA	analysis of variance
AtAHAS	acetohydroxyacid synthase from *Arabidopsis thaliana*; refers to the AtAHAS enzyme comprising mutations R272K and S653N in the context of this dossier
AtAHASL	acetohydroxyacid synthase from *Arabidopsis thaliana* large subunit
AtSEC61γ	gamma subunit protein 61 of the secretory complex of *Arabidopsis thaliana*
At*SEC61γ*	gamma subunit gene 61 of the secretory complex of *Arabidopsis thaliana*
bla1	gene encoding beta-lactamase
bp	base pair(s)
BPS	BASF Plant Science
CDS	coding sequence
CFIA	Canadian Food Inspection Agency
csr1-2	gene from *Arabidopsis thaliana* encoding an imidazolinone-tolerant AHAS enzyme
CTP	chloroplast transit peptide
CV127	imidazolinone-tolerant soybean plant BPS-CV127-9
dw	dry weight
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EMBRAPA	Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuaria)
EST	expressed sequence tag
FARRP	Food Allergy Research and Resource Program
fw	fresh weight
FSANZ	Food Standards Australia New Zealand

gDNA	genomic DNA
G-con	simulated mammalian gastric fluid without pepsin
GM	genetically modified
Gm	*Glycine max*
ha	hectare
I-con	simulated mammalian intestinal fluid without pancreatin
ILSI	International Life Sciences Institute
Imi	imidazolinone (herbicide)
kb	kilobase pairs
LD_{50}	dose that is lethal to 50 % of treated animals
LOD	limit of detection
LOQ	limit of quantification
mol. wt.	molecular weight
MG	maturity group
mRNA	messenger RNA
NOEL	no observed effect level
NDF	neutral detergent fibre
nt	nucleotide(s)
OECD	Organization for Economic Cooperation and Development
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppm	part(s) per million
RT-PCR	reverse transcriptase-polymerase chain reaction
R272K	mutation resulting in a substitution of an arginine residue (R) with a lysine (K) at amino acid 272 relative to the *ahas* gene of *Arabidopsis thaliana*
S653N	mutation resulting in a substitution of a serine residue (S) with an asparagine (N) at amino acid 653 relative to the *ahas* gene of *Arabidopsis thaliana*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC61γ	gamma subunit protein 61 of the secretory complex
*SEC61*γ	gamma subunit gene 61 of the secretory complex
SGF	simulated mammalian gastric fluid containing pepsin
SIF	simulated mammalian intestinal fluid containing pancreatin

SPC	soy protein concentrate
SPI	soy protein isolate
Std(s)	standard(s)
TDF	total dietary fibre
U.S.	United States (of America)
UTR	untranslated region

PART I

Information as specified in Section 3.1 (General Requirements)

1. Executive Summary

Soybean (*Glycine max* L.) plants that are tolerant to the imidazolinone class of agricultural herbicides have been developed by BASF Plant Science (BPS) and EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria). The herbicide-tolerant soybean plants BPS-CV127-9 (also referred to as CV127 in this submission) are derived from a single transformation event and were produced by introduction of the imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene *csr1-2*[1] with its native promoter from *Arabidopsis thaliana* into the soybean plant genome via biolistics transformation technology. The AHAS enzymes from plants are composed of a catalytic subunit (or large subunit) and a regulatory subunit (or small subunit) that is required for feedback inhibition by branched-chain amino acids. The *csr1-2* gene from *A. thaliana* encodes an acetohydroxyacid synthase large subunit (AHASL) enzyme that is tolerant to imidazolinone herbicides due to a point mutation that results in a single amino acid substitution in which the serine residue at position 653 is replaced by asparagine (S653N). The AHASL catalytic subunit encoded by the *csr1-2* gene has altered herbicide binding properties such that imidazolinone herbicides do not bind to the enzyme while retaining its normal biosynthetic function in the plant (Pang *et al*., 2002).

The *Arabidopsis* AHAS (AtAHAS) enzyme is a member of the class of AHAS proteins found ubiquitously in plants. The AHAS enzyme catalyses the first step in the biosynthesis of branched-chain amino acids, valine, leucine, and isoleucine. Typically, inhibition of the AHAS enzyme by imidazolinone herbicides leads to a deficiency in branched-chain amino acids and other compounds derived from this pathway that are needed for plant growth and survival, and results in plant death. The herbicide tolerance in CV127 soybean will allow growers to treat the soybean crop with imidazolinone herbicides for weed control without causing injury to the soybean plant at normal field application rates. Soybean CV127 was developed for cultivation primarily in Brazil and Argentina, and introduction of CV127 soybean varieties will offer soybean growers an additional tool for controlling weeds, as well as an important option for weed resistance management. Imidazolinone herbicides control a wide spectrum of grass and broadleaf weeds. The growing use of glyphosate with glyphosate tolerant soybeans in Argentina and Brazil has led to a shift in the species of prevalent weeds with those that are more tolerant to glyphosate predominating. The most common weeds in this category, include Benghal dayflower (*Commelina benghalensis*), morning glory (*Ipomoea* spp.), pusley (*Richardia brasiliensis*), and buttonweed (*Spermacoce alata*). These weeds are sensitive to imidazolinone herbicides and the product concept of an imidazolinone-tolerant soybean fits well with

[1] It should be noted that throughout this dossier, the gene that resides within the genome of CV127 soybean that encodes the imidazolinone-tolerance trait is referred to as the *csr1-2* gene which is derived from *A. thaliana* and it is recognized that the *csr1-2* gene in CV127 soybean differs from the *csr1-2* gene in *A. thaliana* by a single nucleotide change that results in the R272K replacement. By convention, the amino acid numbering of plant AHAS enzymes correlates to the amino acid numbers of the AHAS from Arabidopsis, including the residues comprising the chloroplast transit peptide.

the commercial needs for weed control in South America. Furthermore, it is expected that growers planting CV127 soybeans will be able to reduce the number of herbicides used to control weeds in their soybean fields and benefit from reduced weed control costs. The reduction in herbicide use is also expected to benefit the environment.

Several *ahasl* genes encoding AHASL enzyme subunits that are tolerant to imidazolinone herbicides have been discovered in plants as naturally occurring mutations and through the process of chemically-induced mutagenesis. There are five single point mutations in *ahasl* genes that result in tolerance to imidazolinone herbicides in plants (Tan *et al*., 2005), including the S653N mutation in the *csr1-2* gene. For example, imidazolinone-tolerant maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* and *B. juncea* L.), and sunflower (*Helianthus annuus* L.) were developed through mutagenesis, selection, and conventional breeding technologies and have been commercialized under the Clearfield® brand name since 1996. Therefore, there has been a long history of safe production of crops containing an imidazolinone-tolerant AHAS with the same S653N amino acid substitution as that in the AtAHAS encoded by the *csr1-2* gene present in CV127 soybeans. In addition, these crops have been used to produce food and feed products that have proven to be as nutritious and as safe as similar products produced from conventional crops.

The food, feed and environmental safety of CV127 soybeans was confirmed based on results of a series of inter-related safety assessment studies. First is the detailed molecular characterization of CV127 soybean, which confirmed that CV127 contains a single functional *csr1-2* gene cassette integrated in the soybean genome. Second, biochemical characterization of the imidazolinone-tolerant AtAHAS protein expressed in CV127 soybean showed that the AtAHAS protein is typical of other AHAS proteins in this diverse protein family as well as most dietary proteins with a history of safe use in food and feed products and lacks any of the characteristics associated with known allergenic or toxic proteins. Third, the composition and nutritional equivalence of CV127 soybean compared to conventional soybeans was demonstrated by analysis of key nutrients and antinutrients, and nutritional equivalence to conventional soybeans was further confirmed in a poultry feeding study. The results of these studies show that CV127 soybeans are as safe for food and feed uses as conventional soybeans.

A complete molecular characterization of the transgenic insert in CV127 was conducted using various methods including Southern blot analysis as well as DNA sequencing. The novel *csr1-2* expression cassette was integrated at a single genetic locus in the soybean genome. No DNA sequences from the backbone of the transformation vector were detected in the event. Further, it was demonstrated that there were no additional fragments of DNA derived from the transformation plasmid inserted separately within the genome of CV127 soybeans. In the insert, there is also a 376 base pair (bp) duplication of a portion of the *csr1-2* coding sequence directly before the 3’ integration point. This duplicated 376 bp segment creates a 501 bp open reading frame (ORF) that extends into the 3’ flanking sequence. Reverse

transcription-polymerase chain reaction (RT-PCR) results showed that this 501 bp ORF is not transcribed. It was also discovered that, in addition to the *csr1-2* native gene promoter, the 5' region upstream of the *csr1-2* coding sequence contains the complete coding sequence of the *A. thaliana* SEC61 (AtSEC61) gamma (γ) protein, which is a component of the DNA fragment used for transformation. This protein is part of a multi-protein secretory complex that is ubiquitous in all eukaryotes. The At*SEC61γ* 5' untranslated region (UTR), as annotated by The Arabidopsis Information Resource, begins 18 nucleotides downstream from the 5' transgene integration site. As such, it is extremely unlikely that the insert contains the complete native promoter for the At*SEC61γ* gene. RT-PCR experiments showed that the At*SEC61γ* subunit gene is weakly transcribed in CV127 leaf tissue. However, protein expression studies demonstrated that there are no detectable levels of the AtSEC61 gamma protein present in CV127 soybeans.

Southern blot analysis and DNA sequence verification of the point mutation in the *csr1-2* gene showed that the transgene insert in CV127 is stably integrated in the soybean genome across the breeding generations studied. This conclusion was confirmed in a study of the inheritance of the imidazolinone-tolerance trait over multiple breeding generations of CV127 soybeans. Results of this study demonstrated that the trait is stably inherited according to classical Mendelian genetics, and results were consistent with the presence of a single dominant imidazolinone-tolerant gene in the soybean genome.

The food and feed safety of the AtAHAS protein expressed in CV127 soybeans was demonstrated by studies that confirmed that the AtAHAS protein is equivalent to other AHAS proteins found ubiquitously among plant species and have a history of safe use in food and feed products. Soybean CV127 plants were grown in multi-location replicated field trials in two different growing seasons in Brazil. Tissue samples were collected from CV127 and the control at different growth stages during the growing season. These samples were analysed by enzyme-linked immunosorbent assay (ELISA) to determine the quantity of AHAS enzyme, including both the AtAHAS encoded by the *csr1-2* gene and the endogenous soybean AHAS, in the tissues. Levels of total AHAS protein were typically much less than 1 ppm (part per million) on a tissue dry weight basis, and declined with age of the plant. The results of this study demonstrated that total AHAS levels in tissues of CV127 are very low. In addition, grain from CV127 and the control soybean were processed using standard soybean grain processing methods to produce soybean oil, meal, and protein isolate and concentrate fractions. Each processed fraction was analysed by ELISA for AHAS protein expression levels. The results of these analyses demonstrated that AHAS protein was not detected in any of the processed fractions produced from the grain of CV127 soybean. These results further confirmed that the AtAHAS protein is present at extremely low levels in processed fractions from CV127 soybean grain that are used in human foods as well as animal feeds.

The safety of the AtAHAS protein for human and animal consumption was assessed by several different approaches. Purified *Escherichia coli*-produced AtAHAS protein, chemically and functionally similar to AtAHAS protein produced in CV127 soybean, was used in these studies. First, the AtAHAS protein was readily degraded in simulated mammalian digestive conditions, and was readily inactivated by heat treatment, properties typical of most dietary proteins with a history of safe use in food and feed products. Second, the AtAHAS protein did not elicit any adverse effects when administered to mice by gavage with a No Observable Effect Level (NOEL) of equal to or greater than 2620 mg AtAHAS protein/kg body weight, the highest dose level tested. This dosage corresponds to greater than a 4.2×10^{10} -fold safety factor relative to the average human daily consumption of protein derived from soybean in Australia, assuming that all soybeans consumed contain the AtAHAS protein. Finally, bioinformatic searches of databases of all known proteins, including protein toxins, as well as of known allergenic proteins were conducted and it was confirmed that the AtAHAS protein expressed in CV127 soybean does not share immunologically relevant amino acid sequence segments or structure with known allergens or have sequence homology to known protein toxins. Therefore, the AtAHAS protein expressed in CV127 soybean tissues does not possess any attributes of known protein food allergens, is not toxic to mammals, and therefore presents no risks for human or animal consumption.

Composition analyses of the grain and forage demonstrated that CV127 is compositionally and nutritionally equivalent to and as safe as the control as well as other conventional soybeans. Samples of grain or forage were harvested from CV127, the near-isogenic comparator (control), and two conventional varieties from multi-location replicated field trials conducted in Brazil. Grain samples were collected from trials conducted in two separate growing seasons and analysed for a comprehensive range of important nutrients and antinutrients of soybean. Forage samples were collected from trials in one growing season and were analysed for proximates and fibre content. Statistical analysis of composition data from grain and forage of CV127 soybean treated with imidazolinone herbicide and from the control and two other conventional soybean varieties, demonstrated that the composition of grain and forage from CV127 soybeans is comparable to that of the control and conventional soybean varieties. Also, in a separate study, a proteomic analysis focusing on the known endogenous allergens present in soybean grain was conducted using grain from CV127 soybean and the parental line Conquista. Within the error level inherent to this study, no significant differences in the levels of the detected allergens of soybean grain were found between grain from CV127 soybean and Conquista.

In addition, grain from CV127 and the control soybean were processed using standard soybean grain processing methods to produce soybean oil, meal, and protein isolate and concentrate and the composition of these fractions was analysed. This study demonstrated that the processed fractions derived from grain of CV127 soybean are equivalent in composition to those from control soybean grain, and that CV127 soybeans are appropriate for use in food and feed products. Further, the nutritional equivalence of CV127 soybean to the control and conventional soybeans was

confirmed in a poultry feeding study. A 42-day feeding trial with broiler chickens was conducted to compare the performance of the animals fed with soybean meal from CV127 soybeans to those fed soybean meal from conventional soybean grain. This study demonstrated that there were no significant differences in the performance of the animals fed a feed containing soybean meal from CV127 soybean compared to those fed a feed containing soybean meal from the grain of conventional soybeans. Collectively, these results demonstrated that the grain and forage produced by CV127 soybean are compositionally equivalent to, and as nutritious as, grain and forage produced by the control and other conventional soybean varieties.

In summary, an extensive range of studies have been conducted that evaluated the food and feed safety and nutritional qualities of grain and forage of CV127 soybeans. In each of these studies, CV127 was compared to the control variety and other conventional soybean varieties. Taken together, these studies demonstrate that grain and forage of CV127 soybeans are compositionally equivalent to the control and other conventional soybeans and have the same nutritional characteristics as conventional soybeans that have a long history of cultivation and safe use in food and feed products.

2. Purpose of the application

The applicant BASF Plant Science Company GmbH is seeking amendment to Standard 1.5.2. (Food produced using Gene Technology) in the Australia New Zealand Standards Code to approve food derived from imidazolinone-tolerant CV127 soybean.
In conjunction with this application, BPS is also seeking a variation to Standard 1.4.2. (Maximum Residue Limits) to establish import tolerance levels for the imidazolinone herbicides that will be used on CV127 soybean. Import tolerances (MRLs) are being requested in the United States of America (U.S.), Canada, Europe, and other importers of soybean including Australia and New Zealand. Applications for the active ingredients imazapic and imazapyr proposing soybean MRLs were submitted to Food Standards Australia New Zealand (FSANZ) in April 2011.

BASF Plant Science, in partnership with EMBRAPA, has developed a genetically modified soybean that is tolerant to the imidazolinone class of herbicides. The herbicide-tolerant BPS-CV127-9 soybean (also referred to as CV127 soybean) was produced by introduction of the imidazolinone-tolerant acetohydroxyacid synthase (*ahas*) large subunit gene *csr1-2* from *Arabidopsis thaliana*. CV127 soybean is being developed primarily for cultivation in Brazil and Argentina, and it is not planned to cultivate the soybean commercially in Australia or New Zealand. The herbicide tolerance in CV127 soybean will allow growers to treat the soybean crop with imidazolinone herbicides for weed control without causing injury to the soybean plant at normal field application rates. Introduction of CV127 soybean varieties will offer soybean growers an additional tool for controlling weeds, as well as an important option for weed resistance management. Furthermore, it is expected that growers planting CV127 soybeans will be able to reduce the number of herbicides used to control weeds in their soybean fields and benefit from reduced weed control costs.

BPS is seeking approval for cultivation as well as food and feed use of CV127 soybean in Brazil and Argentina. Both countries are exporting soybeans and soybean products worldwide, and also Australia and New Zealand import a considerable quantity of soybean and soybean products from Brazil and Argentina.
The regulatory approval for food, feed and commercial growing of CV127 soybean in Brazil, the primary country of production, was granted by the Biosafety National Technical Commission (CTNBio) December 2009. The general release permit application in Argentina is still pending approval.
Additional submissions for the import approval of CV127 soybean for use in food and feed were made to those countries that import significant volumes of soybeans or soybean products. Regulatory approvals for import and food and feed uses of CV127 soybeans were granted in the Philippines by the Bureau of Plant Industry in October 2010 and in Mexico by the Federal Commission for the Protection against Sanitary Risk in May 2011. An overview of regulatory submissions and approvals is presented below.

Country	Authority	Submission	Status
Brazil*	National Technical Committee for Biosafety	Dec 2008	Approved Dec 2009
Japan	Ministry of Agriculture, Fisheries and Food – Environment Agency	Nov 2007	Review concluded in Aug 2010
	Ministry of Agriculture, Forestry and Fisheries	Jan 2009	Under evaluation
	Ministry of Health, Labour and Welfare	Jan 2009	Under evaluation
EU	European Commission of the European Union	Jan 2009	Under evaluation
U.S.	U.S. Department of Agriculture, Animal and Plant Health Inspection Services	Jan 2009	Under evaluation
	U.S. Food and Drug Agency	Jan 2009	Under evaluation
Canada	Health Canada	Feb 2009	Under evaluation
	Canadian Food Inspection Agency (Env)	Mar 2009	Under evaluation
	Canadian Food Inspection Agency (Feed)	Apr 2009	Under evaluation
Korea	Rural Development Administration	Dec 2009	Review concluded in May 2011
	Korea Food and Drug Administration	Dec 2009	Under evaluation
	National Institute of Environmental Research	Jan 2010	Review concluded in June 2011
	National Fisheries Research and Development Institute	Jan 2010	Review concluded in Aug 2010
	Korea Centers for Disease Control and Prevention	Jan 2010	Under evaluation
China	Ministry of Agriculture of China	Feb 2010	Under evaluation
Philippines	Bureau of Plant Industry	Feb 2010	Approved Oct 2010
Argentina*	National Health Service and Food Quality	May 2010	Under evaluation
	National Advisory Committee on Agricultural Biotechnology	June 2010	Under evaluation
Colombia	Colombian Agricultural Research Institute	Sep 2010	Under evaluation
	National Institute of Food and Drug Monitoring	Sep 2010	Under evaluation
Mexico	Federal Commission for the Protection against Sanitary Risk	Sep 2010	Approved May 2011
India	The Genetic Engineering Approval Committee	Nov 2010	Under evaluation
Taiwan	Department of Health of Taiwan	Dec 2010	Under evaluation
Russia	Institute of Nutrition	Mar 2011	Under evaluation
South Africa	Department of Agriculture, Forestry and Fisheries	June 2011	Under evaluation

*application includes cultivation

3. Justification for the application

a) The advantages of the genetically modified food

Soybean is the leading oilseed crop produced and consumed in the world today. The current world production of soybean greatly exceeds that of all other edible oilseed crops (Wilcox, 2004; Soy Stats, 2008). In 2007, 220 million metric tons of soybeans were produced worldwide representing 56 % of the world's total oilseed production (Soy Stats, 2008). Soybean has been the dominant oilseed crop produced in the world since the 1960s (Smith and Huyser, 1987) and in the past twenty years soybean production has increased by 75 million metric tons. For the past 50 years, the U.S. has been the world's leading producer of soybeans and in 2007 approximately 70.4 million metric tons were produced in the U.S., or about 32 % of the world's total production. The second and third largest producers of soybean are Brazil and

Argentina who produced 61 and 47 million metric tons of soybeans, respectively, in 2007. Soybean production in Brazil has increased dramatically in recent years with production from the 2007/2008 season surpassing 60 million metric tons (Soy Stats, 2008). The two largest soybean producers in Asia are the People's Republic of China (14.3 million metric tons in 2007) and India (9.3 million metric tons). Soybean yield can greatly impact the economic value of the crop, and increased yield can be achieved by recommended weed control and other crop management practices as well as by using soybean varieties that have enhanced yield potential.

For many years, genetically modified (GM) soybean varieties have constituted the largest percentage of genetically modified crops planted worldwide. In 2007 the area planted to GM soybeans was 58.6 million hectares or 64 % of the global area planted to soybeans (James, 2007). The vast majority of this acreage was planted with herbicide tolerant soybean varieties. Herbicide tolerant soybeans were planted on 24.2 million of the total 30.3 million hectares planted to soybeans in the U.S. in 2007 (James, 2007). In Argentina, virtually the entire area planted to soybeans in 2007, equal to 16 million hectares, was planted with herbicide tolerant GM soybeans (James, 2007). Cultivation of herbicide tolerant GM soybeans is also increasing rapidly in Brazil accounting for about 64 % of the total area planted to soybean in 2007. The rapid and widespread adoption of herbicide tolerant soybeans by growers worldwide has been due to increased yields, the reduced cost of effective weed control, and a simplified, more flexible weed control program (Reddy, 2001; Gianessi, 2005; USDA-ERS, 2005) that is offered by the use of herbicide tolerant crops.

The primary herbicide tolerant soybeans grown today are tolerant to the herbicide glyphosate. Glyphosate-tolerant soybeans were first grown commercially in 1996 and the cultivation of this crop has steadily increased every year since introduction (Gianessi and Reigner, 2006). In recent years, several weed species have evolved resistance to glyphosate (Heap, 2011; Nandula *et al*., 2005). It should be noted that most of the cases of glyphosate resistant weeds occurred in non-transgenic crops. Exposure to glyphosate started well before the introduction of glyphosate-tolerant crops and so, attributing the development of resistance to glyphosate in weed species to the use of glyphosate tolerant crops is not warranted (Dill, 2005).

In addition, soybeans that are tolerant to sulfonylurea herbicides due to a mutation in the acetohydroxyacid synthase (*ahas*) gene have been developed by conventional mutagenesis procedures (Sebastian *et al*., 1989). The sulfonylurea-tolerant soybeans do not provide cross tolerance to imidazolinone herbicides and are currently marketed in the U.S. as a stacked trait together with glyphosate tolerance.

The introduction of imidazolinone-tolerant soybeans will provide the grower with the opportunity to cultivate soybeans that are tolerant to yet a different herbicide with a different mode of action compared to glyphosate as a means to control weeds that may be tolerant to glyphosate. The rotation of herbicide tolerant crops that are resistant to different herbicide classes in combination with the application of the

corresponding herbicides is an effective strategy for managing the development of herbicide tolerance in weed populations.

Although soybeans are naturally tolerant to some imidazolinone herbicides due to an ability to metabolize specific imidazolinones, there are certain imidazolinone compounds, for example imazapyr and imazapic, that are active ingredients in a number of imidazolinone herbicide products and are not readily metabolized in soybeans. As a result, conventional soybeans are very sensitive to imazapyr and imazapic and the product concept for CV127 soybeans includes the use of imidazolinone herbicides containing these active ingredients for effective weed control. Imidazolinone herbicide tolerance has been developed by either conventional seed mutagenesis procedures or by natural mutation, for several crops like wheat, canola and sunflower currently marketed by BASF under the Clearfield® brand name. However, commercial levels of tolerance to imidazolinone herbicides containing either imazapyr and/or imazapic could not be achieved by these methods in soybeans. Therefore, BPS used a recombinant DNA technology to introduce the imidazolinone herbicide tolerant gene *csr1-2* from *Arabidopsis thaliana* into soybeans to develop the imidazolinone-tolerance trait of CV127 soybean. BPS is developing the imidazolinone-tolerant CV127 soybean for cultivation primarily in Brazil and Argentina. The major weeds in soybean cultivation in these countries are sensitive to the imidazolinone herbicides containing imazapyr and imazapic, making this product concept an attractive proposition for soybean cultivation in South America. CV127 soybean are not planned to be grown in either Australia or New Zealand. Food from CV127 soybean will therefore be entering the Australian and New Zealand food supply as imported and processed food products. Regulatory approval will be sought in Australia and New Zealand for importation of CV127 soybean products for food uses.

The introduction of soybean CV127 in Brazil and Argentina will offer growers excellent weed control options which in turn is expected to provide enhanced soybean yield potential. Furthermore, cultivation of soybean CV127 is expected to build on the environmental benefits realized as a result of cultivation of herbicide-tolerant soybeans. The imidazolinone herbicides possess several environmentally beneficial characteristics compared to other herbicide classes (Tan *et al*., 2005). Imidazolinone herbicides are effective at low application rates, possess residual activity in the soil and are absorbed by roots and translocated throughout the plant, resulting in fewer herbicide applications.

In addition, imidazolinone herbicides have a very favourable toxicology profile. Due to the fact that imidazolinones are rapidly removed from the blood stream and excreted in urine and since animals do not possess the AHAS enzyme that is the target for imidazolinones, imidazolinone herbicides have very low mammalian toxicity. Finally, imidazolinone herbicides are readily degraded in the soil to nontoxic, naturally occurring compounds by the activity of soil microbes.

Grower adoption of imidazolinone-tolerant CV127 soybeans is expected to have other benefits to the environment. The adoption of herbicide tolerant soybeans has resulted in a significant reduction in the adverse environmental impacts of intensive agricultural practices. One of the greatest negative environmental impacts from agriculture comes from the practice of tilling the soil. Soil tillage is practiced to reduce weeds in crops but it leads to reductions in topsoil by promoting erosion from wind and water, loss of soil moisture, and soil compaction (Holland, 2004). The use of a broad spectrum herbicide to control weeds without affecting the crop has facilitated the adoption of reduced- or zero-tillage practices in which seeds are planted directly into unploughed soil. Reduced tillage has resulted in improved soil structure, reduced soil erosion, and reduced risk from runoff and pollution of surface waters with sediment, nutrients, and pesticides. It is anticipated that grower adoption of imidazolinone-tolerant CV127 soybeans will similarly promote the adoption of no-tillage and reduced-tillage practices by soybean growers.

The use of herbicide tolerant crops, including soybean, has also resulted in a documented reduction in the consumption of tractor fuel. The amount of time spent by farmers on their tractors in fields of herbicide tolerant crops compared to fields of conventional crops has been reduced since fewer herbicides are being applied and due to the adoption of reduced- or no-tillage practices. In addition, the volume of herbicides used due to the cultivation of herbicide tolerant soybeans has decreased by 51 million kg since 1996, representing a 4.1 % reduction in herbicide application (Brookes and Barfoot, 2006). The adoption of no-till farming practices and the reduction in herbicide applications has been estimated to save 32.5 litres of fuel per hectare compared to conventional tillage (Jasa, 2002; CTIC, 2002). It has been estimated that the conversion from conventional to no-tillage agricultural practices results in the sequestration of as much as 1,000 kg carbon per hectare per year (Lal, 2004; West and Post, 2002). The estimated combined reduction in carbon dioxide emissions in 2006 due to fuel and tillage reductions is 14.8 billion kg that is equivalent to removing 6.6 million cars from the road for a year (Brookes and Barfoot, 2008). Grower adoption of imidazolinone herbicide tolerant soybeans will continue to promote reductions in fuel use and tillage and promote reductions in carbon dioxide emissions.

b) Potential public health and safety issues

A comprehensive food and feed safety and nutritional assessment of CV127 soybean was conducted which assessed the genetic modification of the plant, the potential toxicity and allergenicity of the novel proteins, the composition of soybean CV127 compared with that of the near-isogenic control and conventional soybean varieties, and the nutritional equivalence of soybean CV127. No potential health or safety concerns were identified. Therefore, the grain from CV127 is considered as safe and nutritious as grain from conventional soybean varieties that have a long history of safe use as food and feed ingredients.

c) The potential impact on trade

BASF Plant Science is developing the imidazolinone-tolerant CV127 soybean for cultivation primarily in Brazil and Argentina and hence will seek approval for the cultivation as well as food and feed use in both countries. Since Brazil and Argentina are exporting soybeans and soybean products worldwide, additional submissions for the import approval of CV127 soybean for use in food and feed were made to the U.S., Canada, Japan, the European Union and those countries that import significant volumes of soybeans or soybean products. Australia and New Zealand import a considerable quantity of soybean and soybean products from Brazil and Argentina.

Australia imported in total 1,304,807 t (metric tons) soybean oil cake from Brazil from 2004 to 2009 (40,323 – 403,291 t per year), and 1,018,741 t of soybean oil cake from Argentina from 2007 to 2010. Also, considerable amounts of soybean oil are imported, annually between 1,000 and 8,950 t from Argentina (2002 – 2010), and between 100 and 2,515 t from Brazil (2004 – 2009). New Zealand imported 7,000 t soybean meal from Argentina in 2010, and 18,971 t and 26,121 t from Brazil in 2006 and 2007, respectively. 956 t of soybean oil were imported by New Zealand from Brazil in 2008 and 948 t from Argentina in 2010 (United Nations, 2010). Upon approval in key markets as described above CV127 soybean is anticipated to represent a portion of the soybean and soybean products traded globally and therefore food from CV127 soybean may be entering the Australian and New Zealand food supply as imported and processed food products. Therefore, after regulatory approvals have been granted in the production countries as well as in countries that import soybeans from Brazil and Argentina, no impacts on trade, e.g. on food importers as well as processors and manufacturers of soybean-containing food products, are anticipated.

d) The costs and benefits for the industry, consumers and governments associated with the use of the genetically modified food

Consumers: If CV127 and derived products were approved in Australia and New Zealand there would be no restriction on imported soybean derived foods containing CV127. This would extend the availability of soybean products.

Industry: Importers, processors and retailers of soybean and derived products in Australia and New Zealand would benefit from an approval of CV127 soybean because this would enable them to choose from a broader choice of soy products available on the market, especially from South America.

Government: Since CV127 has already received cultivation approval in Brazil, an approval in Australia and New Zealand would ensure no trade disruption once the CV127 soybean will be commercially cultivated. Presence of CV127 soybean in commodity imports would not pose any potential regulatory issue and an approval would permit foods containing CV127 soybean to be imported into Australia and New Zealand.

Growers in South America: The introduction of soybean CV127 in Argentina and Brazil will offer growers in these countries excellent weed control options which in turn are expected to provide enhanced soybean yield potential. Also, this technology will offer growers in these countries an additional tool for managing herbicide resistance in weed populations in the soybean crop. Grower adoption of imidazolinone herbicide tolerant soybeans will continue to promote reductions in fuel use and tillage and promote reductions in carbon dioxide emissions.

4. Assessment procedure

BASF Plant Science, after consultation with FSANZ, considers the General Procedure appropriate to assess the application.

5. Exclusive capturable commercial benefit (ECCB)

The application is expected to confer an exclusive capturable commercial benefit to the applicant as outlined in section 2.1.4 of the FSANZ Application Handbook.

6. International and other standards

The studies supporting this application have been conducted according to international standards. In the safety assessment of the CV127 soybean, BPS refers primarily to the Codex Alimentarius Commission weight-of-evidence approach (Codex, 2003). Other guidelines and recommendations were also considered including, e.g., those of the World Health Organisation and the United Nations Food and Agriculture Organisation (FAO, 2001), the Organisation for Economic Cooperation and Development (OECD, 1993, 2000, 2001), and the European Food Safety Agency (EFSA, 2006).

Part II

Information as specified in Section 3.5 (Standards Related to New Foods)

A. Technical information on the genetically modified food

1. Nature and identity of the genetically modified food

a) A description of the GM organisms from which the new GM food is derived

BASF Plant Science, in partnership with EMBRAPA, has developed a genetically modified soybean that is tolerant to the imidazolinone class of herbicides. CV127 soybean is intended primarily for cultivation in Brazil and Argentina. CV127 soybean was produced by introduction of the *Arabidopsis thaliana* acetohydroxyacid synthase (also known as acetolactate synthase) large subunit gene *csr1-2* under the control of its native promoter into the soybean plant genome via biolistics transformation technology. The soybean variety Conquista was used as the recipient variety to develop CV127 soybean. Conquista is a highly productive, commercial Brazilian soybean variety in maturity group VIII, developed by EMBRAPA for cultivation in Brazil. The *csr1-2* gene from *Arabidopsis thaliana* encodes an acetohydroxyacid synthase large subunit (AHASL) enzyme that is tolerant to imidazolinone herbicides due to a point mutation that results in a single amino acid substitution at position 653 at which the serine residue is replaced by asparagine (S653N). The AHASL catalytic subunit encoded by the *csr1-2* gene, while retaining its normal biosynthetic function in the plant, confers altered herbicide binding properties such that imidazolinone herbicides do not bind to the enzyme.

b) The name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived

Throughout the application the genetically modified imidazolinone-tolerant soybean is referred to as CV127 soybean. According to the OECD guidance for the designation of a unique identifier for transgenic plants (OECD, 2002) the unique identifier assigned to CV127 soybean is BPS-CV127-9.

c) The name the food will be marketed under (if known)

After regulatory approvals, CV127 soybeans will be comingled with other commercial soybean varieties and will enter the same commercial processing channels for production of food and feed products. No unique commercial name will be associated with the food derived from CV127 soybean.

d) The types of products likely to include the food or food ingredient

Food and food ingredients derived from CV127 soybean are equivalent to those derived from other commercial soybean varieties. The products likely to include the food and food ingredients derived from CV127 soybean will be those that are currently in use and derived from conventional soybean varieties. An overview of those products is presented in **Section A.2.**

2. History of use of the host and donor organisms

a) A description of all the donor organism(s) from which the genetic elements are derived

(1) Common and scientific names and taxonomic classification

The genes integrated into the genome of CV127 soybean are derived from *Arabidopsis thaliana*. Arabidopsis is classified as follows:

Order	Brassicales
Family	Brassicaceae (mustard family)
Genus	*Arabidopsis* Heynh.
Species	*Arabidopsis thaliana* (L.) Heynh.
Common name	thale cress, wall cress or mouse-ear cress

Arabidopsis thaliana is a small flowering plant that is widely used as a model organism in plant biology. Arabidopsis is a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. Arabidopsis is not of major agronomic significance, but it offers important advantages for basic research in genetics and molecular biology (NCBI, 2008).

(2) Information about any known pathogenicity, toxicity or allergenicity of relevance to the food

Arabidopsis thaliana is not known to be pathogenic to humans or animals, nor is it known to be allergenic or the source of toxins. It has been handled for many years in research with no known toxicity or allergenicity issues. Both of the genes contained within the transformation DNA fragment that is derived from Arabidopsis encode proteins that are ubiquitous in plants and are not known to be allergenic or toxic.

(3) Information about the history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant)

Arabidopsis thaliana is not a typical food plant; therefore there is not a history of human consumption of the plant. But, since the 1940s Arabidopsis is used widely as one of the model organisms in plant sciences and genetics in research laboratories worldwide. The plant is native to Europe, Asia and north-western Africa.

b) A description of the host organism into which the genes were transferred and its history of safe use for food, including

(1) Any relevant phenotypic information

The biology of soybean described herein is based upon the consensus document for *Glycine max* (L.) Merr. prepared by the Organization for Economic Co-operation and Development (OECD, 2000; OECD 2001), a summary document prepared by the USDA-APHIS (2008), and a biology document published by The Plant Biosafety Office of the Canadian Food Inspection Agency (CFIA, 1996) unless indicated

otherwise. The cultivated soybean, *Glycine max* (L.) Merr., a diploidized tetraploid (2n = 40) that belongs to the family Fabaceae and is further classified taxonomically as follows:

Order	Fabales
Family	Fabaceae
Genus	*Glycine* Willd. (soybean)
Species	*Glycine max* (L.) Merr. (soybean)
Cultivar	Conquista
Common name	Soybean, soy, soya, soya bean

Conquista is a highly productive, commercial, Brazilian soybean variety in maturity group VIII. It is well adapted to cultivation in regions of less than 25 degrees latitude and was developed by EMBRAPA for cultivation mainly in Brazil.

The genus *Glycine* Willd. contains two subgenera, *Glycine* and *Soja* (Moench) F.J. Herm. The cultivated soybean, *G. max* (L.) Merr. and its wild annual relatives from Asia, *G. soja* Sieb. and Zucc. are classified in the subgenus *Soja*. *Glycine soja* is an annual that grows in the wild in fields, hedgerows, roadsides, and riverbanks in many countries of East Asia. This plant has a slender build with narrow trifoliate leaves. The flowers are purple, or on rare instances white, and are found on short, slender racemes. The pods are short and tawny with hirsute pubescence and contain oval-oblong seeds (Hermann, 1962).

In addition to *G. max* and *G. soja*, the subgenus *Soja* also contains a form known as *G. gracilis*. This semi-cultivated or weedy plant is found only in Northeast China and is intermediate in morphology between *G. max* and *G. soja*. Recent examinations have concluded it to be a variant of *G. max* (Hermann, 1962; Shoemaker *et al*., 1986). The three species of the subgenus *Soja* are capable of cross-pollination and the hybrid seed that is produced can germinate normally and produce plants with fertile pollen and seed (Singh and Hymowitz, 1989). The wild, weedy relatives of *G. max, G. soja* and *G. gracilis* are indigenous to Asia. Since *G. max* is the only *Glycine* species found in Brazil, there is no possibility for cross pollination between *G. max* and *G. soja* and *G. gracilis* in this country.

Within the tribe Phaseoleae, the genus *Glycine* is the only genus containing species that have diploid chromosome numbers of 40 and 80 and not 20 (Lackey, 1980). Based on taxonomic, cytological, and molecular systematics evidence, it has been proposed that the unique chromosome number of *Glycine* is most likely derived from an unknown progenitor species with a chromosome base number of 11. From this ancient progenitor, a putative ancestor of *Glycine* arose in Southeast Asia with 2n = 20 (Kumar and Hymowitz, 1989; Singh and Hymowitz, 1989; Lee and Hymowitz, 2001). Tetraploidization (2n = 2x = 40) through auto- or allopolyploidy of this ancestor species occurred at some time to produce a species in which 2n = 40. The sequence of events in the development of *G. max* from the ancient progenitor species is proposed by Singh *et al*. (2001) to be: wild perennial (2n = 4x = 40; unknown or

extinct) to wild annual (2n = 4x = 40; *G. soja*) to soybean (2n = 4x = 40; *G. max*). Soybean is regarded as a stable tetraploid with diploidized genome (Gurley *et al*., 1979; Lee and Verma, 1984; Skorupska *et al*., 1989).

Morphology of cultivated soybean
The cultivated soybean is an annual that has an erect, sparsely branched, bushy growth habit and can reach a height of 1.5 meters (OECD, 2000). The primary leaves are unifoliate, opposite and ovate, the secondary leaves are trifoliolate and alternate, and compound leaves with four or more leaflets are occasionally present. The nodulated root system consists of a taproot from which emerges a lateral root system. The plants of most cultivars are covered with fine trichomes, but glabrous types also exist. The purple, pink, or white flowers are situated on short axillary racemes or reduced peduncles. The flower consists of a tubular calyx of five sepals, a corolla of five petals (one banner, two wings and two keels), one pistil and nine fused stamens with a single separate posterior stamen. The pod is straight or slightly curved, varies in length from two to seven centimetres, and consists of two halves of a single carpel which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary amongst cultivars from almost spherical to elongate and flattened. The pods typically produce one to three seeds.

There are three types of growth habit found amongst *G. max* (L.) Merr. soybean cultivars: determinate, semi-determinate and indeterminate (OECD, 2000). Determinate growth is characterized by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both axillary and terminal racemes. Determinate genotypes are typically grown in Brazil and the southern United States (Maturity Groups V to X). Indeterminate genotypes continue vegetative activity throughout the flowering period and are grown primarily in central and northern regions of North America (Maturity Groups 000 to IV). Semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period.

Soybean is a self-pollinating species that is propagated by seed (OECD, 2000). The papilionaceous flowers consist of a tubular calyx of five sepals, a corolla of five petals, one pistil, and nine fused stamens with a single separate posterior stamen. One day before pollination, the stamens elongate and form a ring around the stigma that is receptive to pollen for a period from approximately 24 hours before anthesis to 48 hours after anthesis (OECD, 2000). The anthers mature in the bud and directly pollinate the stigma within the same flower. Pollination typically occurs on the same day that the flower opens and depending on environmental conditions, anthesis normally occurs in the late morning. Pollen typically comes in contact with the stigma during the process of anthesis. Pollen viability lasts for a short time of two to four hours and no viable pollen is present by late afternoon. Natural or artificial cross-pollination is only possible during the short time when the pollen is viable. Accordingly, soybean exhibits a very low level of cross-pollination below one percent and is primarily self-pollinating (Caviness, 1966; Yoshimura *et al*., 2006).

Due to the strong propensity for self-fertilization described above, the frequency of cross-pollination is very low. This is true even under conditions designed to optimize cross-fertilization including bringing synchronous flowers into close proximity to one another. Between plants in the field located in adjacent rows, outcrossing has been measured in the range of 0.03 to 3.62 % (Beard and Knowles, 1971; Caviness, 1966; Yoshimura *et al*., 2006). When plants are located more than 4.5 meters from one another, natural cross-pollination in soybean is undetectable or extremely low (less than 0.02 %) (Caviness, 1966). More recent studies of soybean cross-pollination were conducted by Ray *et al*. (2003) and Yoshimura *et al*. (2006) and they report similar findings. Plants grown in close proximity to each other (15 cm) were found to have average outcrossing rates of 1.8 %, while plants separated by distances of 0.9 m and 5.4 m had outcrossing rates of 0.41 and 0.03 %, respectively (Ray *et al*., 2003). Soybeans are generally not a preferred plant for insect pollinators and insect activity has been found not to increase the outcrossing rate (Erickson, 1975; Erickson, 1984).

In addition to the subgenus *Soja*, the genus *Glycine* also contains the subgenus *Glycine*. The subgenus *Glycine* contains twelve wild perennial species. These members of the subgenus *Glycine* are indigenous to Australia, South Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan. Hybridization between the diploid perennial *Glycine* species produces normal meiosis and fertile offspring. Attempts at hybridization between members of the annual subspecies *Soja* and the perennial subspecies *Glycine* have proved unsuccessful. Although hybridization results in the initiation of pod development, the pods eventually abort and abscise. Soybean is not able to cross with other plants outside of the *Glycine* subgenus *Soja* and so intergeneric hybridization does not occur.

Soybean plants are not weedy and are not found outside of cultivation. Soybeans are annuals that reproduce solely from seeds. Cultivated soybean rarely displays any dormancy characteristics (a desirable trait that is selected for in commercial varieties) (TeKrony *et al*., 1987) and are sensitive to cold temperatures (Raper and Kramer, 1987). Soybean seeds normally germinate quickly under the appropriate environmental conditions that include adequate moisture and moderate temperatures and could potentially grow as a volunteer. However, any volunteers that grow after harvest would be destroyed by the low and freezing temperatures encountered during the following winter. Were volunteers to become established, they would not compete well with succeeding crops and they could be controlled by either mechanical or chemical means (OECD, 2000).

(2) How the organism is typically propagated for food use

Soybean has long history of cultivation for food and feed uses in many different countries. The following summarizes the history of cultivation of soybean as well as provides a description of typical cultivation practices, using Brazil as an example, since CV127 soybeans were developed to address grower needs for weed control in this country.

Soybean is commonly considered one of the oldest cultivated crops, native to North and Central China (Hymowitz, 1970). Historic and archaeological evidence suggests that the soybean [*Glycine max* (L.) Merr.] first emerged as a domesticate during the Zhou Dynasty in north-eastern China (Hymowitz, 2004). Soybean domestication most likely occurred during the Shang Dynasty (ca 1500 - 1100 B.C.) or earlier. It is thought that by the first century A.D., the soybean had spread to Central and South China and Korea. The subsequent movement of soybean germplasm within the primary gene centre was probably associated with the development and consolidation of territories and the collapse of the Chinese dynasties (Ho, 1969; Hymowitz, 1970).

Due to the development of land races, the cultivation of soybeans spread from the first century A.D. to the 15th to 16th centuries to other regions in East Asia, including Japan, Indonesia, Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal, and north India. The establishment of trade routes over sea and land during this time facilitated the movement of soybeans from China and the rapid acceptance of seeds as a stable food by other cultures (Hymowitz, 1990; Hymowitz and Newell, 1981). Beginning in the late 16th century and throughout the 17th century, European visitors to China and Japan noted the use of soybeans from which the Chinese and Japanese produced various food products. By the late 17th century, soy sauce had become a common item of trade between the East and Europe.

The soybean was first introduced into North America in 1765 by Samuel Bowen who obtained the soybean seed while he was a seaman employed by the East India Company on a trade ship sailing between China and London. Soybeans were first introduced from the U.S. into Brazil in 1882 (Dall'Agnol, 2004). However, the germplasm that was introduced in the state of Bahia at latitude 12°S was not adapted to such low latitudes and did not perform well. A decade later and after the development of later maturing varieties, soybeans were planted further south in the state of Sao Paulo at 23°S and improved production was realized. In the first years of the 20th century, soybeans were grown at 30°S in Rio Grande do Sul, the most southerly state in Brazil, with climatic conditions similar to those in the southern regions of the U.S. Commercial production of soybeans in Brazil increased dramatically in the mid-1950's following a government decision that provided incentives to grow wheat. Since soybeans are a good rotational crop with wheat and use similar farm machinery and infrastructure, this policy has also led to increased cultivation of soybeans in Brazil. The primary centres of soybean cultivation in Brazil range from southern Brazil to the savannas (Cerrado) of Central Brazil. Today Brazil is the third largest producer of soybeans behind the U.S. and Argentina.

CV127 soybean is intended for production primarily in Brazil and Argentina, therefore the following is a brief description of soybean cultivation practices in Brazil as an example of general soybean cultivation practices. Soybeans can be produced on almost all arable land in Brazil from latitudes 5°N up to 33°S, except for the semi-arid regions of the northeast, and the mountains in the east of the country (Guidelines for GAP, 2002). Factors including precipitation, temperature and soil type further define the regions most suited for soybean production. For example, the soybean crop

typically requires from 450 to 800 mm precipitation throughout the growing season, and the crop does not perform well in temperatures less than 10°C or greater than 40°C. Furthermore, the crop grows well in soils of medium texture, so should not be grown in soils containing less than 15 % clay content. Different tillage practices are used throughout the soybean growing regions of Brazil, with a trend toward grower adoption of the no-tillage practice. The no-tillage system typically allows a longer sowing period, requires less precipitation for seed germination, reduces the crop susceptibility to water stress, and provides other environmental benefits including minimal soil compaction and less topsoil erosion.

Due to the ability of soybean to support a nitrogen fixing symbiosis with *Bradyrhizobium japonicum* and to convert atmospheric nitrogen to fixed forms of nitrogen, soybeans are often planted in rotation with corn, wheat, cotton, rice and other crops. Studies have demonstrated that the rotation of soybean with other crops generally leads to increased yields for the soybean and subsequent crops (Johnson, 1987; Wesley, 1999). Other benefits for the farmer from rotation with soybeans include reduced nitrogen input in the following crop, increased residue cover, and reduction of pest and weed cycles.

Prior to planting, fertilizer is applied as needed according to soil test recommendations. For example, it is estimated that soil content of phosphorus and potassium should correspond to the availability of at least 20 kg of each element for each ton of estimated grain harvested. It is recommended that nitrogen supply should be exclusively through biological N_2 fixation by bacteria from the *Bradyrhizobium* genus. Inoculants containing the bacteria typically contain more than 10^8 cells per gram, and are applied directly to the soybean seeds immediately prior to planting. In Brazil, the optimal planting time is from mid-October to mid-December. Mean ambient temperatures at the time of planting typically range from 20 to 30°C. Seeding rate typically ranges from 220 to 320 thousand plants per hectare, and row spacing is 0.4 to 0.5 meters.

Soybeans grow quickly to close the crop canopy, after which they are very competitive with weeds. However, soybeans are very sensitive to weed competition in the first weeks after emergence from the soil. It is recommended by weed specialists that weeds be controlled in soybeans within three to five weeks after emergence in order to prevent irreversible yield losses due to early competition from weeds. The use of post-emergence herbicides has greatly facilitated the adoption of reduced or no-tillage practices since farmers could effectively control weeds with one or two early treatments. This has both economic and environmental advantages, including more effective weed control leading to enhanced yield. In Brazil, Argentina and the U.S., the cultivation of glyphosate-tolerant soybeans treated with glyphosate has been widely adopted. Therefore, the use of glyphosate herbicide to control weeds in soybeans is the most common practice in these countries. However, the growing use of glyphosate with glyphosate-tolerant soybeans in Brazil has led to a shift in the species of prevalent weeds with those that are more tolerant to glyphosate predominating. Therefore, to manage and reduce the development of weed resistance,

growers are encouraged to use the rotation of herbicides with different modes of action for weed control.

In Brazil, the disadvantage of a climate with warm winters is higher levels of insect and disease infestations. Insect pests of soybean in Brazil include velvetbean caterpillars, southern stinkbug, and the ceratoma beetle. Some growers use biological control for velvetbean caterpillars, but it is not uncommon for growers to apply insecticides several times during the growing season for insect pest control. Diseases in Brazil include stem canker, frogeye leaf spot and Asian rust. To date, genetic resistance to Asian rust has not been identified and so control relies on the use of foliar fungicides where infestations are severe.

Soybean grain is harvested when the plants reach the R_8 stage of development, and grain moisture is between 13 % and 15 %. The grain is stored at a moisture level below 12.5 %, at temperatures less than 25°C and relative humidity less than 70 %. The major commodity products of soybean include the seeds and the major processed products oil and meal. One of the major food uses for soybean includes the production of soybean oil that is utilized in margarines, shortenings, salad and cooking oils. Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50 - 75 % of its overall value. Most soybean meal (97 %) is used in feed rations for livestock, including poultry, swine, and dairy and beef cattle.

(3) What part of the organism is typically used as food

There are two different types of soybeans, commonly referred to as oil beans and food beans (Liu *et al.*, 1995; Orthoefer and Liu, 1995; Wilson, 1995). Whereas food beans are varieties of soybeans that have been bred for specific qualities required in the production of traditional soy foods, oil beans make up the majority of soybeans grown for producing oil and high-protein meal. A large array of different manufacturing processes is applied to obtain the many soy products used in animal and human nutrition (Berk, 1992; van Eys *et al*., 2004).

Soybean processing involves a series of steps to produce commodities for food, industrial, and animal feed uses. **Figure A.2-1** provides an exemplary schematic representation of the manufacturing steps involved. After cleaning the harvested crop from impurities, soybeans are cracked to remove the hull, rolled into full-fat flakes to disrupt the oil cells, and steam conditioned. The resulting full-fat flakes are conveyed to oil extraction where crude oil is obtained as a major product. Oil extraction can be achieved either mechanically by a screw press (expeller process) or by extraction with non-polar solvents such as hexane (solvent extraction). Solvent extraction is the most efficient and widely used method at present. The crude oil is separated into lecithin and refined oil used in human and animal nutrition. After oil extraction defatted soy flakes remain that are processed further into various end products.

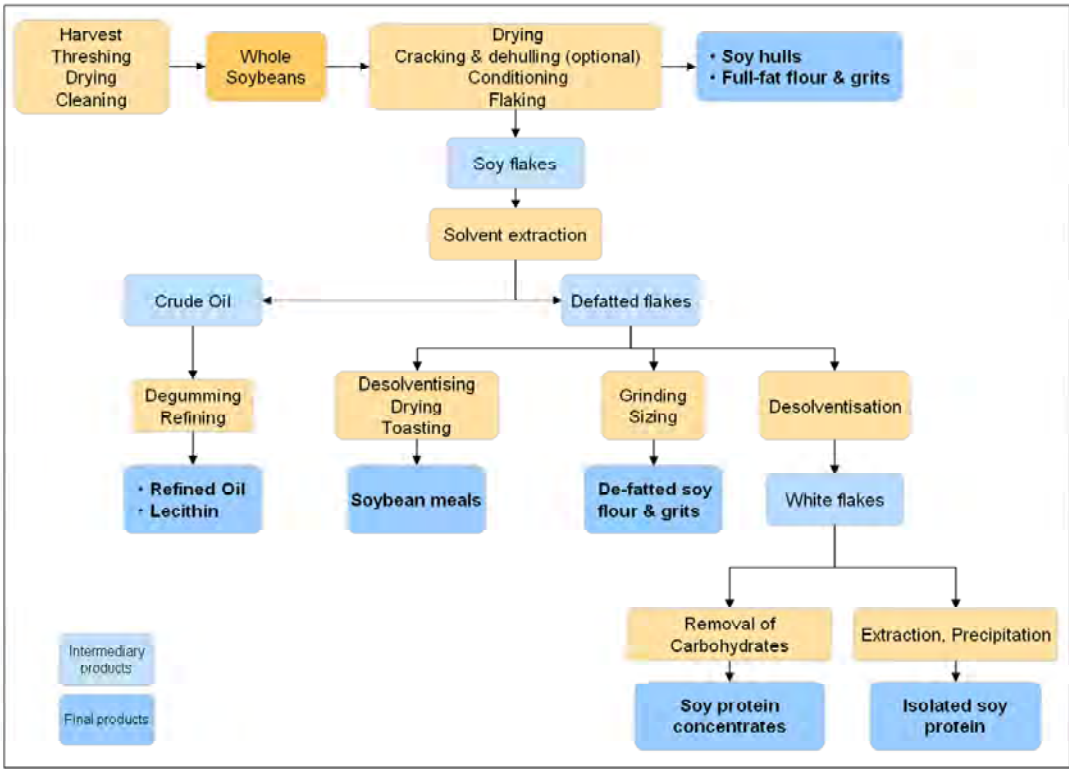
The most important products obtained from soybeans on a volume basis are the soybean meals for animal feeding, predominantly for poultry diets. To obtain soybean meals the defatted soy flakes are steam toasted in order to remove residual solvent and

to inactivate enzymes such as trypsin inhibitors and urease that may reduce the digestibility and nutritional value of the meal. Urease activity is typically used as an indicator of reduction of trypsin inhibitor activity in meal as a result of heat treatment during soybean processing. This has been a reliable method because trypsin inhibitor is readily reduced by heat as is urease activity. Soybean meals may contain variable levels of fibre and crude protein depending on the amount of hulls added back to the toasted flakes. Alternatively the defatted flakes can be ground to produce soy flour or sized to produce soy grits for food uses.

Flash desolventization or heat vacuum drying of the defatted flakes produces the untoasted white flakes that are high in protein quality. White flakes are generally the starting materials for high protein food ingredients such as soy protein concentrate (SPC) and soy protein isolate (SPI) preparation. Other soybean meals or flours besides white flakes may also be used as starting materials, though. Soy protein concentrate is made by removing a portion of the soluble carbohydrates. It is very digestible and widely used as functional or nutritional ingredient in a wide variety of food products. Soy protein isolate contains 90 percent protein and is used mainly by the food industry. SPI and SPC have functional and nutritional applications in various types of bakery, dairy and meat products, and infant formulas. They are also used in animal nutrition but are limited to specialty diets due to the relatively high cost.

Other food products derived from food soybeans are divided in to two groups, fermented and non-fermented products. Soy sauce is an example of a fermented product. Whole soybeans are typically soaked and cooked in water. The cooked soybeans are dusted with *Aspergillus oryzae* as a fungal starter, and then shaped in to koji nuggets. The nuggets are incubated to mature the fungi on the soybeans, and enzymes and nutrients are produced that are vital to the later fermentation steps. The mature nuggets are mixed with salt and water in fermentation vats to produce a mash. The mature mash is pressed to yield a raw liquid, the oil is removed which leaves the fermented soy sauce. Tofu is an example of a non-fermented soybean product. The soybeans are typically soaked in water, and after decanting the water, the soybeans are ground to a pulp, which is cooked in water. The resulting soymilk is coagulated with calcium or magnesium, and curds and whey are formed. The whey is drained off, and the curds are pressed in perforated boxes to form a cohesive block of tofu.

Figure A.2-1. Outline of the manufacturing of soybean products



(4) Whether special processing is required to render food derived from the organism safe to eat

Soybeans do not produce any known toxic compounds, but do contain several antinutrients (OECD, 2001). However, the processing methods described above, particularly the different heat treatments, either inactivate or destroy the antinutrients, and this processing allows safe use of the processed products as food or feed. Soybeans harvested from CV127 soybean plants will undergo existing methods of production and processing used for commercial soybean. No novel method of production and processing will be required.

(5) The significance to the diet in Australia and New Zealand of food derived from the host organisms

The majority of soybeans are processed for soybean meal used in animal feed, and soybean oil for human food uses. In Western societies such as in Australia, the U.S., Canada and Europe, soybeans do not constitute a significant portion of the diet. In these regions, soybeans are typically processed into oil and soybean meal. Soybean oil is used for cooking and to produce edible food products such as margarine, salad dressings, shortening, cooking oil and mayonnaise. Lecithin, derived from crude soybean oil, is used as a natural emulsifier, lubricant and stabilizing agent. Soybean meal is primarily used as an animal feed ingredient and is also not a significant source of protein in human diets in Western societies. Australians, Americans, Canadians and Europeans as a whole consume very little soy protein. Foods that contain soybean protein include bakery products, confections, meat products, textured foods and

nutritional supplements. Soybean protein isolate is also the protein source for soy-based infant formula. Based on data from a dietary survey conducted by the UN Food and Agriculture Organization (FAO, 2003), the per capita consumption of soybean protein in these regions is less than 1 g per day.

In the Asia-Pacific countries, soybeans constitute a more significant part of the human diet compared to societies in the West. In the Asia-Pacific countries, soybeans are used to produce a variety of foods, including non-fermented soy foods such as tofu, soymilk, and soy sprouts, and fermented soy foods such as soy sauce, miso, tempeh, and natto. As a result, the database from the FAO shows that the per capita consumption of soybean protein in Asian-Pacific societies ranges from 3 to nearly 10 g per day.

3. The nature of the genetic modification

a) A description of the method used to transform the host organism

The herbicide-tolerant CV127 soybean plants are derived from a single transformation event and were produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* (L.) Heynh. The Arabidopsis *ahasl* (S653N) allele, which confers tolerance to imidazolinone herbicides, has been referred to in the literature as *csr1-2* (Sathasivan *et al.,* 1990; Jander *et al*., 2003). It should be noted that throughout this dossier, the gene that resides within the genome of CV127 soybean that encodes the imidazolinone-tolerance trait is referred to as the *csr1-2* gene which is derived from *Arabidopsis thaliana* and it is recognized that the *csr1-2* gene in CV127 soybean differs from the *csr1-2* gene in Arabidopsis by a single nucleotide change that results in the R272K replacement. By convention, the amino acid numbering of plant AHAS enzymes correlates to the amino acid numbers of the AHAS from Arabidopsis, including the residues comprising the chloroplast transit peptide.

A purified, linear DNA fragment derived from plasmid pAC321 was used to transform embryogenic axis tissue derived from the apical meristem of a single soybean seed of the commercial variety Conquista. This soybean variety and tissue type was chosen because it responds well to particle bombardment transformation and tissue culture regeneration. In addition, Conquista is a highly productive Brazilian soybean variety with wide adaptation to different Brazilian growing environments. Biolistic transformation (microprojectile or particle bombardment) (Aragão *et al.,* 1996) was used to produce soybean transformation events containing the *csr1-2* gene. This DNA delivery system is well documented to transfer and integrate new DNA into a plant genome (Klein *et al.,* 1987; Lee *et al.,* 1996; Sanford *et al.,* 1993). Prior to bombardment, the purified PvuII DNA fragment containing the *csr1-2* gene cassette was precipitated onto microscopic gold particles. The precipitated DNA and particles were then placed onto a plastic macrocarrier and accelerated at high velocity such that a stopping screen retained the macrocarrier. The particles with DNA were permitted to continue their flight and eventual penetration and incorporation into the soybean

plant cells. These cells were transferred to a selective media containing the equivalent of 100 g ai/ha imazapyr, an imidazolinone herbicide, and only those cells transformed with the *csr1-2* gene continued to grow.

b) Information about the intermediate host organisms (e.g. Bacteria) used for all laboratory manipulations prior to transformation of the host organism

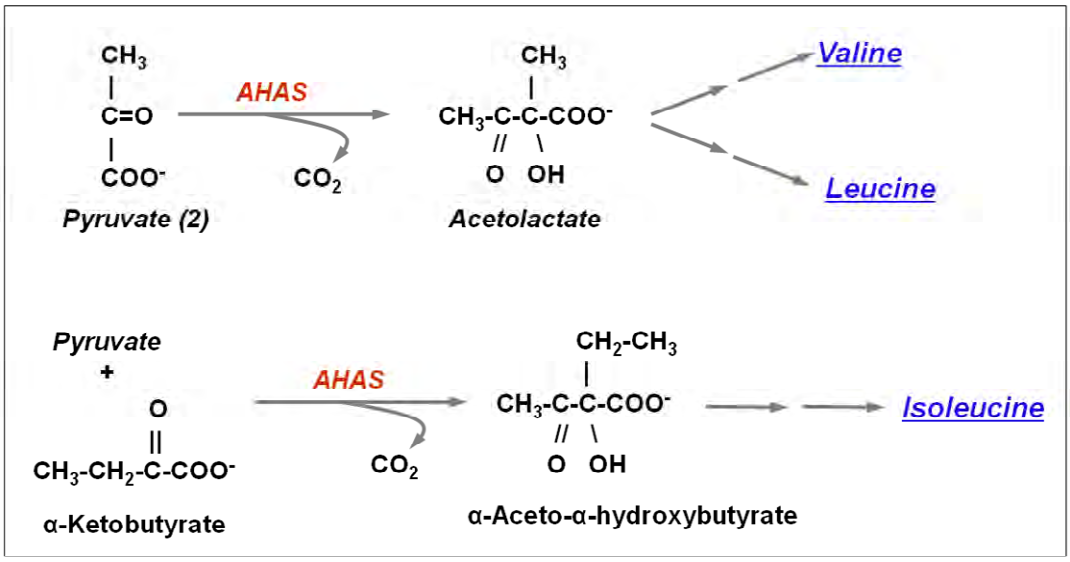
The approximately 6.2 kb linear fragment PvuII DNA fragment, used for the transformation of the soybean tissue, is derived from plasmid pAC321 containing the *csr1-2* gene cassette. The plasmid was assembled and propagated in *E. coli.*

c) A description of the gene construct and the transformation vectors used, including

(1) The size, source and function of all the genetic components including marker genes, regulatory and other elements

The complete gene cassette that was inserted into the genome of CV127 soybeans to impart the imidazolinone-tolerance trait was derived from *Arabidopsis thaliana*. The DNA fragment used to transform soybeans contains the Arabidopsis *csr1-2* gene that encodes an imidazolinone-tolerant form of the AHAS large subunit. The AHAS enzyme is ubiquitous in all plants and microbes. The AHAS enzyme catalyses the first step in the biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine (**Figure A.3-1**).

Figure A.3-1. Enzymatic reactions catalysed by acetohydroxyacid synthase (AHAS)



The *csr1-2* gene in CV127 soybeans is under the regulatory control of the native *csr1-2* gene promoter and the termination of translation is also by the native *csr1-2* translation termination signal. The *csr1-2* gene sequence in CV127 soybean differs from the *csr1-2* gene in Arabidopsis by a single nucleotide change that results in a

R272K replacement. In addition to the *csr1-2* gene, the transformation fragment also includes the Arabidopsis gene encoding the AtSEC61*γ* subunit, a protein of the endoplasmic reticulum secretory system, and two regions of unannotated Arabidopsis genomic DNA. A 5717 bp XbaI fragment from Arabidopsis that contains the *csr1-2* gene was cloned into vector pBluescript SK(-) to create plasmid pAC321. In order to encompass the complete and functional Arabidopsis promoter and terminator regions flanking the *csr1-2* coding sequence the 5717 bp XbaI fragment contained approximately 2.5 kb of unannotated DNA 5' to the start of the *csr1-2* coding sequence and 5' untranslated region (UTR) and approximately 1 kb of unannotated DNA 3' to the end of the *csr1-2* coding sequence. For transformation, the 6.2 kb PvuII fragment from pAC321 was used which contained the entire 5717 bp XbaI fragment from Arabidopsis as well as the multiple cloning site and other portions of pBluescript SK (-). At the time of transformation the complete Arabidopsis genome sequence was not available and the *csr1-2* gene promoter region was not described more precisely. As the Arabidopsis sequence information was revealed, the presence of the At*SEC61γ* subunit gene as part of the unannotated DNA flanking the 5' end of the *csr1-2* coding sequence could be described. The 6.2 kb PvuII transformation fragment contained about 1 kb of unannotated DNA 5' to the At*SEC61γ* subunit 5' UTR, most of which is not present in CV127 soybeans. The At*SEC61γ* 5' UTR, as annotated by The Arabidopsis Information Resource (TAIR, 2008), begins 18 nucleotides downstream from the 5' transgene integration site. As such, it is extremely unlikely that the insert contains the complete native promoter for the At*SEC61γ* gene.

The size, intended function, and origin of each genetic element of the region intended for insertion are described in **Table A.3-1**. The genetic elements contained within the 5.7 kb XbaI fragment that was derived from the genome of Arabidopsis and integrated into plasmid pAC321 are listed beginning with nucleotide 1 and continuing to nucleotide 5717.

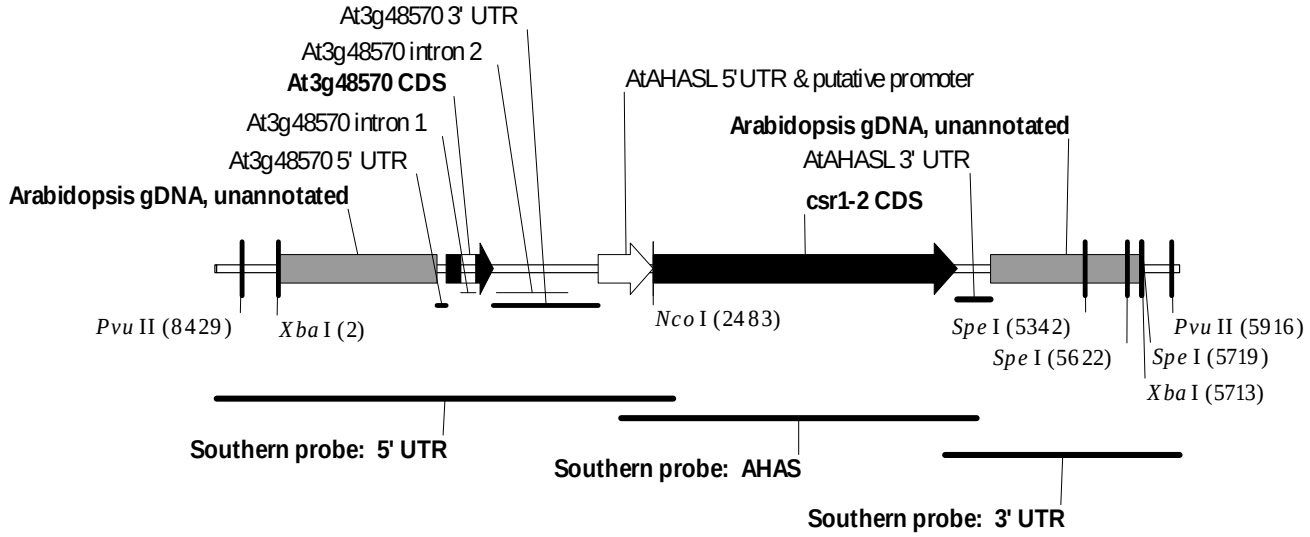
Table A.3-1. DNA components of the linear PvuII fragment of pAC321 that was used for transformation. The numbering of base pairs refers to the position in plasmid pAC321. A complete list of all genetic elements of plasmid pAC321 is given in Appendix 1.

Genetic Element	Range (bp)	Function
pBluescript SK (-) phagemid	8429-8669	Stratagene Corporation; La Jolla, CA (Short *et al.*, 1988)
PvuII restriction site	8429	
lacZ' CDS, interrupted	8590-8669	*E. coli* β-galactosidase alpha fragment coding sequence, interrupted by Arabidopsis genomic DNA in pAC321; allows blue-white screening for DNA inserts in pBluescript SK(-) multiple cloning site by alpha-complementation
T3 promoter	8632	Bacteriophage T3 promoter transcription initiation site; allows in vitro synthesis of RNA from DNA cloned in phagemid by T3 polymerase
Arabidopsis gDNA, unannotated	1-1051	*Arabidopsis thaliana* genomic DNA: no genes currently annotated in this region
Arabidopsis locus At3g48570	1052-2119	Protein translocation complex SEC61 GAMMA CHAIN-LIKE protein from *Arabidopsis thaliana*
At3g48570 5' UTR	1052-1113	5' untranslated region for putative Arabidopsis *SEC61 GAMMA CHAIN*
At3g48570 CDS	1114-1207, 1307-1422	Putative Arabidopsis *SEC61 GAMMA CHAIN* coding sequence
At3g48570 intron 1	1208-1306	Putative Arabidopsis *SEC61 GAMMA CHAIN* intron 1, interrupts CDS
At3g48570 3' UTR	1423-1442, 1916-2119	3' untranslated region for putative Arabidopsis *SEC61 GAMMA CHAIN*
At3g48570 intron 2	1443-1915	Putative Arabidopsis *SEC61 GAMMA CHAIN* intron 2
At *AHASL* 5' UTR and putative promoter	2120-2483	Putative promoter and 5' untranslated region for Arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*
csr1-2 CDS	2484-4496	Coding sequence for *Arabidopsis thaliana acetohydroxyacid synthase large subunit* with (S653N) point mutation (*csr1-2*) which confers tolerance to imidazolinones (Sathasivan *et al*., 1990)
At *AHASL* 3' UTR	4497-4714	3' untranslated region for Arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*
Arabidopsis gDNA, unannotated	4715-5717	*Arabidopsis thaliana* genomic DNA: no genes currently annotated in this region
pBluescript SK(-) phagemid	5718-5916	Stratagene Corporation; La Jolla, CA (Short *et al.*, 1988)
lacZ' CDS, interrupted	5718-5916	*E. coli* β-galactosidase alpha fragment coding sequence, interrupted by Arabidopsis genomic DNA in pAC321; allows blue-white screening for DNA inserts in pBluescript SK(-) multiple cloning site by alpha-complementation
PvuII restriction site	5916	

(2) A detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites

A molecular map of the PvuII DNA fragment containing the *csr1-2* gene cassette that was used in the transformation is shown in **Figure A.3-2**. The molecular map and a table with all genetic elements of plasmid pAC321 are described in **Appendix 1.**

Figure A.3-2. Linear map of the PvuII fragment of pAC321 containing the AHASL 5' UTR, *csr1-2* coding sequence and AHASL 3' UTR that was used for transformation. The restriction sites of the enzymes (NcoI, XbaI, SpeI) and DNA probes used for Southern blot analyses of copy number, absence of backbone and intergenerational stability are indicated.



d) A full molecular characterization of the genetic modification in the new organisms, including

(1) Identification of all transferred genetic material and whether it has undergone any rearrangements

The insert integrity was determined by Southern analysis as well as DNA sequence analysis. The detailed molecular characterization of the insert including a description of the methods employed is presented in **Appendix 1**. The insert in CV127 soybean comprises a single functional copy of the *csr1-2* gene, the majority of At*SEC61γ* subunit gene locus on the 5' end of the *csr1-2* gene, and a 376 base pair duplicated segment of the *csr1-2* coding sequence directly before the 3' integration point. DNA sequence analysis revealed that certain parts of the PvuII transformation fragment are not contained within the transgene insert in CV127 soybean. Deletions of unannotated Arabidopsis genomic DNA (see **Table A.3-1**) occurred both at the 5' end (1274 bp) and 3' end (500 bp) upon the insertion into the soybean genome. An alignment of the

transformation fragment with the DNA insert present in CV127 soybean is shown in **Appendix 1,** Figure 3.

(2) A determination of the number of insertion sites, and the number of copies at each insertion site

The PvuII fragment derived from plasmid pAC321 was integrated at a single locus and as one single copy into the soybean genome. The complete structure of the single insert of 4758 bp in length is presented in **Section A.3.d)(4)** below.

No DNA sequences other than those derived from the PvuII transformation fragment were integrated into the CV127 genome. Southern blot analyses clearly indicated that no elements derived from the backbone of the plasmid pAC321 either linked or unlinked to the insert were detected in the genome of CV127 soybean (**Appendix 1,** Figure 5).

(3) Full DNA sequence data of each insertion event, including junction regions with the host DNA, sufficient to identify any substances expressed as a consequence of the inserted material, or where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food

The complete DNA sequence of the insert in CV127 soybean was determined based on the polymerase chain reaction (PCR) amplification of six overlapping amplicons. The DNA sequence of the insert including a detailed description of the methods employed is presented in **Appendix 1**.

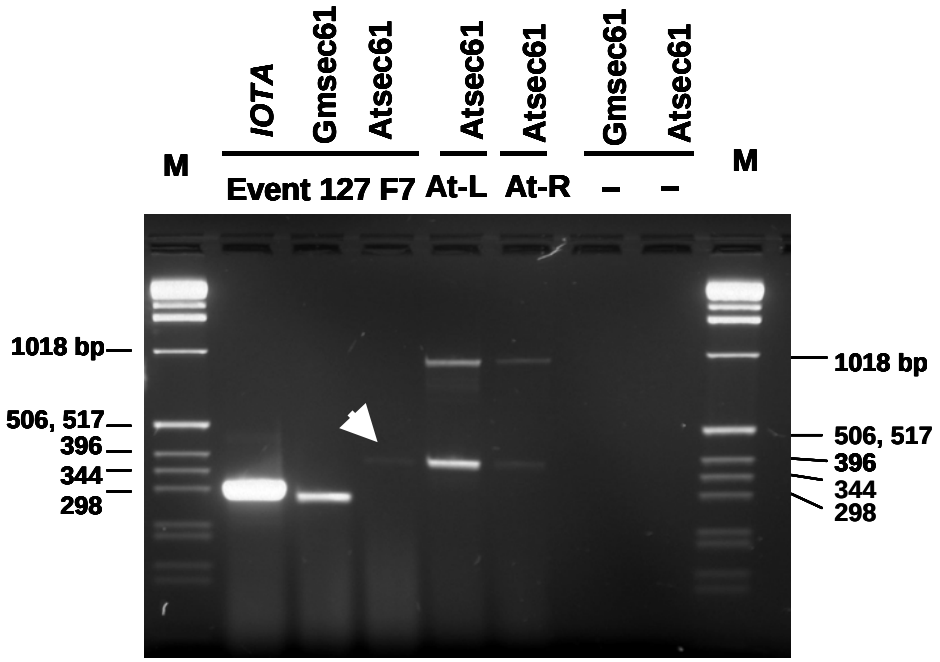
DNA sequence analysis revealed that the *csr1-2* gene cassette contains three point mutations relative to the PvuII linear DNA fragment of pAC321 used to produce CV127 soybean. One of the point mutations is a G to A mutation at position 272 in the *ahasl* coding sequence, which results in an amino acid change from R to K. This is a conservative amino acid substitution and has no impact on the herbicide tolerance or enzymatic properties of the AtAHAS protein as shown in **Section B.2.a)** of this application. The other two mutations are located downstream of the At*AHASL* 3' untranslated region (UTR) and so are genetically silent.

As described above, the complete DNA sequence of the *Arabidopsis thaliana* gene encoding the Arabidopsis SEC61γ (AtSEC61γ) subunit protein resides in the insert of CV127 soybean near the 5' junction with genomic soybean DNA. Although it is assumed that the At*SEC61γ* gene in CV127 soybean lacks its complete native promoter, experiments were performed to evaluate its possible expression. These demonstrated that a very low amount of mRNA is produced from this gene that, and if translated, would encode the native AtSEC61γ subunit protein.

The possible transcription of the At*SEC61γ* subunit gene located in the insert in soybean CV127 was evaluated using RT-PCR. RT-PCR was carried out using DNase-treated total RNA extracted from the F7 generation of soybean CV127 as a template (refer to **Figure A.3-6** for a breeding diagram of CV127 soybean). Primers specific to

two endogenous soybean genes, *Iota* and Gm*SEC61γ*, were used as positive controls to confirm the quality of the template RNA. Total RNA from Arabidopsis leaf and root tissues without DNase treatment was also used as a positive control. Results showed that a very low amount of mRNA specific to the At*SEC61γ* subunit gene in the CV127 F7 generation was present; indicating that the gene is only weakly transcribed (**Figure A.3-3)**. Details on the methods used are described in **Appendix 1**. The 393 bp At*SEC61γ* subunit DNA band amplified by RT-PCR is the same size as that amplified from Arabidopsis leaves and roots (**Figure A.3-3**).

Figure A.3-3. RT-PCR analysis of transcription of the At*SEC61γ* subunit in soybean CV127. Total RNA from CV127 F7 generation plant leaf tissue was extracted with Qiagen RNeasy Mini Kit and treated with DNase and total RNA from Arabidopsis leaf (At-L) and root (At-R) was extracted with TRIzol reagent without DNase treatment. Primers specific to the soybean proteasomal *Iota* subunit gene and the endogenous soybean *SEC61γ* subunit gene (Gm*Sec61γ*) are used in positive control reactions. Reactions without template RNA using At*SEC61γ* subunit-specific primers and Gm*Sec61γ* subunit-specific primers are used as negative controls. M: 1 kb DNA ladder. The arrow indicates the faint RT-PCR product corresponding to the At*SEC61γ* subunit amplified from CV127.



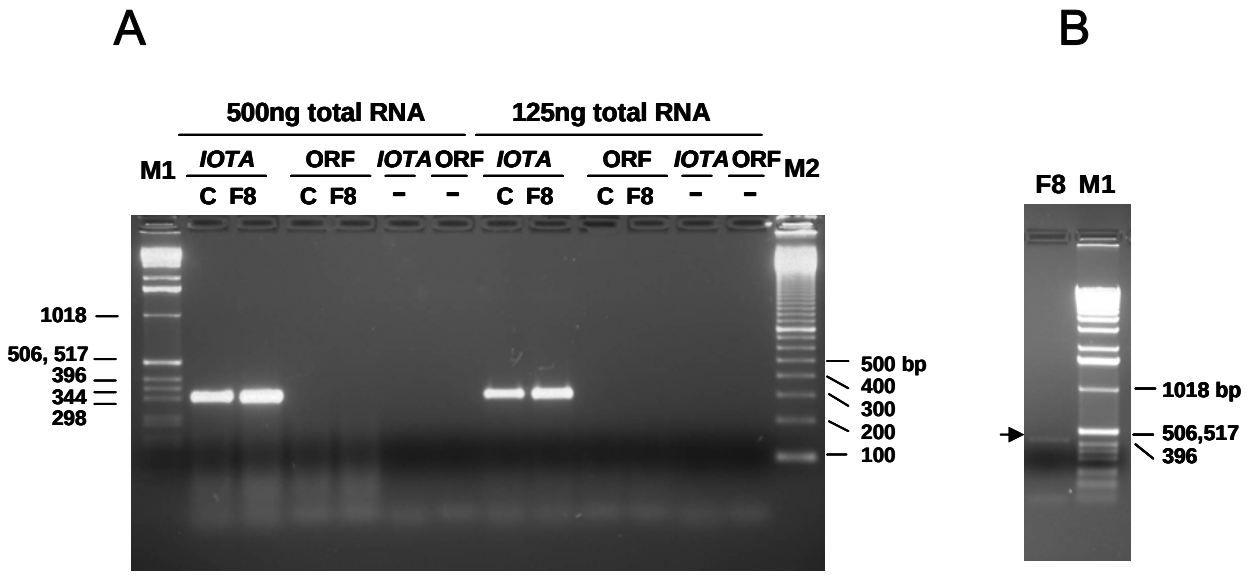
To confirm the identity of the soybean CV127 At*SEC61γ* subunit gene transcript, the 5' end of the Arabidopsis *SEC61γ* subunit transcript in CV127 soybean was determined by RNA-ligase mediated rapid amplification of 5' complementary DNA ends (**Appendix 2**). The resulting PCR product was sequenced and compared with the predicted mRNA sequence of the At*SEC61γ* subunit gene. The sequence data

obtained indicated that the Arabidopsis *SEC61γ* subunit transcript in soybean CV127 is a transcriptional fusion with a short segment (89 nucleotides) of adjacent soybean flanking sequence. However, the first "ATG" start codon in the sequence coincides with the predicted translation initiation site of the native Arabidopsis *SEC61γ* subunit gene. As there are no changes at the nucleotide level, the predicted protein sequence of the Arabidopsis SEC61γ subunit in soybean CV127 does not differ from the native SEC61γ subunit in Arabidopsis.

Based on results of the studies described above, it was concluded that a transcript corresponding to the Arabidopsis *SEC61γ* subunit gene is detected at very low levels in CV127 soybean leaves. Since the transcript was highly amplified by RT-PCR to maximize detection capability, and only a faint band corresponding to the Arabidopsis *SEC61γ* subunit transcript was detected, it was presumed that either no SEC61γ subunit protein is produced in CV127, or if produced, the protein would be present at extremely low levels in the plant.

The insertion of a 376 bp portion of the *csr1-2* coding sequence near the 3' flanking sequence junction as described above created an open reading frame of 501 bp in length. The possible transcription of this ORF was investigated by RT-PCR analysis. RT-PCR was carried out with two different amounts of RNA template, 500 ng and 125 ng. CV127 F8 generation genomic DNA was also used in a positive control reaction with ORF-specific primers. Primers specific for the soybean *Iota* gene were used in positive control reactions to confirm the quality of the template RNA. The ORF-specific primers amplified a 435 bp fragment from CV127 genomic DNA. However, no detectable RT-PCR product was observed using total RNA from young leaf tissue as a template, suggesting that the ORF is not expressed in CV127 soybean (**Figure A.3-4**).

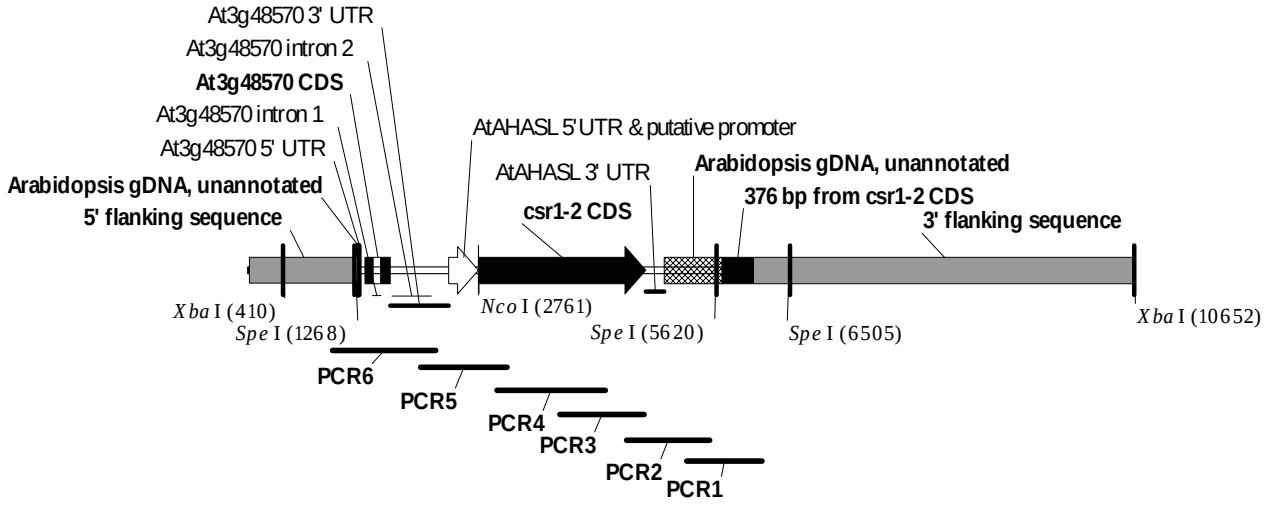
Figure A.3-4. RT-PCR analysis of a 501 bp ORF created by insertion of a 376 bp portion of the *csr1-2* coding sequence at the 3' flanking sequence junction in CV127 soybean. Total RNA was extracted from CV127 F8 generation and non-transgenic Conquista leaf tissues with the Qiagen RNeasy Mini Kit and treated with DNase. Primers specific for the soybean proteasomal subunit *Iota* gene were used in positive control reactions. Reactions without RNA using both *Iota*- and ORF501-specific primers are used as negative controls (panel A). Genomic DNA from the CV127 F8 generation was used as template in a PCR to test the primers for ORF501 (panel B). Of the 25 µl reaction, 5 µl was loaded on the gel (panel B). The band with an expected size of 435 bp is indicated with an arrow in panel B. M1: 1 kb ladder and M2: 100 bp ladder.



(4) A map depicting the organization of the inserted genetic material at each insertion site

The complete structure of the single insert is presented in **Figure A.3-5**. The corresponding detailed molecular characterization of the insert is described in **Appendix 1**.

Figure A.3-5. Diagram of insert and flanking sequence in CV127 soybean. The insert and flanking sequences are displayed. Six amplicons used for the DNA sequence analysis of the insert are also indicated.



(5) The identification and characterization of any unexpected open reading frames within the inserted DNA or created by insertion with contiguous genomic DNA, including those that could result in fusion proteins or unexpected protein expression products

Inverse PCR from genomic CV127 soybean DNA and subsequent sequencing was applied to obtain 3' and 5' soybean flanking genomic sequence (**Appendix 1)**. The sequence of the entire transgene insert, as well as the junctions with the CV127 soybean genomic DNA, were analysed for predicted open reading frames (ORFs) (see **Appendix 3** and **Appendix 4**).

For analysis of the transgene insert of CV127, the bioinformatics searches were conducted start codon-to-stop codon in all three reading frames on both DNA strands (i.e. a six-frame search). Any ORF from any of the six potential reading frames with a deduced amino acid sequence of 29 amino acids or greater (Bannon *et al.*, 2002) which is located either entirely or partly within the transgene insert was selected for further bioinformatics analyses to assess their allergenicity and toxicity potential and to establish that the protein does not share potentially immunologically relevant amino

acid sequence segments or structure with known allergens or sequence homology with known protein toxins. The assessment of potential amino acid sequence homology to known protein allergens followed the guidelines described in the Codex Alimentarius Commission (Codex, 2003) report. 27 ORFs were identified within the CV127 transgene insert. The deduced amino acid sequences of these ORFs identified within the transgene insert of CV127 and the location of these ORFs within the transgene insert are shown in **Appendix 3**. It was confirmed that none of the ORF deduced amino acid sequences in the CV127 soybean transgene insert share immunologically relevant amino acid sequence segments with known allergens or have sequence homology to known protein toxins.

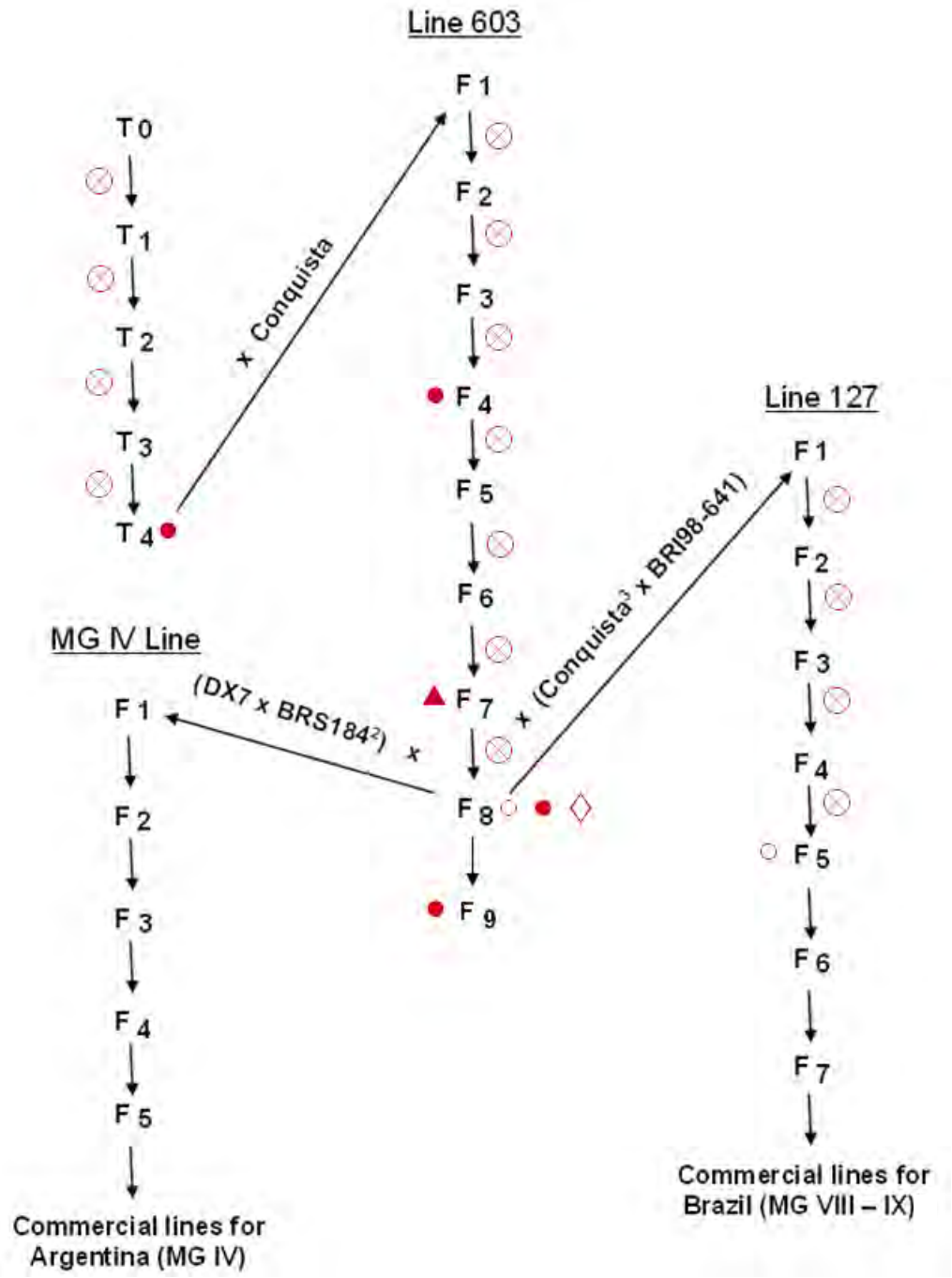
The only ORF with a complete coding region for a known protein is an Arabidopsis gene fragment encoding the gamma subunit of SEC61 (AtSEC61γ). This gene segment upstream of the *csr1-2* coding region was part of the linear DNA fragment used in the transformation and was originally annotated as the AtAHASL promoter and 5' untranslated region. Sequence analysis has revealed that this segment also contains the previously unannotated sequence information for AtSEC61γ. SEC61 is a small 69 amino acid heterotrimeric complex which forms a protein-conducting channel involved in transport across the endoplasmic reticulum membrane in eukaryotes and which is ubiquitous in all plants and other eukaryotes (Hartmann *et al.*, 1994). Two of the three subunits of SEC61, the α and γ subunits, are found in all organisms (Osborne *et al.*, 2005). The sequence was not found to share significant homology to any known allergen or protein toxin sequences. Thus, the bioinformatics analysis does not provide any indication of a potential allergenicity or toxicity concern.

In addition, the junctions between the transgene insert of CV127 soybean and the soybean genome were analysed for potential additional newly created ORFs of at least one amino acid (**Appendix 4**). The search covering the 5' junction produced 24 putative ORFs; the 3' junction produced six putative ORFs. None of the 30 ORF deduced amino acid sequences showed significant homology to a known protein toxin or shared amino acid a potential allergen. Thus, the putative ORFs newly created by the CV127 transgene insertion into the contiguous soybean genomic DNA do not provide any indication of a potential allergenicity or toxicity concern. Further the analysis of the 3' flanking sequence showed that there is a 376 bp duplication of a portion of the *csr1-2* coding sequence directly before the 3' integration point of the insert. The sequence differs from the respective portion of the *csr1-2* coding sequence by only a single nucleotide. The insertion of this duplicated 376 bp segment created an ORF of 501 bp that extends from the transgene insert into the 3' flanking sequence. The possible transcription of this ORF was investigated by RT-PCR, however, no detectable RT-PCR product was observed, suggesting that the ORF is not expressed in CV127 soybean (**Section A.3.d)(3)**).

e) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process)

The herbicide-tolerant CV127 soybean was derived from a single transformation event produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit gene with its native promoter from *Arabidopsis thaliana* into the genome of soybean variety Conquista via biolistics. An initial imidazolinone tolerant transformation event (T0 generation) was advanced to the T4 generation via self-pollination and crossed with Conquista. The resultant progeny (F1 generation) was advanced to the F8 generation by successive rounds of self-pollination and selection and designated the imidazolinone tolerant CV127 line 603. Line 603 was used as a parent for crosses in a breeding program to further the development of varieties containing imidazolinone tolerance. One of these crosses was to a conventional soybean breeding line with the pedigree, Conquista^{3} x BRI98-641. From this cross, individual plants from within the same F3 family were evaluated by PCR to identify those plants that were either homozygous for the *csr1-2* gene (transgene that provides tolerance to imidazolinone) or were null-segregants (did not contain either *csr1-2* alleles). From these selected plants, two F4 seed bulks were created, one bulk for progeny of F3 plants homozygous for *csr1-2* (line CV127) and one bulk for progeny of null F3 plants (near-isogenic control). Each F4 bulk was multiplied in a greenhouse to produce the F5 seed of CV127 line 127 and the near-isogenic control used in the field studies. Line 127 continues to undergo additional cycles of self-pollination and selection as it advances towards commercial cultivation in Brazil and other tropical climates (maturity group [MG] VIII - IX). Another cross was made with a breeding line selected from the cross of DX x BRS184^{2} and resulted in progeny with maturities adapted to the higher latitudes of Argentina (MG IV). The breeding steps are presented schematically in **Figure A.3-6** below.

Figure A.3-6. Breeding history of CV127 soybean. The original transformed event is designated as T0 and the 4th selfed generation as T4. Key molecular and inheritance data was collected at the points in the history as indicated in the legend. Two elite lines are shown. CV127 soybean line 603 was the product of a cross between the T4 generation and the conventional soybean variety Conquista. CV127 soybean line 127 is the product of a cross between CV127 soybean line 603 F8 and a conventional soybean variety closely related to Conquista (Conquista^3 x BRI98-641). Key molecular and inheritance data were collected at the indicated points in the history: ⊗ = self-pollination, ● = intergenerational stability, ▲ = DNA sequence analysis, ◊ = Southern blot analysis for molecular characterization, and ○ = Southern blot analysis



f) Evidence of the stability of the genetic changes

(1) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored

The genetic stability of the transgene insert in CV127 across nine breeding generations was demonstrated by molecular methods and by progeny segregation analysis using traditional breeding methods.

Southern blot analyses were conducted to monitor genetic stability of the insert in CV127 across multiple generations and are presented in **Appendix 1**. Genomic DNA samples from four different generations, T4, F4, F8 and F9 were digested with identical restriction enzymes and hybridized with probes spanning the entire DNA fragment used for transformation. The combination of these restriction enzymes and probes provides a unique fingerprint for the DNA insert in CV127 (**Appendix 1,** Figure 4). Multiple bands from CV127 soybean T4 generation DNA were detected with all three probes, indicating that the T4 generation contains multiple copies of the *csr1-2* cassette. However, DNA from the F4, F8 and F9 generations of CV127 line 603 all showed the same Southern pattern (**Appendix 1**, Figure 6) previously observed in the insert and copy number analyses. This result clearly indicates that the multiple copies of the insert in the T4 generation segregated in the progeny of the cross between T4 and Conquista and that only one single copy is retained in the segregant selected and is stably inherited in subsequent generations.

CV127 line 127 was derived from CV127 line 603 by traditional breeding methods and used to generate the safety data in regulatory field trials in Brazil. The breeding steps are depicted in **Figure A.3-6**. To extend the intergenerational stability data a bridging study demonstrated by Southern blot analysis that the transgene insert in CV127 line 603, from which some commercial varieties of CV127 soybean will be derived, is identical to the transgene insert in CV127 line 127 and thus, the transgene insert is stably inherited between these lines and the regulatory safety data generated from CV127 line 127 is equally applicable to CV127 line 603 (**Appendix 5**).

The integration of one single insert into the nuclear soybean genome was substantiated by segregation studies that are consistent with the molecular characterization data. Determination of the inheritance pattern of the *csr1-2* gene was performed by crossing the F8 generation of CV127 line CV603, homozygous for *csr1-2* gene, with a commercial non-GM line (Conquista^{3}xBRI98-641). The heterozygous F1 progeny was selfed to generate an F2 generation of segregating individuals. The seeds from each individual F2 parent plant were used to plant separate rows in the greenhouse. Each row of F3 plants was considered to be a discrete family and six to eight plants form each family were sprayed with imazapyr to assign the families to three genetic groups, including homozygous null, heterozygous or homozygous for the *csr1-2* gene. Two F3 families (family 12 and family 13) that were found to be segregating for the *csr1-2* gene were selected for further studies. Individuals were analysed by PCR to determine the number of null, heterozygous, and homozygous *csr1-2* plants. The results of the chi-square test as

presented in **Table A.3-2** for the expected 1:2:1 segregation pattern supports the single gene hypothesis for determination of herbicide tolerance, and the herbicide tolerance gene is stably inherited according to the principles of Mendelian genetics.

The F3 plants of the selected families were selfed and seed was planted to generate an F4 population of each family. The F4 plants were spayed with imazapyr and plants were rated as resistant or sensitive to the herbicide. As all the families were derived from plants heterozygous for the *csr1-2* gene, a ratio of three tolerant plants to one sensitive plant was expected within the F4 progeny. The observed ratio was confirmed by the chi-square test (**Table A.3-3**) and supported the expectation the introduced herbicide tolerance trait segregated in normal Mendelian fashion as expected for a single dominant gene. The results are consistent with the conclusions of the molecular characterization studies of CV127 soybean that showed a single functional *csr1-2* gene is integrated in the soybean genome and is stably inherited across breeding generations.

Table A.3-2. Chi-square analysis demonstrating single gene inheritance (1:2:1 segregation) in a Mendelian fashion of the *csr1-2* gene in two F3 families of CV127 soybean.

Reaction	Observed number of plants	Expected number of plants	Chi-square value for 1:2:1 hypothesis
Family 12			
Segregating	30	28.50	
Homozygote Positive	18	14.25	$X^2 = 3.00$
Homozygote Negative	9	14.25	P = 0.22
Total	57	57	
Family 13			
Segregating	23	26	
Homozygote Positive	14	13	$X^2 = 0.73$
Homozygote Negative	15	13	P = 0.69
Total	52	52	

Table A.3-3. Chi-square analysis demonstrating single gene Mendelian inheritance (3:1 segregation) of the *csr1-2* gene in two F4 families of CV127 soybean.

Reaction	Observed number of plants	Expected number of plants	Chi-square value for 3:1 hypothesis
Family 12			
Tolerant	493	508.5	$X^2 = 1.89$
Sensitive	185	169.5	P = 0.17
Total	678	678	
Family 13			
Tolerant	330	340.5	$X^2 = 1.30$
Sensitive	124	113.5	P = 0.26
Total	454	454	

(2) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

In addition, phenotypic stability of CV127 soybeans was demonstrated by investigating the expression of the *csr1-2* gene and the production of the AtAHAS protein in CV127 soybean tissues. Levels of total AHAS in different plant parts of CV127 soybean, including both the AtAHAS and the endogenous soybean AHAS, were measured in two different generations of CV127 (F5 and F6), that were grown in two separate field test seasons (see **Section B.3.c)**) at multiple locations representative of different commercial soybean growing regions in Brazil. Results showed that expression of AHAS in different plant tissues was comparable between the two generations of CV127 thereby confirming the stable integration of the *csr1-2* gene in the soybean genome as well as the phenotypic stability and the stable inheritance of the trait across breeding generations.

4. Information on the labelling of the GM food

Food derived from the genetically modified CV127 soybean that contains novel DNA or novel protein will be labelled according to section 18(1)(b) of the Act. In the following a method is described that allows for the event-specific detection and quantification of CV127 soybean.

The event-specific method for the quantification of CV127 soybean is based on a real-time quantitative TaqMan PCR procedure for the determination of the relative content of CV127 to total soybean DNA in a sample. The protocol was shown to be capable of detecting 0.04 % CV127 soybean DNA in a mixture of non-transgenic soybean DNA. The protocol consists of a method module for DNA extraction from soybeans and a method module for event-specific quantification of CV127 soybean DNA. For quantitative event-specific detection of CV127 soybean two real-time PCR systems are used: a soybean-specific reference PCR system and a PCR system for event-specific detection of CV127 soybean. The soybean-specific reference PCR system is based on the sequences of the soybean *Le1* (lectin) gene. The event-specific real-time PCR system for CV127 soybean is located at the 3' insert-to-genomic soybean DNA junction. The forward primer binding site is located in the CV127 insert, the binding site of the reverse primer is within genomic soybean DNA, and the binding site of the probe spans the junction between the CV127 insert and genomic DNA. A description of development and validation of the DNA extraction method and the event-specific real-time PCR system including a detailed protocol is presented in **Appendix 6**.

B. Information related to the safety of the genetically-modified food

1. Information on antibiotic resistance marker genes (if used)

This section is not applicable to the safety assessment of CV127 soybean since there are no antibiotic resistance marker genes integrated in the genome of CV127 soybean. There was no antibiotic resistance marker gene present in the *csr1-2* gene cassette used to transform soybean to produce CV127. Selection of soybean transformants was achieved by the use of imidazolinone herbicide for which tolerance is encoded by the *csr1-2* gene. The *bla1* gene encoding β-lactamase that provides resistance to ampicillin is included in the vector plasmid as a selectable marker for maintenance in *E. coli*. However, the *bla1* gene is not included in the DNA fragment containing the *csr1-2* gene cassette that was used to transform soybeans to produce CV127 soybean. The DNA transformation fragment was cleaved from plasmid pAC321 with restriction endonucleases and purified by agarose gel electrophoresis prior to transformation, and none of the *bla1* gene and other sequences of the vector plasmid backbone are present in the genome of CV127 soybean. This was confirmed by Southern blot analyses, details of which are presented in **Appendix 1** of this application.

2. The characterization of novel proteins or other novel substances

a) A full description of the biochemical function and phenotypic effects of all novel substances (e.g. protein or an untranslated RNA) that could potentially be expressed in the new GM organisms, including those resulting from the transfer of marker genes

Function of the AHAS protein

Soybean CV127 contains the *csr1-2* gene from *Arabidopsis thaliana* that encodes an imidazolinone-tolerant acetohydroxyacid synthase large subunit enzyme (AHASL, also known as acetolactate synthase) (Sathasivan *et al*., 1990). The AHAS enzyme (EC 2.2.1.6) is ubiquitous in plants and microbes and catalyses the first step in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Stidham and Singh, 1991). The AtAHAS enzyme catalyses the condensation of two molecules of pyruvate to form acetolactate, the precursor of valine and leucine, or the condensation of a molecule of pyruvate with a molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (**Figure A.3-1**), an intermediate in isoleucine biosynthesis (Delfourne *et al*., 1994; Singh and Shaner, 1995; Duggleby and Pang, 2000). AHAS is under feedback regulation by these amino acids in plants. The nuclear encoded AHASL enzyme is targeted to the chloroplast, the cellular location of amino acid synthesis, via an amino-terminal transit peptide of 85 amino acids as presumed by Mazur *et al.* (1987).

The AtAHASL protein encoded by the *csr1-2* gene is structurally and functionally identical to the native AtAHASL, except for its tolerance to imidazolinone herbicides due to a single point mutation that consists of a guanine to adenine change that results in a substitution of a serine with an asparagine at residue 653 (S653N). This amino acid change in plant AHAS proteins is known to prevent the binding of imidazolinone herbicides and thereby to result in tolerance to these herbicides with no effect on feedback regulation by branched-chain amino acids or normal biosynthetic function (Newhouse *et al*., 1992). In addition, the *csr1-2* gene that resides in the genome of CV127 soybean carries a second mutation. This mutation, in which arginine at position 272 is replaced by lysine, does not impact the enzymatic function of the AHAS enzyme or herbicide tolerance properties. A study was conducted to compare the AtAHASL subunit protein expressed in CV127 soybean that contains both the S653N and R272K mutations with the AtAHASL encoded by the *csr1-2* gene that contains only the S653N mutation. This study demonstrated that both AtAHAS enzymes had equivalent levels of catalytic activity and tolerance to imidazolinone herbicides (**Appendix 7**). Feedback regulation of AHAS activity by the branched-chain amino acids is effected through the AHAS small subunit (Chipman *et al*., 2005). The AtAHASL enzyme encoded by the *csr1-2* gene in CV127 soybean interacts with the endogenous soybean AHAS small subunit protein (AHASS) to achieve this regulation. Therefore, the feedback regulation of the AtAHASL encoded by the *csr1-2* gene in CV127 soybean is expected to be identical to that of the endogenous soybean AHAS. This conclusion is supported by results of grain compositional analyses that showed levels of branched-chain amino acids in CV127 soybean are comparable to levels in the control soybean (**Section B.5.** and **Appendix 19**), indicating that regulation of this biosynthetic pathway is equivalent between CV127 soybean and its near-isogenic control. The AtAHAS enzyme expressed in CV127 has the same enzymatic function and substrate specificity as the endogenous soybean AHAS, and only differs with respect to tolerance to imidazolinone herbicides.

Characterization of the AtAHAS Protein Produced in CV127 Soybean

The biochemical characterization of the AtAHAS protein produced in CV127 soybean comprised the molecular weight, the immunoreactivity with AHAS-specific antibodies, the enzymatic activity and the inhibition of the activity by imidazolinone herbicide and feedback inhibition by branched-chain amino acids, the glycosylation, and the determination of the amino acid sequence of several peptide fragments derived from purified AHAS protein preparations. Due to the low expression level of the AHAS protein in CV127 soybean and the difficulty to isolate sufficient quantity of purified protein from the soybean plant, protein characterization and safety studies were conducted with an AtAHAS protein expressed in *E. coli.* The biochemical and immunological equivalence of the AtAHAS protein generated in *E. coli* to the AtAHAS protein expressed in CV127 soybean was determined based on various parameters.

AtAHAS protein was purified from an *E. coli* over-expression system by affinity chromatography followed by precipitation by ammonium sulfate and dialysis to remove salts. The *E. coli*-produced AtAHAS protein was characterized to determine

the identity, functionality, purity, concentration and solubility of the protein (**Appendix 8**) used in several product safety studies including an acute mouse gavage. Several biochemical parameters were used to confirm the identity of the *E. coli*-produced AtAHAS protein. SDS-PAGE analysis of the protein sample revealed a major protein band of molecular weight approximately 64,000 corresponding to the predicted molecular weight of AtAHAS. In addition, western blot analysis using polyclonal antibodies specific for the AtAHAS protein showed a single immunoreactive protein band corresponding to the predicted molecular weight of the AtAHAS protein, confirming immunoreactivity as well as protein identity. Finally, identity of the *E. coli*-produced AtAHAS protein was demonstrated by amino acid sequence analysis of both the N-terminal and internal peptide fragments. The major protein band at approximately 64,000 is AtAHAS since the amino acid sequences obtained from peptide fragments were identical to the corresponding sequences encoded by the expression vector. The functionality of the *E. coli*-produced AtAHAS protein was demonstrated by the enzymatic activity of the protein, which catalysed the production of acetolactate from two moles of pyruvate. The specific enzymatic activity of the protein was 0.790 ± 0.216 units per mg protein. Furthermore, the enzymatic activity showed reduced sensitivity to inhibition by an imidazolinone herbicide which also confirmed the identity of the test substance (**Appendix 8**). The purity and concentration of the AtAHAS protein preparation was assessed using ELISA and protein quantification. The preparation was shown to contain approximately 52.4 % AtAHAS by weight, and corresponded to approximately 90.6 % of the total protein in the preparation. The solubility of the AtAHAS test substance was approximately 10 mg/ml of buffer.

In order to establish the equivalence of the *E. coli-produced* protein to the plant expressed AtAHAS protein the following studies were performed. The summary of the findings of the protein characteristics and the equivalence of both proteins is presented below and a detailed description of the methods and results employed is presented in **Appendix 9**.

AHAS protein was purified from young leaf tissue of CV127 soybean plants and the biochemical characteristics of the protein were assessed and compared to that of the *E. coli*-produced AtAHAS protein. In order to confirm that the AHAS produced in CV127 soybean plants had the predicted molecular weight of AtAHAS (*ca.* 64,000) the protein samples were subjected to SDS gel electrophoresis followed by a western blot analysis. The molecular weight and immunoreactivity of the AHAS enzyme produced in CV127 soybean was shown to be similar to that of the endogenous AHAS from the conventional near-isogenic control, and the reference AtAHAS protein produced in an *E. coli* over-expression system.

The specific activity of the AHAS produced in CV127 soybean was very similar to that of the endogenous soybean AHAS and was higher than the specific activity of the AtAHAS produced in *E. coli.* AHAS is a very unstable protein *in vitro* and loses activity easily upon purification (Chang and Duggleby, 1997). Therefore, it is not unexpected that the AHAS activity in the impure state such as the ammonium sulfate

AHAS-enriched fraction from plant tissues is higher than that retained in the much more pure preparation purified from the *E. coli* expression system. Intermediate sensitivity to inhibition by the imidazolinone herbicide, imazethapyr, was found for the mixture of AHAS proteins (endogenous and transgenic) in extracts of CV127 soybean leaves as compared to that observed for the *E. coli*-produced AtAHAS and the imidazolinone-sensitive AHAS found in the conventional comparator variety Conquista. Feedback inhibition of the AHAS proteins by branched-chain amino acids in CV127 soybean was the same as the inhibition observed in the control soybean, showing that the endogenous small subunit of AHAS interacts with the transgenic AtAHAS protein in CV127 soybean to provide the appropriate feedback regulation. The *E. coli*-produced large subunit AtAHAS protein was not sensitive to branched-chain amino acid feedback inhibition because this protein lacked the small subunit responsible for conferring feedback regulation.

The predicted amino acid sequence of the AtAHAS protein comprises four potential N-glycosylation sites (**Figure B.2-1**). In order to determine if post-translational glycosylation of the AtAHAS as expressed in CV127 soybean occurred, the protein sample was subjected to a glycosylation assay, using transferrin and creatinase as the positive and the negative control, respectively. The protein samples were separated via SDS gel electrophoresis followed by western blot analysis detecting labelled glycoconjugates. No evidence of glycosylation was found associated with the AtAHAS protein in CV127 soybean leaves.

To compare the amino acid sequence of the AtAHAS protein produced in CV127 soybean with that produced in the *E. coli* expression system, the amino acid sequence of the AtAHAS protein of CV127 soybean was investigated. Attempts to obtain N-terminal amino acid sequence using Edman degradation methods were unsuccessful. Therefore, a combination of SDS-PAGE, liquid chromatography coupled with tandem mass spectroscopy (LC/MS/MS) was performed. Peptides derived from trypsin-treated AtAHAS protein that was prepared from CV127 soybean were isolated and their amino acid sequence determined by LC/MS/MS. The amino acid sequence obtained covered approximately 24 % of the entire AtAHAS amino acid sequence. All amino acid sequence data obtained from the AtAHAS of CV127 soybeans was identical to the corresponding amino acid sequence of the *E. coli*-produced AtAHAS with the exception of a region near the N-terminus that is predicted to contain the chloroplast transit peptide (CTP) (**Figure B.2-1**). Since AHAS proteins are present at very low levels in plant tissues, the amino acid sequence of the AtAHAS has not been determined before, and the precise location of the cleavage site for the CTP is unknown. However, based on a sequence comparison of the deduced amino acid sequence of plant and microbial AHAS proteins, Mazur *et al.* (1987) predicted that the CTP of the AtAHAS is cleaved at the C-terminal side of residue 85 (**Figure B.2-1**). This is consistent with the results of N-terminal amino acid sequencing of the mature AHAS from maize that indicated that the CTP of the maize AHAS is cleaved at the same location (B. Singh, BPS, personal communication). Based on this information, the *E. coli* expression system for the production of the AtAHAS protein was designed to yield an AtAHAS protein with the same length that was expected to

be produced in soybean tissues. Therefore, the genetic fragment to be transferred to *E. coli* was designed to express the mature AtAHAS protein lacking the amino acids of the predicted CTP and beginning at the N-terminus with amino acid 86 of the immature form of the enzyme. The presence of sequences matching the CTP-related amino acids in the AtAHAS protein from CV127 soybean indicates that the CTP of the AtAHAS is either partially cleaved or not cleaved in soybean tissues, or that the cleavage site is different in soybeans, or that the CTP cleavage site in the AtAHAS is different in Arabidopsis than in other organisms. Nonetheless, the presence of the CTP amino acid residues on the AtAHAS protein does not have an impact on the biological activity of the AtAHAS in CV127 soybeans since the AtAHAS protein produced in CV127 soybeans has been shown to be biologically active and to have all of the enzymatic characteristics of the native enzyme.

In summary, comparison of the AtAHAS proteins derived from leaf tissues of CV127 soybeans and from the *E. coli* expression system demonstrated that the two proteins have an identical molecular weight, are immunoreactive with AtAHAS-specific antibodies, have identical enzymatic activity that is tolerant to imidazolinone herbicide, and are not glycosylated. The only difference between the two AtAHAS proteins is the presence of some amino acids of the AtAHAS CTP on the N-terminus of the protein from CV127 soybean. These results confirm the substantial biological equivalence of these proteins and justify the use the AtAHAS protein produced by *E. coli* as a surrogate for the AtAHAS from CV127 soybeans in safety studies.

Figure B.2-1. The predicted amino acid sequence of AtAHAS protein showing sequences that were obtained by LC/MS/MS of the AtAHAS protein isolated from CV127 soybean (underlined). The four potential N-glycosylation sites [NX(S, T)X] are indicated by shading. The glutamate (residue 85) at the predicted cleavage site of the CTP is indicated in black shading. The two amino acids that are different from wild type Arabidopsis AHAS are indicated in bold (K^{272}, N^{653}).

```
1   MAAATTTTTT SSSISFSTKP SPSSSKSPLP ISRFSLPFSL NPNKSSSSSR
51  RRGIKSSSPS SISAVLNTTT NVTTTPSPTK PTKPETFISR FAPDQPRKGA
101 DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSSIRNVL PRHEQGGVFA
151 AEGYARSSGK PGICIATSGP GATNLVSGLA DALLDSVPLV AITGQVPRRM
201 IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRIIEEAFF LATSGRPGPV
251 LVDVPKDIQQ QLAIPNWEQA MKLPGYMSRM PKPPEDSHLE QIVRLISESK
301 KPVLYVGGGC LNSSDELGKF VELTGIPVAS TLMGLGSYPC DDELSLHMLG
351 MHGTVYANYA VEHSDLLLAF GVRFDDRVTG KLEAFASRAK IVHIDIDSAE
401 IGKNKTPHVS VCGDVKLALQ GMNKVLENRA EELKLDFGVW RNELNVQKQK
451 FPLSFKTFGE AIPPQYAIKV LDELTDGKAI ISTGVGQHQM WAAQFYNYKK
501 PRQWLSSGGL GAMGFGLPAA IGASVANPDA IVVDIDGDGS FIMNVQELAT
551 IRVENLPVKV LLLNNQHLGM VMQWEDRFYK ANRAHTFLGD PAQEDEIFPN
601 MLLFAAACGI PAARVTKKAD LREAIQTMLD TPGPYLLDVI CPHQEHVLPM
651 IPNGGTFNDV ITEGDGRIKY
```

Function of the SEC61γ protein

The 5' untranslated region (UTR) of the *csr1-2* gene contains a previously unannotated Arabidopsis gene encoding the gamma subunit of SEC61 (AtSEC61γ), a small (69 amino acid) multimeric transport protein of the endoplasmic reticulum that is ubiquitous in all plants and other eukaryotes (Hartmann *et al*., 1994; Stephenson, 2005). Close homology (86 %) exists between the soybean SEC61γ subunit and that from Arabidopsis as presented in the alignment in **Figure B.2-2**.

Figure B.2-2. Alignment of the deduced amino acid sequences of the SEC61γ subunit proteins from soybean (*Glycine max* L.) and *Arabidopsis thaliana*. The nucleotide sequence for *G. max* was obtained from the GenBank nucleotide database and the sequence for *A. thaliana* was taken from the sequence of the gene encoding the SEC61γ subunit protein in CV127 soybeans. Identical amino acids are unshaded, conservative replacements are shaded, and different amino acids are in bold.

```
Soybean      MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT
Arabidopsis  MEAIDSAIDP LRDFAKSSVR LVQRCHKPDR KEFTKVAVRT

Soybean      AIGFVVMGFV KIIFIPINNI IVGSG
Arabidopsis  AIGFVVMGFV KLVFIPINNI IVGSS
```

The CV127 soybean insert sequence contains the majority of the At*SEC61γ* subunit gene including the complete coding sequence. The At*SEC61γ* 5' UTR, as annotated by The Arabidopsis Information Resource (TAIR, 2008), begins 18 nucleotides downstream from the 5' transgene integration site. As such, it is extremely unlikely that the insert contains the complete native promoter for the At*SEC61γ* gene. RT-PCR experiments showed that the At*SEC61γ* subunit gene is only weakly transcribed in CV127 soybean leaf tissue (**Section B.2.b)** and **Appendix 1**).

Characterization of the SEC61γ protein

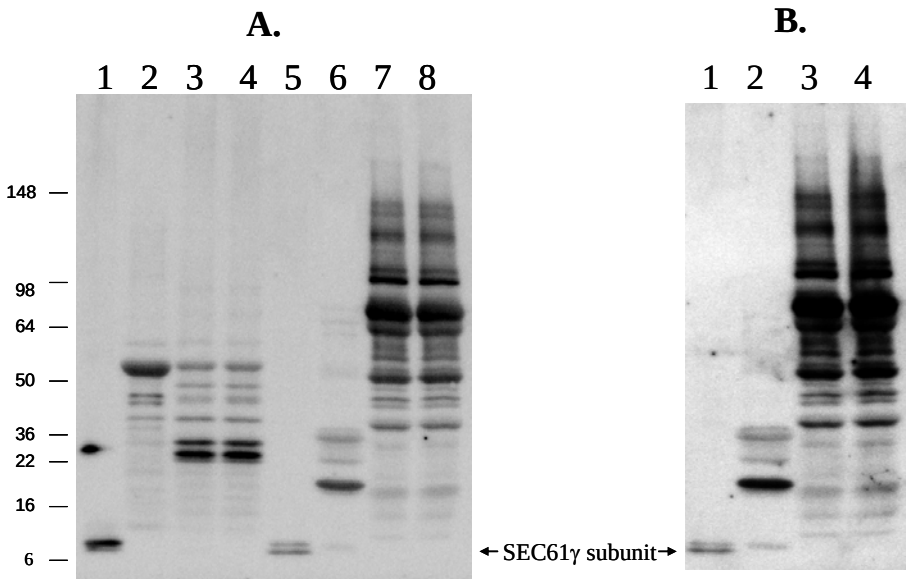
Based on results of the studies described above **(Section A.2.d)**, it was concluded that a transcript corresponding to the Arabidopsis *SEC61γ* subunit gene is detected at very low levels in CV127 leaves. Since the transcript was highly amplified by RT-PCR to maximize detection capability, and only a faint band corresponding to the Arabidopsis *SEC61γ* subunit transcript was detected, it was presumed that either no SEC61γ subunit protein is produced in CV127, or if produced the protein would be present at extremely low levels in the plant. To determine if AtSEC61γ subunit protein is expressed in CV127 soybean tissues, western blot analysis of microsomal membrane protein fractions prepared from leaf and grain samples of CV127 was conducted. Protein samples were derived from leaf and grain of CV127 soybean plants as well as leaf and grain tissues from the parental soybean variety Conquista. Both CV127 soybean and control plants were grown under greenhouse conditions. Arabidopsis leaf and seed tissue were also harvested from greenhouse grown plants. The AtSEC61γ subunit protein used as a standard in these studies was produced and purified from an *E. coli* expression system. An ECL Plus Western Blotting Detection method was used

for protein detection on the western blot. The description of the methods employed for these studies are presented in **Appendix 10**.

The *E. coli*-produced AtSEC61γ subunit protein was readily detected by western blot analysis in the standard protein lanes at a molecular weight of approximately 7,000 (**Figure B.2-3**). Furthermore, expression of the endogenous AtSEC61γ subunit protein in Arabidopsis was detected in microsomal membrane protein preparations from seed but was not detected in Arabidopsis leaf tissue (**Figure B.2-3** lane 6 and lane 2, respectively). This result showed that the AtSEC61γ subunit protein can be detected in tissues of the plant species which served as the donor of the At*SEC61γ* subunit coding sequence introduced in the genome of CV127 soybean, and served as a positive control for this study. In contrast, western blot analysis detected no AtSEC61γ subunit protein in leaf or grain microsomal membrane protein preparations of CV127 (**Figure B.2-3** lane 4 and lane 8). Because of the high amino acid sequence homology of SEC61γ subunit protein across species (e.g., 86% homology between soybean and Arabidopsis) it was expected that the antibody would detect the endogenous soybean SEC61γ subunit protein, however no protein band corresponding to the soybean SEC61γ subunit protein was detected in tissue extracts of the control soybean Conquista. Therefore, AtSEC61γ subunit protein was not detectable at levels greater than 30 and 110 ppb for leaf and grain of CV127 soybean, respectively. This result is not unexpected given the extremely low levels of the transcript corresponding to the At*SEC61γ* subunit gene detected in CV127.

These results demonstrate that no detectable amounts of the AtSEC61γ subunit protein are produced in either leaf tissues or grain of CV127 soybean. However, in the unlikely event that the AtSEC61γ subunit protein is present in tissues of CV127 at extremely low levels, below the limit of detection of the assay, the studies described in **Sections C.3.** and **C.4.** of this application were conducted to confirm the food and feed safety of the AtSEC61γ subunit protein.

Figure B.2-3. SEC61γ subunit protein western blot analysis of leaf and grain microsomal membrane protein preparations from CV127 and Conquista soybeans. Microsomal membrane proteins were prepared from leaf and grain samples and subjected to electrophoresis on an 8 - 16% polyacrylamide gel. Western blot analysis was conducted using rabbit anti-AtSEC61γ subunit polyclonal antibodies and developed using a chemiluminescent substrate. The molecular weight of SEC61γ subunit protein is approximately 7,000. All samples except for the AtSEC61γ subunit protein standard are from microsomal membrane protein preparations. Panel A. Lanes 1, and 5, purified SEC61γ subunit protein standard, 2.4 and 1.2 µg, respectively; lane 2, 20 µg protein from Arabidopsis leaf microsomal membrane protein preparation; lane 3, 20 µg protein from Conquista soybean leaf microsomal membrane protein preparation, lane 4, 20 µg protein from CV127 leaf microsomal membrane protein preparation; lane 6, 15 µg protein from Arabidopsis seed microsomal membrane protein preparation; lane 7, 15 µg protein from Conquista soybean seed microsomal membrane protein preparation and lane 8, 15 µg from CV127 soybean seed microsomal membrane protein preparation. Panel B. Longer exposure of same samples (different blot) from Panel A, lanes 5 - 8.



b) The identification of any other novel substances (e.g. metabolites) that might accumulate on or in the GM organism as a result of the genetic modification, and their levels and site of accumulation

The mechanism of action of imidazolinone herbicides on weeds and non-tolerant plants is by inhibition of AHAS and branched-chain amino acid biosynthesis. AHAS catalyses the first common step in branched-chain amino acid biosynthesis that is specific to plants and microorganisms. The enzyme catalyses the condensation of two molecules of pyruvate to form acetolactate, the precursor of valine and leucine, or the condensation of a molecule of pyruvate with a molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate, an intermediate in isoleucine biosynthesis (Delfourne *et al*., 1994; Singh and Shaner, 1995; Duggleby and Pang, 2000). AHAS is a highly specific anabolic enzyme that is found exclusively in the valine, leucine, isoleucine

amino acid biosynthetic pathway of plants and microorganisms. The regulation of the biosynthesis of branched-chain amino acids is specific, complex and carefully controlled. This regulation involves the presence of multiple isozymes, different mechanisms controlling the expression of the enzymes, allosteric effects on activity such as end product feedback inhibition, and compartmentalization of the biosynthetic pathway in the case of eukaryotes. AHAS is the key control enzyme within the biosynthetic pathway which can be feedback inhibited by the presence of the end product amino acids, valine, leucine and isoleucine. Imidazolinone herbicides such as imazamox, once inside the plant cells, readily inhibit the activity of AHAS (Duggleby and Pang, 2000), resulting in plant death.

CV127 soybean is tolerant to imidazolinone herbicides as a result of introduction of the *csr1-2* gene into the soybean genome, and subsequent expression of the AtAHAS protein in CV127 soybean. The AtAHAS protein expressed in CV127 soybean contains an amino acid modification of the enzyme that confers resistance to inhibition by this class of herbicides. Data presented showed that the AHAS enzyme activity extracted from CV127 soybean was less sensitive to the imidazolinone herbicide when compared to the inhibition curve for the AHAS enzyme extracted from the near-isogenic control. However, AHAS enzyme activity of CV127 soybean exhibited *in vitro* feedback inhibition by valine and leucine that was indistinguishable from feedback sensitivity of the AHAS enzyme activity from the near-isogenic control soybean. Therefore, the AtAHAS enzyme present in CV127 soybean has the same enzymatic activity as the endogenous AHAS enzyme present in soybean and has the same sensitivity to feedback regulation by end-products of the branched-chain amino acid pathway. For this reason, no differences were expected in the branched-chain amino acid biosynthesis pathway between CV127 soybean and the control soybean.

No secondary effects were anticipated and none have been observed or identified. No novel constituents from the intentional modification to CV127 soybean have been identified and none were anticipated.

c) Data on the site of expression of all novel substances, particularly whether they are likely to be present in the edible portions of the organism, and levels of expression

Expression levels of the AHAS protein in different tissues of CV127 soybean plants were determined by enzyme-linked immunosorbent assay (ELISA). Tissues were harvested from plants grown in multi-location replicated field trials in two different growing seasons in Brazil. In addition, grain from CV127 and the control soybean were processed using standard soybean grain processing methods to produce soybean oil, meal, and protein isolate and concentrate fractions, and each processed fraction was analysed by ELISA for AHAS protein expression levels. Results of these studies are presented below.

To assess the level of expression of the AtAHAS enzyme in CV127 soybean, the amount of AHAS protein was determined in tissues of CV127 soybean and the near-isogenic control that were grown in multi-location replicated field trials in two different growing seasons in Brazil. Seven field trials in the 2006/2007 growing season (Season 1) and six in the 2007 short growing season (Season 2) were conducted in geographically distinct locations that were representative of commercial soybean production areas in Brazil. The experimental setup of the expression analysis including trial locations, plot treatment, sampling, and methods is detailed in **Appendices 11** and **12**.

Each trial location had two replications (two separate plots) of the imidazolinone tolerant CV127 soybean and the imidazolinone-sensitive near near-isogenic control soybean. All plots of the CV127 soybean were sprayed with an imidazolinone herbicide. All plots of the imidazolinone-sensitive control were treated with conventional herbicides. In each of the two field trial seasons, leaf and grain samples were collected at all field trial sites at the V2 and R8 growth stages, respectively. In addition, six whole plants per plot, including roots, were collected at two of the trial sites at three different developmental stages, including the V2 (plants 15 - 20 cm tall with three nodes and two unfolded leaflets), R2 (plants in full bloom), and R8 (full maturity) stages. Three whole plants were maintained as such, and the remaining three whole plants were dissected in to plant parts, including leaves, stems, roots, flowers, and pods, depending on the stage of plant development.

Expression levels of the AHAS protein in different tissues of CV127 soybean plants were determined by enzyme-linked immunosorbent assay (ELISA) using AHAS-specific antibodies. Due to the high amino acid homology between the AtAHAS enzyme encoded by the *csr1-2* gene and the endogenous soybean AHAS enzyme, the antibody used in the ELISA assay is not capable of distinguishing between these two enzymes but measures the total AHAS protein in the samples. Therefore, it was expected that tissues from CV127 soybean that are expressing both the AtAHASL protein and the endogenous soybean AHASL protein would have higher levels of total AHASL protein compared to the near-isogenic control that only expresses the endogenous soybean AHAS protein. The difference between the AHAS amount in

CV127 plants and the near-isogenic control is attributed to the expression of the *csr1-2* gene.

The lower limit of quantification (LOQ) and limit of detection (LOD) for AHAS by the ELISA assay was determined experimentally for each tissue type in each experiment. These values are listed in **Table B.2-1**. The LOQ determined for samples analysed from the Seasons 1 and 2 were 13 and 14 ng AHAS/g on a fresh weight basis, respectively. The LOQs and LODs a dry weight basis varied depending on the moisture content of the fresh tissues (**Table B.2-1**).

Table B.2-1. The calculated limit of detection (LOD) and limit of quantification (LOQ) for AHAS protein by ELISA presented for different tissues derived from soybeans during the field trials conducted in Brazil during the 2006/2007 (Season 1) and 2007 (Season 2) growing seasons.

LOQ (ng AHAS/g fw)		Season 1 13		Season 2 14	
Tissue	Growth Stage	LOQ (ng AHAS/g dw)		LOD (ng AHAS/g dw)	
		Season 1	Season 2	Season 1	Season 2
Whole Plants	V2	68	78	16	17
	R2	65	66	15	14
	R8	15	28	3	6
Leaves	V2	70	76	16	16
	R2	62	59	14	13
Roots	V2	41	51	9	11
	R2	38	41	9	9
	R8	17	37	4	8
Flowers	R2	77	83	18	18
Pods	R8	15	17	3	4
Grain	R8	15	15	3	3

AHAS enzyme activity in general is known to be highest in young and growing plant tissues where the need for branched-chain and other amino acids is greatest due to the higher level of *de novo* protein synthesis and declines as tissues mature (Stidham and Singh, 1991). Therefore, AHAS enzyme expressed in tissues of CV127 soybean is expected to follow the same changes in activity throughout the developmental stages of the plant. Indeed the AHAS protein expression results for CV127 soybean and the near-isogenic control from both trial seasons reflect this since the highest levels of AHAS protein were detected in leaves and whole plants at the V2 growth stage (**Table B.2-2** and **B.2-3**). During Season 1 (2006/2007) AHAS protein levels in leaves of CV127 soybean plants from all sites at the V2 stage ranged from 53 to 128 ng/g fresh weight (fw) and 335 to 714 ng/g dry weight (dw), respectively (**Table B.2-2**). During Season 2 (2007) AHAS levels of 18 to 59 ng/g fresh tissue and <LOQ to 254 ng/g dry weight were determined for the leaves of CV127 soybean plants at the V2 stage (**Table B.2-3**). In the near-isogenic control leaf tissues at the V2 stage, the AHAS protein levels on a fresh weight basis at all sites ranged from below the LOQ

(13 ng/g) to 16 ng/g during Season 1 (2006/2007) and were equal to or below the LOQ (14 ng/g) at all locations during Season 2 (2007) (**Table B.2-3**). Expression of AHAS decreased with age of the plant. Tissues from CV127 soybean collected at two locations each in the R2 and R8 growth stages during Season 1 (2006/2007) had low levels of AHAS protein ranging from below the LOQ (13 ng/g fresh weight) to 34 ng/g fresh weight found in whole plants in Season 1 (2006/2007) (**Table B.2-2**). Similar results were determined during Season 2 (2007) with AHAS levels in all tissues tested at both locations mostly below the LOQ.

AHAS protein levels were generally higher at all field locations in CV127 soybean than in the near-isogenic control, which is consistent with the fact that the ELISA assay detects both the AtAHASL protein encoded by the *csr1-2* gene and the endogenous soybean AHAS enzyme (**Tables B.2-2, B.2-3 and B.2-4**). The mutation in the *csr1-2* gene conferring herbicide tolerance does not affect levels of AHAS enzyme in plant tissues.

The expression levels of the AtAHAS enzyme were not only determined in leaves but also in roots, flowers, grain, and whole plants (**Table B.2-2)**. Highest levels of AHAS protein were detected in young leaves, whereas AHAS protein amounts in roots were generally very low and either below or around the LOQ. In roots from both the CV127 and the near-isogenic control plants at the V2 stage AHAS protein levels were below the LOQ on a fresh weight and a dry weight basis, respectively. In roots from plants at the R2 stage AHAS levels of CV127 plants were just above the LOQ (13 ng/g) in one out of two locations in Season 1 (2006/2007) and below the LOQ (14 ng/g) at all locations in Season 2 (2007). Near-isogenic control plants all had AHAS amounts below the LOQ. At the R8 growth stage levels of AHAS enzyme in roots were below the LOQ in Season 1 (2006/2007) both on a fresh weight and a dry weight basis and slightly above the LOQ in Season 2 (2007). Near-isogenic controls showed comparable amounts of AHAS in the latter case. In flowers, AHAS protein levels were in general low and only at one location amounts above the LOQ could be detected in one season. It is noteworthy that in all grain samples AHAS protein levels were at or below the LOQ both in CV127 soybean and the near-isogenic control on a fresh weight and dry weight basis at all locations and from both seasons (**Table B.2-4**).

In summary, expression levels of the AtAHASL protein in CV127 soybean are extremely low, especially at later stages of plant growth and development, however sufficient to confer herbicide tolerance to CV127 soybean plants. Expression levels of the AHAS protein in the grain, the edible portion, of CV127 soybean are at or below the level of quantification.

Table B.2-2. AHAS protein levels on a fresh and dry weight basis in different tissues and growth stages of CV127 soybean and the near-isogenic control. Plants were harvested from plots at Londrina (Lond), Santo Antônio de Posse (SAP), Brasília (Bras), and Santo Antônio de Goiás (SAG) during the 2006/2007 and 2007 growing seasons (Season 1 and Season 2, respectively).*

Growth Stage	Soybean Line	Tissue	Mean ng AHAS/g fw				Mean ng AHAS/g dw[1]			
			Season 1		Season 2		Season 1		Season 2	
		Location	Lond	SAP	Bras	SAG	Lond	SAP	Bras	SAG
V2	CV127	Whole Plant	**61**	**40**	<LOQ	<LOQ	**314**	**214**	<LOQ	<LOQ
		Leaves	**103**	**128**	**27**	**18**	**511**	**714**	**133**	**113**
		Roots	<LOQ[2]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		1st Trifoliate	**55**	**60**	**22**	**21**	**278**	**300**	**111**	**126**
	Isogenic Control	Whole Plant	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		Leaves	**15**	**14**	<LOQ	<LOQ	**78**	**73**	<LOQ	<LOQ
		Roots	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		1st Trifoliate	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
R2	CV127	Whole Plant	ND[3]	**34**	<LOQ	<LOQ	ND	**160**	<LOQ	<LOQ
		Leaves	<LOQ	**24**	<LOQ	<LOQ	<LOQ	**106**	<LOQ	<LOQ
		Roots	<LOQ	**17**	<LOQ	<LOQ	<LOQ	**50**	<LOQ	<LOQ
		Flowers	<LOQ	**22**	<LOQ	-[4]	<LOQ	**125**	<LOQ	-
	Isogenic Control	Whole Plant	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ
		Leaves	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ
		Roots	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		Flowers	<LOQ	<LOQ	<LOQ	-	<LOQ	<LOQ	<LOQ	-
R8	CV127	Whole Plant	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		Leaves	**24**	**26**	<LOQ	<LOQ	**26**	**30**	<LOQ	<LOQ
		Roots	<LOQ	<LOQ	**15**	**17**	<LOQ	<LOQ	**42**	**48**
		Grain	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Isogenic Control	Whole Plant	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		Leaves	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
		Roots	ND	ND	**15**	<LOQ	ND	ND	**41**	<LOQ
		Grain	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

* Whole plants at three different developmental stages (V2, R2, and R8) were collected at two of the seven trial sites in 2006/2007 and two of the six trial sites in 2007. For tissue specific AHAS expression analysis, plants were dissected in to plant parts.

[1] The limit of quantification (LOQ) on a dry weight basis varied for each tissue type and growth stage based on the moisture content of the fresh tissues. Values below the LOQ are reported as less than the LOQ for that tissue and growth stage. LOQ values for different tissues are shown in **Table B.2-1**.

[2] Sample had detectable but non-quantifiable amounts of AHAS below the limit of quantification (13 or 14 ng/g fresh weight in the 2006/2007 and 2007 seasons, respectively).

[3] ND = AHAS was considered non-detectable because the result generated by ELISA was below the limit of detection (LOD) of the method. LOD values for different tissues are shown in **Table B.2-1.**

[4] Not tested due to insufficient amount of flower tissues at this site.

Table B.2-3. AHAS protein levels on a fresh and dry weight basis in trifoliate leaves (V2 stage) of CV127 soybean and the isogenic control cultivated at field locations in Brazil during the 2006/2007 (Season 1) and 2007 (Season 2) growing seasons.

Growth Stage/ Tissue	Location	Soybean Line	Mean ng AHAS/ g fw		Mean ng AHAS/ g dw[1]	
			Season 1	Season 2	Season 1	Season 2
V2 / **Leaves**	Uberaba	CV127	**80**	**59**	**427**	**254**
		Isogenic Control	**16**	<LOQ	**86**	<LOQ
	Sete Lagoas	CV127	**53**	**59**	**335**	**220**
		Isogenic Control	<LOQ[2]	<LOQ	<LOQ	<LOQ
	Sto. Ant. de Goiás	CV127	**61**	**18**	**337**	**113**
		Isogenic Control	<LOQ	<LOQ	<LOQ	<LOQ
	Brasília	CV127	**61**	**27**	**363**	**133**
		Isogenic Control	<LOQ	<LOQ	**<70**	<LOQ
	Ponta Grossa	CV127	**92**	-[3]	**478**	-
		Isogenic Control	<LOQ	-	<LOQ	-
	Teresina	CV127	-	**<14**	-	**<80**
		Isogenic Control	-	<LOQ	-	<LOQ
	Vilhena	CV127	-	**39**	-	**207**
		Isogenic Control	-	**14**	-	**<83**
	Londrina	CV127	**103**	-	**511**	-
		Isogenic Control	**15**	-	**78**	-
	Sto. Ant. de Posse	CV127	**128**	-	**714**	-
		Isogenic Control	**14**	-	**<73**	-

[1] The limit of quantification (LOQ) on a dry weight basis varied for each tissue type and growth stage based on the moisture content of the fresh tissues. Values below the LOQ are reported as less than the LOQ for that tissue and growth stage. LOQ values for different tissues are shown in **Table B.2-1**.
[2] Sample had detectable but non-quantifiable amounts of AHAS. The value is indicated as below the limit of quantification (13 or 14 ng/g fresh weight in the 2006/2007 and 2007 seasons, respectively).
[3] Sample was not tested at this location.

Table B.2-4. AHAS protein levels on a fresh and dry weight basis in grain (R8 stage) of CV127 soybean and the isogenic control cultivated at field locations in Brazil during the 2006/2007 (Season 1) and 2007 (Season 2) growing seasons.

Growth Stage / Tissue	Location	Soybean Line	Mean ng AHAS/ g fw**t**		Mean ng AHAS/ g dw	
			Season 1	Season 2	Season 1	Season 2
R8 / **Grain**	Uberaba	CV127	<LOQ[1]	<LOQ	<LOQ	<LOQ
		Isogenic Control	<LOQ	<LOQ	<LOQ	<LOQ
	Sete Lagoas	CV127	**13**	<LOQ	**14**	<LOQ
		Isogenic Control	<LOQ	<LOQ	<LOQ	<LOQ
	Sto. Ant. de Goiás	CV127	<LOQ	<LOQ	<LOQ	<LOQ
		Isogenic Control	<LOQ	<LOQ	<LOQ	<LOQ
	Brasília	CV127	<LOQ	<LOQ	<LOQ	<LOQ
		Isogenic Control	<LOQ	<LOQ	<LOQ	<LOQ
	Teresina	CV127	-[2]	<LOQ	-	<LOQ
		Isogenic Control	-	<LOQ	-	<LOQ
	Vilhena	CV127	-	<LOQ	-	<LOQ
		Isogenic Control	-	<LOQ	-	<LOQ
	Londrina	CV127	<LOQ	-	<LOQ	-
		Isogenic Control	<LOQ	-	<LOQ	-
	Sto. Ant. de Posse	CV127	<LOQ	-	<LOQ	-
		Isogenic Control	<LOQ	-	<LOQ	-

[1] Sample had detectable but non-quantifiable amounts of AHAS below the limit of quantification (13 or 14 ng/g fresh weight in the 2006/2007 and 2007 seasons, respectively).
[2] Sample was not tested at this location.

d) Information on whether any newly expressed protein has undergone any unexpected post-translational modification in the new host

In order to determine if post-translational glycosylation of the AtAHAS as expressed in CV127 soybean occurred, the protein sample was subjected to a glycosylation assay, using transferrin and creatinase as the positive and the negative control, respectively. The protein samples were separated via SDS gel electrophoresis followed by western blot analysis detecting labelled glycoconjugates. No evidence of glycosylation was found associated with the transgenic AHAS in CV127 soybean leaves. A description of the method and detailed results are presented in **Appendix 9.**

e) Evidence of non-expression of a gene, in the case where a transferred gene is not expected to express any novel substances (e.g. because it has a 'silencing' role or is in a non-functional form)

This point is not applicable, since the trait of imidazolinone tolerance is conferred to CV127 soybean via the transfer the *csr1-2* gene and resultant expression of the AtAHAS enzyme.

f) Information about prior history of human consumption of the novel substances, if any or their similarity to substances previously consumed in food

A history of safe consumption of a protein with no indications of allergenicity or toxicity is a strong indication that the protein is safe for human consumption and is not allergenic or toxic. The AtAHAS encoded by the *csr1-2* gene is highly similar to the native soybean AHASL enzyme and to other AHASL enzymes from common food crops. A comparison of the amino acid sequence of the AtAHASL encoded by the *csr1-2* gene with the deduced amino acid sequence of the endogenous AHASL protein from *G. max* shows that these two proteins share 81.8 percent amino acid identity and, when conservative amino acid replacements are considered, they share 90.2 percent amino acid homology. Similar levels of amino acid identity and homology are present between the AtAHASL encoded by the *csr1-2* gene and the AHASL proteins from Arabidopsis, corn and wheat. The AHAS proteins present in soybean, corn and wheat have a history of safe use in food and feed products, and the AtAHAS protein expressed in CV127 soybean is highly homologous to the AHAS proteins present in these food crops (**Figure B.2-4**).

Several *ahasl* genes encoding AHASL enzymes that are tolerant to imidazolinone herbicides have been discovered in plants as naturally occurring mutations and through the process of chemically-induced mutagenesis. Including the S653N mutation in the *csr1-2* gene, there are five single point mutations in AHAS genes that have been found to result in tolerance to imidazolinone herbicides in plants (Tan *et al.*, 2005). For example, imidazolinone-tolerant maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* and *B. juncea* L.), and sunflower (*Helianthus annuus* L.) were developed through mutagenesis, selection, and conventional breeding technologies and have been commercialized under the Clearfield® brand name since 1992. Therefore, there has been a long history of safe production of crops containing an imidazolinone-tolerant AHAS with the same S653N amino acid substitution as that in the AtAHAS encoded by the *csr1-2* gene that has been used to develop imidazolinone-tolerant CV127 soybeans. In addition, these crops have been used to produce food and feed products that have proven to be as nutritious and as safe as similar products produced from conventional crops.

Figure B.2-4. Alignment of the AHAS large subunit proteins from CV127 soybean and other crop plants. The AHAS large subunit proteins lacking the chloroplast transit peptides (first 85 amino acids in **Figure B.2-1**) were aligned using the Vector NTI version 9.0.0 software. The AHAS proteins are from CV127 soybean (the AtAHAS encoded by the *csr1-2* gene), *Glycine max* (Gmax, BPS database), *Arabidopsis thaliana* (Athal, Accession ABJ80681), *Zea mays* (Zmays, Accession CAA45116), and *Triticum aestivum* (Taes, Accession AAO53549). The consensus sequence for all the AHAS proteins is shown on the bottom line. The S653N mutation in the CV127 soybean AHAS that imparts resistance to the imidazolinone herbicides is indicated at residue 567 (in this sequence alignment) of the mature protein by an asterisk. Amino acids that are identical in all organisms compared are highlighted in light grey, those that are identical in most of the organisms are highlighted in dark grey, and those that are different but conserved compared to the residues from other organisms are indicated in bold.

```
                  1                                                   50
   CV127      (1) FISRFAPDQPRKGADILVEALERQGVETVFAYPGGASMEIHQALTRSSSI
     Gmax     (1) FVSRFASGEPRKGADILVEALERQGVTTVFAYPGGASMEIHQALTRSAAI
    Athal     (1) FISRFAPDQPRKGADILAEALERQGVETVFAYPGGASMEIHQALTRSSSI
    Zmays     (1) PLRPWGPTDPRKGADILVESLERCGVRDVFAYPGGASMEIHQALTRSPVI
     Taes     (1) ALRPWGPSEPRKGADILVEALERCGIVDVFAYPGGASMEIHQALTRSPVI
Consensus     (1) FISRFAP EPRKGADILVEALERQGV TVFAYPGGASMEIHQALTRSSSI
                  51                                                  100
   CV127     (51) RNVLPRHEQGGVFAAEGYARSSGKPGICIATSGPGATNLVSGLADALLDS
     Gmax    (51) RNVLPRHEQGGVFAAEGYARSSGLPGVCIATSGPGATNLVSGLADALMDS
    Athal    (51) RNVLPRHEQGGVFAAEGYARSSGKSGICIATSGPGATNLVSGLADALLDS
    Zmays    (51) ANHLFRHEQGEAFAASGYARSSGRVGVCIATSGPGATNLVSALADALLDS
     Taes    (51) TNHLFRHEQGEAFAASGYARASGRVGVCVATSGPGATNLVSALADALLDS
Consensus    (51) RNVLPRHEQGGVFAAEGYARSSGK GVCIATSGPGATNLVSGLADALLDS
                  101                                                 150
   CV127    (101) VPLVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRIIE
     Gmax   (101) VPVVAITGQVXRRMIGTDAFQETPIVEVSRSITKHNYLILDVDDIPRVVA
    Athal   (101) VPLVAITGQVPRRMIGTDAFQGTPIVEVTRSITKHNYLVMDVEDIPRIIE
    Zmays   (101) VPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVDDIPRVVQ
     Taes   (101) IPMVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVIQ
Consensus   (101) VPLVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRVI
                  151                                                 200
   CV127    (151) EAFFLATSGRPGPVLVDVPKDIQQQLAIPNWEQAMRLPGYMSRMPKPPED
     Gmax   (151) EAFFVATSGRPGPVLIDIPKDVQQQLAVPNWDEPVNLPGYLARLPRPPAE
    Athal   (151) EAFFLATSGRPGPVLVDVPKDIQQQLAIPNWEQAMRLPGYMSRMPKPPED
    Zmays   (151) EAFFLASSGRPGPVLVDIPKDIQQQMAVPVWDKPMSLPGYIARLPKPPAT
     Taes   (151) EAFFLASSGRPGPVLVDIPKDIQQQMAVPVWDTPMSLPGYIARLPKPPST
Consensus   (151) EAFFLATSGRPGPVLVDIPKDIQQQLAVPNWD PM LPGYIARLPKPPAD
                  201                                                 250
   CV127    (201) SHLEQIVRLISESKKPVLYVGGGCLNSSDELGRFVELTGIPVASTLMGLG
     Gmax   (201) AQLEHIVRLIMEAQKPVLYVGGGSLNSSAELRRFVELTGIPVASTLMGLG
    Athal   (201) SHLEQIVRLISESKKPVLYVGGGCLNSSDELGRFVELTGIPVASTLMGLG
    Zmays   (201) ELLEQVLRLVGESRRPVLYVGGGCAASGEELRRFVELTGIPVTTTLMGLG
     Taes   (201) ESLEQVLRLVGESRRPILYVGGGCAASGEELRRFVELTGIPVTTTLMGLG
Consensus   (201) S LEQIVRLI ESKKPVLYVGGGCLNSSDELRRFVELTGIPVASTLMGLG
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```
                251                                                 300
   CV127   (251) SYPCDDELSLHMLGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFA
     Gmax  (251) TFPIGDEYSLQMLGMHGTVYANYAVDNSDLLLAFGVRFDDRVTGKLEAFA
    Athal  (251) SYPCDDELSLHMLGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFA
    Zmays  (251) NFPSDDPLSLRMLGMHGTVYANYAVDKADLLLALGVRFDDRVTGKIEAFA
     Taes  (251) NFPSDDPLSLRMLGMHGTVYANYAVDKADLLLAFGVRFDDRVTGKIEAFA
Consensus  (251) SFP DDELSL MLGMHGTVYANYAVD SDLLLAFGVRFDDRVTGKLEAFA
                301                                                 350
   CV127   (301) SRAKIVHIDIDSAEIGKNKTPHVSVCGDVKLALQGMNKVLENRAEELKLD
     Gmax  (301) SRAKIVHIDIDSAEIGKNKQAHVSVCADLKLALKGINMILEEKGVEGKFD
    Athal  (301) SRAKIVHIDIDSAEIGKNKTPHVSVCGDVKLALQGMNKVLENRAEELKLD
    Zmays  (301) SRAKIVHVDIDPAEIGKNKQPHVSICADVKLALQGMNALLEGSTSKKSFD
     Taes  (301) SRSKIVHIDIDPAEIGKNKQPHVSICADVKLALQGLNALLNGSKAQQGLD
Consensus  (301) SRAKIVHIDIDSAEIGKNKQPHVSVCADVKLALQGMN LLE RA E KLD
                351                                                 400
   CV127   (351) FGVWRNELNVQKQKFPLSFKTFGEAIPPQYAIKVLDELTDGKAIISTGVG
     Gmax  (351) LGGWREEINVQKHKFPLGYKTFQDAISPQHAIEVLDELTNGDAIVSTGVG
    Athal  (351) FGVWRNELNVQKQKFPLSFKTFGEAIPPQHAIKVLDELTDGKAIISTGVG
    Zmays  (351) FGSWNDELDQQKREFPLGYKTSNEEIQPQYAIQVLDELTKGEAIIGTGVG
     Taes  (351) FGPWHKELDQQKREFPLGFKTFGEAIPPQYAIQVLDELTKGEAIIATGVG
Consensus  (351) FG WR ELNVQK KFPLGFKTFGEAIPPQYAI VLDELT GEAIISTGVG
                401                                                 450
   CV127   (401) QHQMWAAQFYNYKKPRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDID
     Gmax  (401) QHQMWAAQFYKYKRPRQWLTSGGLGAMGFGLPAAIGAAVANPGAVVVDID
    Athal  (401) QHQMWAAQFYNYKKPRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDID
    Zmays  (401) QHQMWAAQYYTYKRPRQWLSSAGLGAMGFGLPAAAGASVANPGVTVVDID
     Taes  (401) QHQMWAAQYYTYKRPRQWLSSSGLGAMGFGLPAAAGAAVANPGVTVVDID
Consensus  (401) QHQMWAAQFY YKRPRQWLSSGGLGAMGFGLPAAIGASVANPGAIVVDID
                451                                                 500
   CV127   (451) GDGSFIMNVQELATIRVENLPVKVLLLNNQHLGMVMQWEDRFYKANRAHT
     Gmax  (451) GDGSFIMNVQELATIRVENLPVKILLLNNQHLGMVVQWEDRFYKSNRAHT
    Athal  (451) GDGSFIMNVQELATIRVENLPVKVLLLNNQHLGMVMQWEDRFYKANRAHT
    Zmays  (451) GDGSFLMNVQELAMIRIENLPVKVFVLNNQHLGMVVQWEDRFYKANRAHT
     Taes  (451) GDGSFLMNIQELALIRIENLPVKVMILNNQHLGMVVQWEDRFYKANRAHT
Consensus  (451) GDGSFIMNVQELATIRVENLPVKVLLLNNQHLGMVVQWEDRFYKANRAHT
                501                                                 550
   CV127   (501) FLGDPAQEDEIFPNMLLFAAACGIPAARVTKKADLREAIQTMLDTPGPYL
     Gmax  (501) YLGDPSSESEIFPNMLKFADACGIPAARVTKKEELRAAIQRMLDTPGPYL
    Athal  (501) FLGDPAQEDEIFPNMLLFAAACGIPAARVTKKADLREAIQTMLDTPGPYL
    Zmays  (501) YLGNPENESEIYPDFVTIAKGFNIPAVRVTKKNEVRAAIKKMLETPGPYL
     Taes  (501) YLGNPENESEIYPDFVTIAKGFNVPAVRVTKKSEVTAAIKKMLETPGPYL
Consensus  (501) YLGDPANESEIFPNML FA ACGIPAARVTKKAELRAAIQKMLDTPGPYL
                551             *             584
   CV127   (551) LDVICPHQEHVLPMIPNGGTFNDVITEGDGRIKY
     Gmax  (551) LDVIVPHQEHVLPMIPSNGSFKDVITEGDGRTRY
    Athal  (551) LDVICPHQEHVLPMIPSGGTFNDVITEGDGRIKY
    Zmays  (551) LDIIVPHQEHVLPMIPSGGAFKDMILDGDGRTVY
     Taes  (551) LDIIVPHQEHVLPMIPSGGAFKDMIMEGDGRTSY
Consensus  (551) LDVIVPHQEHVLPMIPSGGTFKDVITEGDGRTKY
```

As outlined before the 5' untranslated region of the *csr1-2* gene contains a previously unannotated Arabidopsis gene encoding the gamma subunit of SEC61 (AtSEC61γ, a small (69 amino acid) multimeric transport protein of the endoplasmic reticulum that is ubiquitous in all plants and other eukaryotes. The SEC61γ subunit, together with the α and β subunits, is part of the protein translocation complex associated with the endoplasmic reticulum and is ubiquitous and highly conserved in eukaryotes as well as being structurally related to analogous proteins in prokaryotes (Hartmann *et al.*, 1994). There is a high degree of amino acid sequence homology (86 %) between the soybean SEC61γ subunit and that from Arabidopsis (see **Figure B.2-5** and **Table B.2-5**). The amino acid sequence of the Arabidopsis SEC61γ subunit protein is 86 % identical and 93 % homologous to the corresponding protein in soybean. Therefore the Arabidopsis SEC61γ subunit protein is highly homologous to the same protein produced in a crop with a history of safe use in food and feed products.

Figure B.2-5. Amino acid sequence comparison of the SEC61γ subunit protein from Arabidopsis with SEC61γ subunit-like proteins encoded by four genes from soybean. Identical amino acids are shaded, conservative replacements are in bold, and different amino acids are unshaded.

```
                1                                                   50
At3g48570/Sec61 MEAIDSAIDP LRDFAKSSVR LVQRCHKPDR KEFTKVAVRT AIGFVVMGFV
   Soy_Sec61g_1 MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61g_2 MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61g_3 MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AMGFVVMGFV
   Soy_Sec61g_4 MDAIDSVFDP LLEFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV

                51                  69
At3g48570/Sec61 GFFVKLVFIP INNIIVGSS
   Soy_Sec61g_1 GFFVKLIFIP INNIIVGSG
   Soy_Sec61g_2 GFFVKIIFIP INNIIVGSG
   Soy_Sec61g_3 GFFVKLIFIP INNIIVGSG
   Soy_Sec61g_4 GFFVKLIFIP INNIIVGSG
```

Table B.2-5. Percent identical amino acids between Arabidopsis SEC61γ and the corresponding soybean protein.

	At3g48570/Sec61	Soy Sec61γ1	Soy Sec61γ2	Soy Sec61γ3	Soy Sec61γ4
At3g48570/Sec61	100	87	86	86	86
Soy Sec61γ1		100	98	98	98
Soy Sec61γ2			100	97	97
Soy Sec61γ3				100	97
Soy Sec61γ4					100

3. The potential toxicity of novel proteins or other novel substances

Imidazolinone herbicide tolerant CV127 soybean plants were produced by introduction of the imidazolinone-tolerant acetohydroxyacid synthase large subunit gene *csr1-2* into the soybean plant genome. The *csr1-2* gene from *A. thaliana* encodes the AtAHAS protein that confers tolerance to imidazolinone herbicides due to a point mutation at amino acid position 653 (Sathasivan *et al.*, 1990). In addition to the *csr1-2* native gene promoter, the 5' region upstream of the *csr1-2* coding sequence contains the complete coding sequence of the *A. thaliana* SEC61γ protein, which is a component of the DNA fragment used for transformation. The At*SEC61γ* 5' UTR begins 18 nucleotides downstream from the 5' transgene integration site. As such, it is extremely unlikely that the insert contains the complete native promoter for the AtSEC61γ gene. RT-PCR experiments showed that the At*SEC61γ* subunit gene is weakly transcribed in CV127 soybean leaf tissue. However, protein expression studies demonstrated that there are no detectable levels of the AtSEC61γ protein present in CV127 soybeans. Based on this molecular characterization of the DNA insert in the CV127 soybean genome, studies were conducted to confirm the absence of toxicity associated with the AtAHAS protein and even though the AtSEC61γ subunit protein was not detected in tissues of CV127 soybean, absence of toxicity associated with the AtSEC61γ subunit protein was assessed for the unlikely event that the protein is present in tissues of CV127 soybean at extremely low levels below the level of detection of the assay.

a) A bioinformatic comparison of the amino acid sequence of each of the novel proteins to known protein toxins and antinutrients (e.g. protease inhibitors, lectins)

In an assessment of potential protein toxicity, it is important to establish that the protein does not share relevant amino acid sequence homology with known protein toxins or anti-nutritional proteins; therefore a bioinformatics analysis was performed. The AtAHAS protein was analysed for sequence homology to known toxins (40 CFR Part 725.421) via a protein-protein Basic Local Alignment Search Tool (BLASTP), (version 2.2.18) analysis of the downloaded March 14, 2008 GenBank non-redundant peptide sequence database (containing a total of 6,319,926 sequences from all non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF, excluding environmental samples). All parameters were left at the default setting for this BLASTP analysis with the exception of the Expect (E) value cut-off which was set to 1. The submitted protein sequence (**Figure B.3-1**) did not show significant homology to a toxin from an organism named in 40 CFR Part 725.421 or other proteins that may be potentially toxic or anti-nutritional to humans or animals. The result of these searches is summarized in **Table B.3-1** and presented in detail in **Appendix 13**.

Figure B.3-1. Submitted sequence for AtAHAS protein.

>*ahasl*(R272K,S653N)
MAAATTTTTTSSSISFSTKPSPSSSKSPLPISRFSLPFSLNPNKSSSSSRRRGIKSSSPSSISAVLNTT
TNVTTTPSPTKPTKPETFISRFAPDQPRKGADILVEALERQGVETVFAYPGGASMEIHQALTRSSSIRN
VLPRHEQGGVFAAEGYARSSGKPGICIATSGPGATNLVSGLADALLDSVPLVAITGQVPRRMIGTDAFQ
ETPIVEVTRSITKHNYLVMDVEDIPRIIEEAFFLATSGRPGPVLVDVPKDIQQQLAIPNWEQAMKLPGY
MSRMPKPPEDSHLEQIVRLISESKKPVLYVGGGCLNSSDELGRFVELTGIPVASTLMGLGSYPCDDELS
LHMLGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKTPHVSVCGD
VKLALQGMNKVLENRAEELKLDFGVWRNELNVQKQKFPLSFKTFGEAIPPQYAIKVLDELTDGKAIIST
GVGQHQMWAAQFYNYKKPRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDIDGDGSFIMNVQELATIR
VENLPVKVLLLNNQHLGMVMQWEDRFYKANRAHTFLGDPAQEDEIFPNMLLFAAACGIPAARVTKKADL
REAIQTMLDTPGPYLLDVICPHQEHVLPMIPNGGTFNDVITEGDGRIKY

Table B.3-1. Summary of toxin search results for AtAHAS

Query Sequence	Sequence Length (aa)	Toxin search
ahasl (R272K, S653N)	670	negative

Even though it has been demonstrated that there are no detectable levels of the AtSEC61γ subunit protein produced in CV127 soybean, a similar bioinformatics assessment of the potential toxicity of this protein was also conducted using the same methodology as was used for the AtAHAS protein above. The AtSEC61γ subunit protein is highly homologous to the SEC61γ subunit protein present in other crop plants and none of these proteins are known to be toxic (**Section B.2.** and **Figure B.2-5**). Therefore, it was expected that no homologies to known toxins or anti-nutritional proteins would be detected. The submitted protein sequence (**Figure B.3-2**) did not show significant homology to a toxin from an organism named in 40 CFR Part 725.421 or other proteins that may potentially be toxic or anti-nutritional to humans or animals. The result of these searches is summarized in **Table B.3-2** and presented in detail in **Appendix 13**.

Figure B.3-2. Submitted sequence for AtSEC61γ subunit protein.

> *SEC61γ*
MEAIDSAIDPLRDFAKSSVRLVQRCHKPDRKEFTKVAVRTAIGFVVMGFVGFFVKLVFIPINNIIVGSS

Table B.3-2. Summary of toxin search results for AtSEC61γ subunit protein.

Query Sequence	Sequence Length (aa)	Toxin search
AtSEC61γ	69	negative

The results of the bioinformatics assessment confirmed that neither AtAHAS protein nor the AtSEC61γ subunit protein share any similarities with known toxins.

b) Information on the stability to heat or processing and/or to degradation in appropriate gastric and intestinal model systems

In the following data are presented in regard to the stability of the AtAHAS protein to heat and processing, whereas data for the stability of the protein to gastric and intestinal fluids is described in **Section B.4**.

Information on the stability of the AtAHAS protein to heat

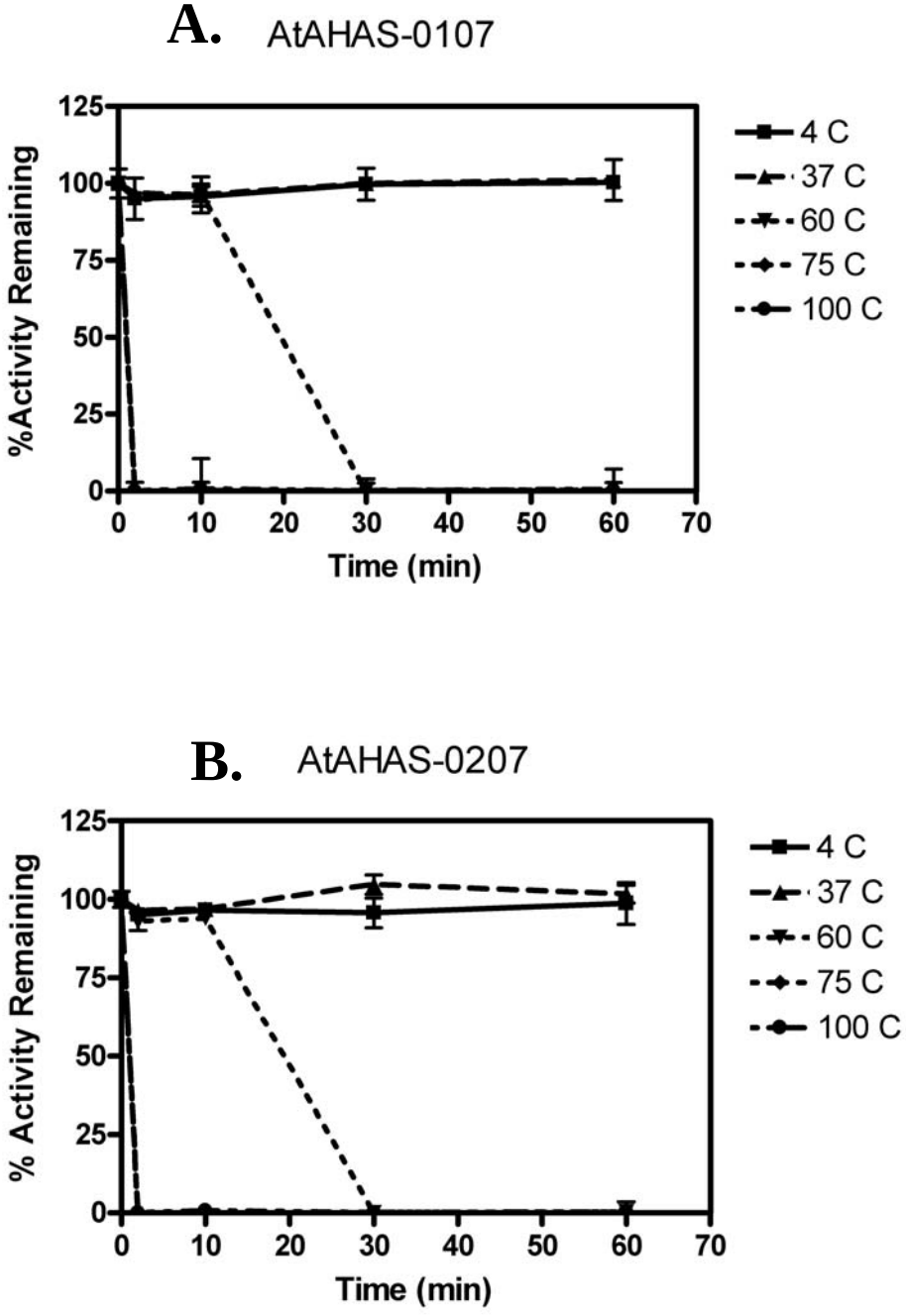
Dietary proteins do not typically represent a hazard and are in fact essential for the growth of most organisms (Delaney *et al.*, 2008). The amino acid sequences of about 2.8 million known polypeptides are available and many of these proteins have been isolated and characterized for their function and structure. Only a very few proteins, the majority of which are from pathogenic bacteria, have toxic properties (Gill, 1987). Compared to the number of bacterial protein toxins, the number of known plant protein toxins and antinutrients is much smaller (Liener, 1994). The toxic properties are related to the specific functional activity or binding activity of the protein that is imparted by its primary and tertiary structure. Upon denaturation or degradation, the protein loses its three dimensional structure and hence its functional activity. Protein denaturation and degradation typically occur during food processing and digestive proteolysis in the mammalian digestive tract, respectively. Therefore, there are examples of proteins that are safely consumed by humans and other mammals because they are readily inactivated by heat during processing or cooking, or are degraded by digestive proteolysis.

In a study to confirm that the enzymatic activity of the AtAHAS protein is sensitive to heat treatment, it was shown that like most non-thermostable enzymes, AtAHAS enzymatic activity is rapidly inactivated at temperatures above 60℃. The complete study is presented in **Appendix 14**. The test substance used for the assessment of the heat stability was the *E. coli*-produced protein, which had been shown to be chemically and functionally similar to the AtAHAS produced in CV127 soybeans. For the characterization of the test substance refer to **Appendix 8**.

The thermal stability of the imidazolinone-tolerant AtAHAS protein containing the R272K and S653N mutations was compared to that of the wildtype AtAHAS protein that lacks these two mutations. The heat stability of AtAHAS gives an indication of the fate of the protein during processing of the soybean grain. Specifically, the enzymatic activity of the AHAS proteins was monitored during incubation of a solution of the protein preparations at various temperatures over a period of 60 minutes (see method described in **Appendix 14**). Samples of both proteins were incubated at 4, 37, 60, 75 and 100°C for 2, 10, 30 and 60 minutes. The AHAS activity of both AtAHAS proteins was found to be stable at both 4 and 37°C over one hour of incubation **(Figure B.3-3**). However at temperatures higher than 37°C, both AtAHAS proteins were found to be unstable. At 60°C, activity had decreased to zero by 30 minutes and within two minutes at both 75 and 100°C. These results indicate that like most non-thermostable enzymes, AHAS activity is rapidly inactivated at temperatures

above 60°C. Therefore activity of the imidazolinone-tolerant AtAHAS protein shows the same sensitivity to heat treatment as the wildtype AHAS as well as other conventional dietary proteins with a history safe consumption in food products. Since the production of soybean processed food and feed products involves heat treatments greater than 60°C, the AtAHAS protein will be functionally inactive in food and feed products derived from processed soybean, and this further confirms that the protein does not pose any risk of toxicity in soybean food and feed products.

Figure B.3-3. Percent AHAS activity remaining as a function of time at different incubation temperatures. Results for both the imidazolinone-tolerant (Panel A, R272K, S653N) and wildtype AtAHAS proteins (Panel B) are shown.



Information on the stability of the AtAHAS protein to processing

As will be described in **Section B.5.a)**, the grain of CV127 soybean and the near-isogenic control variety was processed into fractions using standard methodology for the processing of soybean grain. The processed fractions thus produced included refined oil, toasted and untoasted defatted soybean meal, and protein isolate and concentrate. Each of these processed fractions, except the untoasted defatted soybean meal was analysed to determine the amount of AHAS protein in each of the processed fractions by an AHAS-specific ELISA assay. This assay is described in **Appendix 15** and is the same one used to determine the AHAS levels present in soybean grain and tissues (see **Appendices 11** and **12**). The LOD, LOQ and spike and recovery validation parameters as well as the amount of AHAS detected in the processed fractions from CV127 soybean and the near-isogenic control are presented in **Table B.3-3**. The results of these analyses demonstrate that the AHAS protein, including both the AtAHAS encoded by the *csr1-2* gene and the endogenous soybean AHAS, could not be detected in any of the processed fractions produced from the grain of CV127 soybean. This result was as expected given the instability of the AHAS protein to heat treatment (see **Figure B.3-3**) the protein is not expected to survive the heat treatments involved in producing the processed fractions. For the untoasted defatted soybean meal produced from the grain of the near-isogenic control variety, AHAS was detectable but not quantifiable (LOQ of 13 ng per g fresh weight). In all other processed fractions produced from the near-isogenic control, no AHAS protein was detected. It should be noted that the spike and recovery of AtAHAS from refined oil could not be determined due to an inability to recover the AHAS protein that was spiked into the oil. However, soybean oil typically contains very little, if any, protein and this was confirmed by measurements of total protein in the refined oil fractions that gave values ranging from 1.9 - 4.0 μg per gram oil.

The results of these analyses of the processed fractions for the AHAS protein demonstrate that processing reduces the levels of AHAS protein from the very low levels that are detectable but not quantifiable in soybean grain. These findings are consistent with the conclusions of other studies that compare CV127 soybeans with the near-isogenic control and conventional control varieties that demonstrate that the imidazolinone-tolerance trait of CV127 soybean has no significant impact on the agronomic characteristics, nutrient composition, and other aspects related to the safe use of CV127 soybean for food, feed and other uses.

Table B.3-3.Assay validation parameters and AHAS levels in processed soybean fractions produced from CV127 soybean and the near-isogenic control as determined by ELISA.

Fraction	LOD (ng AHAS/ g dw)	LOQ (ng AHAS/ g dw)	Spike and Recovery Efficiency % ± C.V.	AHAS (ng/g dw)	
				CV127 (N = 4)	Isogenic Control (N = 4)
Defatted Toasted Meal	6	8	75 ± 5	ND[†]	ND
Defatted Untoasted Meal	6	13	88 ± 15	ND	ND - <13
Protein Isolate	6	24	79 ± 9	ND	ND
Protein Concentrate	9	24	79 ± 8	ND	ND
Oil	*	*	*	ND	ND
Grain	3	15	88 ± 7	<14	<14

[†]Not detected

*Extraction efficiency experiments using AtAHAS protein were unsuccessful;, however, recovery of bovine serum albumin (BSA) protein was possible with an extraction efficiency ranging between 2 – 24 % for 10 ppm and 37 – 62 % for 100 ppm.

c) Detailed reports of all available acute or short term oral toxicity studies in animals on the novel proteins or other novel substances

The imidazolinone-tolerant AtAHAS protein that is responsible for the imidazolinone-tolerance trait of CV127 soybean is not expected to be toxic for several reasons. First, the AtAHAS protein is highly homologous to, and has the same biological activity and mode of action as, the AHAS proteins that are ubiquitous in all plants (**Section B.2.a)**). Second, the AtAHAS protein is not present at levels that can be detected in the grain of CV127 soybeans (**Section B.2.c)**). Third, the AtAHAS protein produced in CV127 soybean contains the same S653N mutation that is present in several imidazolinone-tolerant Clearfield® crops that have been cultivated and consumed for many years without any adverse health effects (**Section B.2.a)**). Fourth, the AtAHAS protein has been shown to be rapidly digested in a simulated digestive environment (**Section B.4.c)**). Finally, a bioinformatics assessment of the AtAHAS protein demonstrated that it has no similarities to known protein toxins. As supplemental evidence to confirm the absence of oral toxicity of the AtAHAS protein, it was tested by acute oral administration in mice. A detailed description of the study including methods and results is presented in **Appendix 16.**

The purified AtAHAS protein produced in an *E. coli* expression system (see **Appendix 8** for production and characterization of the test substance) was administered by gavage to groups of five male and five female CD®-1 mice at a dose of 5000 mg/kg body weight per day (corresponding to 2620 mg AtAHAS protein/kg body weight). The dose was given in two equal doses of 2500 mg per kg body weight in an interval of approximately one hour apart. This dose represents the highest feasible dose based on the test system capacity and solubility of the test protein. Vehicle control animals received drinking water containing 0.5 % carboxy-methylcellulose by gavage. After the test substance administration, the animals were maintained over a post observation period of 14 days. The animals were examined for any abnormal clinical signs before the first test substance administration and at regular intervals from 15 minutes to 6 hours thereafter. Subsequently, the animals were observed daily for any abnormal clinical signs. Body weights were determined on the day of the test substance administration (day 0) and on study days 7 and 14. The animals were sacrificed at the end of the study under anaesthesia and assessed by gross pathology. Selected organ weights of all sacrificed animals were determined.

None of the animals died during the study period due to treatment of mice with the test substance, and none of the animals showed any clinical signs of toxicity, and there were no significant differences in body weight of the animals treated with the AtAHAS protein compared to the control group. There were no relevant differences in mean absolute weights of the selected different organs between the test and control groups. Compared to controls, male mice treated with the AtAHAS protein had a statistically significant increase of 6 % in relative liver weights. However, this finding was not considered treatment-related since absolute liver weights were not changed in the treated group compared to the control group and no effect was observed in the female mice. Also the relative liver weights across both treatments and sex were considered to be within the normal biological range of test animals of that age. For all other organs there were no relevant differences in mean relative weight parameters between the treatment and control groups. Also, no treatment related gross lesions on animals were observed in this study. Therefore it is concluded that no test substance-related findings were noted.

Since none of the animals died during the study period due to administration of the test substance, a median lethal dose (LD_{50}) was not achieved. Thus, the LD_{50} can be considered as being higher than 5000 mg/kg body weight (or higher than 2620 mg AtAHAS protein/kg body weight) for male and female CD®-1 mice. In addition, no test substance-related findings were noted. As such, the no observed effect level (NOEL) was the limit dose of 5000 mg/kg body weight (2620 mg AtAHAS protein/kg body weight) for male and female CD-1® mice. These results confirm the nontoxic nature of the AtAHAS protein in mammals.

Conclusion of the toxicity assessment

The conclusions of the toxicological assessment indicate that the food and feed derived from CV127 soybean is as safe and as nutritious as its commercial comparator.

Absence of toxicity associated with the AtAHAS protein expressed in CV127 soybean was demonstrated by the following: *Arabidopsis thaliana*, the source organism of the *csr1-2* gene, is not known to produce toxic substances and is not a known human or animal pathogen. There is a long history of safe human consumption of AHAS proteins in plants. The AtAHASL protein encoded by the *csr1-2* gene is highly similar to the native soybean AHASL enzyme and is structurally and biologically closely related to the AHASL proteins in all plants. Several imidazolinone-tolerant crops that produce AHASL enzymes with the same serine to asparagine substitution at residue 653 that is present in CV127 soybeans have been commercialized and cultivated under the Clearfield® brand name for many years without any adverse environmental or health effects. The AtAHAS protein does not share any amino acid sequence homology with known toxins. The AtAHAS protein is rapidly inactivated by temperatures above 60℃ and is rapidly degraded in simulated digestive fluids. An acute oral toxicity study was conducted with purified AtAHAS protein and the results demonstrated no adverse effects in mice, which confirmed the non-toxic nature of the AtAHAS protein in mammals.

4. The potential allergenicity of novel proteins

a) Source of the introduced protein

There are no definitive methods to assess potential allergenicity of proteins originating from sources not known to produce food allergy. However, there are some recognized procedures that can be used to evaluate a new protein to assess its allergenic potential. Based on a weight of evidence approach the source of the protein, its similarity to known allergens as well as its resistance to heat and digestion are considered in the assessment of potential allergenicity.

The AtAHAS protein as well as the AtSEC61γ subunit protein were derived from *Arabidopsis thaliana* (L.) Heynh. which is a species of the Arabidopsis genus and of the Brassicaceae family. *Arabidopsis thaliana* is not known to be allergenic to humans. However, since it is not a typical food, there is not a history of human consumption of the plant. But, it has been handled for many years in research with no known allergenicity issues. As such, Arabidopsis does not warrant further analytical tests and presents no concerns as a source of allergens. Both proteins are ubiquitous in plants and are not known to be allergenic.

The AHAS enzyme catalyses the first step in the biosynthesis of branched-chain amino acids, valine, leucine, and isoleucine (**Figure A.3-1**). Induced as well as acquired mutations have been known to confer tolerance to imidazolinone herbicides in crop plants for a long time (Chang and Duggleby, 1998). A history of the safe consumption of a protein with no indications of allergenicity is a strong indication that the protein is safe for human consumption and is not allergenic. The AtAHASL protein is structurally and biologically closely related to the AHASL proteins in all plants. For example, there is 81.8 % amino acid identity between the AtAHASL and the endogenous soybean AHASL proteins (**Figure B.2-4**) and there is a long history

of safe human consumption of AHASL proteins in plants. Furthermore, the safety of several imidazolinone-tolerant crops that produce AHASL enzymes with the same serine to asparagine substitution at residue 653 that is present in CV127 soybeans has been assessed by the Canadian Food Inspection Agency. These crops which include maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and oilseed rape (*Brassica napus* L.) have been commercialized and cultivated under the Clearfield® brand name for many years without any adverse health effects. Until 2008, Clearfield crops have been authorized in several countries worldwide including Australia, Argentina, Brazil, Canada, the U.S., and many others.

The AtSEC61γ subunit protein forms part of the SEC61 protein which is a heterotrimeric complex forming a protein-conducting channel involved in transport across the endoplasmic reticulum membrane in eukaryotes. The γ subunit as well as the α subunit are found in all organisms (Osborne *et al*., 2005), therefore the protein has a history of safe consumption and no indication of allergenicity, which is a strong indication that the protein, if it were expressed, is safe for human consumption and not allergenic.

b) Any significant similarity between the amino acid sequence of the protein and that of known allergens

In an assessment of potential protein allergenicity, it is important to establish that the protein does not share potentially immunologically relevant amino acid sequence segments or structure with known allergens. The amino acid sequence of the AtAHAS protein (**Figure B.4-1**) was compared *in silico* to all proteins in the Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (version 8.00) containing 1313 entries via a protein-protein FASTA (version 3.4) analysis. The default parameters of the FASTA program were used with two exceptions: the threshold score for optimization was set to 20 and only the best alignment was shown. In this analysis, any protein showing 35 % or greater identity over 80 amino acids to a known allergen would be identified. The amino acid sequences thus analysed did not show 35 % or greater identity over 80 amino acids to any potential allergen. The amino acid sequence of the AtAHAS proteins was also submitted to a second analysis using a custom program which provides an exhaustive search of all possible eight-amino acid segments of the query protein against all possible eight-amino acid segments in proteins in the FARRP Allergen Protein Database. Regions of at least eight consecutive amino acids which are identical between a submitted protein and a known allergen are identified by this search. The AtAHAS protein sequence was found not to share a sequence of eight or more consecutive identical amino acids with any potential allergen. Proteins that are analysed by these techniques and that are found to contain 35 % or greater identity over any contiguous 80 amino acid sequence or to contain an identical sequence of eight or more consecutive amino acids with a known allergen are considered to be potentially allergenic and would require a more detailed assessment for allergenicity. However, in the case of the AtAHAS protein the results of these bioinformatic analyses (see **Appendix 13**) did not show 35 % identity over 80 amino acids nor did AtAHAS contain a an identical sequence of eight amino

acids with known allergens (**Table B.4-1**). Thus this constitutes further support for the conclusion that the AtAHAS protein is not allergenic.

Table B.4-1.Summary of allergen search results for AtAHAS (R272K, S653N).

Query Sequence	Sequence Length (aa)	Eight-mer Search	35 % Identity over 80 aa
AtAHAS	670	negative	negative

The AtAHAS amino acid sequence that was used in this search is presented in **Figure B.3-1**.

Even though it has been demonstrated that no detectable levels of the AtSEC61γ subunit protein are produced in CV127 soybean, a similar bioinformatics assessment of the potential allergenicity of this protein was also conducted using the same methodology as was used for the AtAHAS protein. The AtSEC61γ subunit protein is highly homologous to the SEC61γ subunit protein present in other crop plants and none of these proteins are known to be allergens (**Section B.2.** and **Figure B.2-2)**. Therefore, it was expected that no homologies to known allergens would be detected.

Table B.4-2. Summary of allergen search results for AtSEC61γ subunit protein.

Query Sequence	Sequence Length (aa)	Eight-mer Search	35 % Identity over 80 aa
AtSEC61γ	69	negative	negative

The AtSEC61γ subunit protein amino acid sequence used in this search is presented in **Figure B.3-2**.

Therefore, bioinformatic searches of databases of all known proteins as well as of known allergenic proteins were conducted and it was confirmed that the AtSEC61γ subunit protein does not share immunologically relevant amino acid sequence segments or structure with known allergens (**Table B.4-2**). The methods and results of the bioinformatics assessment are described in **Appendix 13**.

c) Its structural properties, including but not limited to, its susceptibility to enzymatic degradation (e.g. digestion by pepsin), heat stability and/or, acid and enzymatic treatment

Large quantities of proteins from diverse sources are consumed daily by humans and are required for a well balanced and healthy diet. The instance of allergenicity among the tens of thousands of different proteins consumed by humans is rare but there are no accurate predictive assays available to assess the allergenic potential of new proteins (Taylor, 1992). However, there are some recognized procedures that can be used to evaluate a new protein to assess its allergenic potential. Amongst those are examining the biochemical characteristics of the protein including resistance to digestive degradation and heat stability. Most conventional dietary proteins with a history of safe use in food and feed products are denatured by heat treatment during cooking or processing of the raw agricultural commodity to produce food and feed

products. However, there are examples of major protein allergens that remain stable to processing to food products (Metcalfe *et al.*, 1996). Therefore, heat stability is one of the criteria used in a weight of evidence approach to assess potential allergenicity of protein. It was demonstrated that the activity of the imidazolinone-tolerant AtAHAS protein shows the same sensitivity to heat treatment as the wildtype AtAHAS as well as other conventional dietary proteins with a history of safe consumption in food products (see **Section B.3.b)**). This further confirms that the protein does not pose any risk of allergenicity in soybean food and feed products.

Digestive Fate of the AtAHAS Protein in Simulated Gastric and Intestinal Fluids
A correlation has been established between allergenic proteins and their stability to digestive processes (Fuchs and Astwood, 1996; Kimber *et al.*, 1999). It is believed that protein allergens tend to be more resistant to digestion by the acidic conditions and pepsin present in the stomach, thereby increasing the probability that they will reach the intestinal mucosa more or less intact where an immune response can be initiated. For a food protein to elicit an allergenic response, the protein must survive the proteolytic environment of the gastrointestinal tract and be absorbed by the intestinal mucosa and initiate an IgE-mediated series of responses.

Therefore, a study was conducted to demonstrate that the AtAHAS protein has the same susceptibility to digestion in simulated mammalian digestive fluids as other dietary proteins with a history of safe use in foods. The methods used in this study to determine digestive fate of the AtAHAS protein in simulated gastric (SGF) and intestinal fluids (SIF) were in accordance with procedures described in the United States Pharmacopoeia (2000). The methods used in this study are presented in detail in **Appendix 17**.

The lack of potential allergenicity of the AtAHAS protein was confirmed by showing that the protein is rapidly digested in simulated mammalian gastric fluid (SGF) as well as intestinal fluids (SIF), similar to conventional dietary proteins in food products. AtAHAS protein derived from an *E. coli* expression system (chemically and functionally similar to the AtAHAS protein produced in CV127 soybean) and from CV127 soybean grain and leaf tissues were assessed in these test systems in order to determine whether the plant matrix has an effect on the digestibility and to confirm that the microbially-produced and the plant-produced AtAHAS proteins have a similar digestive fate. The AtAHAS proteins were incubated in SGF with a buffered pH of 1.2 and pepsin at 37°C and aliquots were removed at 0.5, 2, 5, 10, 30 and 60 min and the reactions were stopped by heating at 75°C for 10 min. Similarly, samples of the AtAHAS proteins were incubated in SIF containing a buffer at pH 7.5 and the proteolytic enzyme pancreatin and aliquots were removed at 0.5, 2, 5, 10, 30, and 60 minutes and the reactions were stopped and heated at 75°C for 10 min. To monitor the integrity of the AtAHAS proteins (*ca*. 64,000 mol. wt.) after incubation in the various digestion mixtures, aliquots of the quenched and heated mixes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resulting protein bands were visualized by Coomassie blue staining and western blotting. In addition, reactions that served as controls for pepsin and pancreatin auto-

digestion and test protein stability were prepared containing SGF without test protein and SGF with test protein but without pepsin (G-con), respectively. Similarly, reactions that served as controls for pancreatin auto-digestion and test protein stability were prepared containing SIF without test protein and SIF with test protein but without pancreatin (I-con), respectively. These control reactions were treated exactly as described above except samples were only taken at 0 and 60 minutes after the start of the 37°C incubation.

The microbially produced AtAHAS protein was rapidly degraded in SGF (**Figure B.4-3A**). AtAHAS (molecular weight *ca*. 64,000) is readily detected in the time 0 sample and the G-con samples. However, no full-length protein is visible in the 0.5 min sample. Some low molecular weight staining bands (<6000) were observed at 0.5 min but were degraded by 2 min. There is a low molecular weight band at approx. 6000 that is also visible in the SGF only lane and hence must be associated with the pepsin preparation.

The microbially produced AtAHAS protein was also rapidly degraded in SIF (**Figure B.4-4A**). Again, AtAHAS is detected in the time 0 sample and the I-con samples. However, no full-length protein is visible in the 0.5 min sample. Some degradation of the AtAHAS is observed in the 60 min I-con sample indicating protein sensitivity to incubation at 60 min in simulated intestinal fluid in the absence of pancreatin. Pancreatin is comprised of multiple proteases and lipases and this accounts for the multiple staining bands in the SIF lanes. All of the lower molecular weight bands visible in the digestion time course correspond to those present in the SIF only control sample (without the AtAHAS protein). The results of AtAHAS digestion in SIF using western blot analysis (**Figure B.4-3A**) confirm that no immunoreactive degradation products of AtAHAS were hidden by bands associated with the pancreatin, and confirm that the AtAHAS protein is rapidly degraded in SIF within 0.5 min incubation time.

The AHAS protein produced in CV127 soybean leaves and grain is a mixture of the endogenously encoded soybean protein as well as the AtAHAS transgenic protein encoded by the introduced imidazolinone-tolerant *csr1-2* gene. These two proteins are immunologically indistinguishable. Both are rapidly digested in SGF and although the AHAS protein band is visible at time 0, no AHAS protein band is detectable within two minutes of incubation (**Figure B.4-3B** and **B.4-3C**). An additional immunoreactive band at approximately 50,000 molecular weight is observed in leaf extracts. It is stable in the absence of pepsin (G-con lanes) but is not visible during the digestion time course. A different immunoreactive band at approximately 36,000 molecular weight is observed in the grain extracts, and it is also rapidly digested by pepsin within 0.5 min (**Figure B.4-3C**).

The full-length leaf AHAS protein is rapidly digested and is no longer visible within 0.5 min in SIF **(Figure B.4-4B**). Again, as was observed with the microbially-produced AtAHAS protein, the leaf AHAS protein appears unstable in SIF without pancreatin (I-con). Also, a lower molecular weight protein of approximately 50,000

molecular weight appears to increase in intensity with time of digestion. Furthermore, an immunoreactive band of approximately 16,000 molecular weight. appears to be associated with the pancreatin, since the band was not visible in the absence of SIF (I-con lanes) or at the zero time point where the SIF was inactivated by heating prior to addition of the leaf extract. A smaller immunoreactive band of >6,000 molecular weight is also observed and since it is also present in the SIF only incubations, appears to be associated with pancreatin. This band may be a degradation product of pancreatin. Full length AHAS protein is just barely detected by western blot in the leaf extract of conventional nontransgenic soybean, and the lower molecular weight protein at approximately 50,000 molecular weight (observed in the leaf extract of CV127 soybean) is apparently below the detection limit for this method as the 50,000 molecular weight band was not observed in the conventional nontransgenic leaf extract subjected to digestion in SIF (**Figure B.4-3B).** This result was not unexpected since the AHAS protein expression levels were approximately 600 ng AHAS per gram dry weight in young CV127 leaves as compared to approx. 75 ng AHAS per gram dry weight in young leaves of conventional, nontransgenic soybean. The lower molecular weight bands at approximately 16,000 and >6,000 molecular weights, previously observed in SIF digestion of leaf extracts of CV127, were again observed in SIF digestion of the leaf extract of conventional nontransgenic soybean (**Figure B.4-3B**), supporting the conclusion that these bands are associated with pancreatin and not with the AtAHAS transgenic protein encoded by the introduced imidazolinone-tolerant *csr1-2* gene.

In the grain extracts of CV127, full-length AHAS is rapidly degraded by pancreatin and is not observed at 0.5 min (**Figure B.4-2C**). Full length AHAS protein was not detected in the SIF digest of the conventional nontransgenic soybean grain sample (**Figure B.4-3C**). This result was not unexpected, since the expression levels were below the level of quantification in the conventional nontransgenic soybean grain, and approximately 28 ng/g dry weight in CV127 grain. As observed with digestion of the leaf extract of CV127, where a lower molecular weight (approx. 50,000) protein band was observed, a lower molecular weight immunoreactive band at approximately 36,000 molecular weight appears to accumulate with digestion time in SIF digestions of both CV127 and the conventional nontransgenic soybean grain (**Figures B.4-2C** and **B.4-3C**). Also, the same immunoreactive bands associated with pancreatin (at approximately 16,000 and >6,000 molecular weights) and described in digest of the leaf extracts are also observed in digestion of the grain extracts (**Figures B.4-2C** and **B.4-3C**). The grain AtAHAS appears more stable in the SIF without pancreatin (I-con) compared to the leaf AtAHAS, and this may be due to the presence of other components in the grain extract that stabilize AHAS (**Figure B.4-2C).**

In leaf and grain extracts, protein bands corresponding to approximately 50,000 and 36,000 molecular weights, respectively, appeared to increase in intensity with increasing time of SIF digestion. Because no lower molecular weight protein bands at approximately 50,000 or 36,000 molecular weights were observed to accumulate in SIF digestion of the microbially-produced AtAHAS (**Figure B.4-3A**), it is unlikely that these protein bands are a degradation product of the full-length AHAS protein

produced in leaf or grain tissue, and are more likely immunoreactive peptides generated from digestion of a different protein present in the leaf or grain extracts.

Microbially produced AtAHAS, and both forms of AHAS produced in CV127 leaves and grain (endogenous soybean AHAS and transgenic AtAHAS), are rapidly degraded in simulated mammalian gastric fluid. Full-length AHAS, regardless of source, is also rapidly degraded in simulated mammalian intestinal fluid. Degradation of the AHAS and AtAHAS proteins in plant extracts was slightly slower compared to the purified microbially-produced protein in both SGF and SIF, showing that the plant matrix has only a slight effect on protein digestion. Therefore, AtAHAS expressed in CV127 soybean is digested the same as conventional dietary proteins, and the same as endogenous soybean AHAS. Results of the study show that the AtAHAS protein expressed in CV127 soybean is highly digestible under simulated digestion conditions, which is typical of most proteins exposed to the proteases of the mammalian digestive tract. Furthermore, the transgenic AtAHAS protein expressed in CV127 soybean was immunologically indistinguishable from the endogenous soybean AHAS protein in the current studies, and results suggest that the digestive fate of both proteins in SGF and SIF was identical. Therefore, the AtAHAS protein shares the same digestive fate properties with the endogenous soybean AHAS protein, which has a history of safe consumption in food and feed products.

Figure B.4-1. Susceptibility of AtAHAS to digestion in simulated mammalian gastric fluid (SGF). Incubation time at 37 ºC is indicated in minutes. SGF is simulated mammalian gastric fluid containing pepsin, G-con is SGF without pepsin; SGF only indicates no AtAHAS sample was included. **Panel A**. Coomassie blue stained 4 - 20 % polyacrylamide gel containing digestion reactions carried out with the *E. coli-produced* AtAHAS protein. **Panel B**. Western blot analysis of digestion reactions carried out with leaf extract (Ext.) from CV127 soybean. **Panel C**. Western blot analysis of digestion reactions carried out with grain extract (Ext.) from CV127 soybean. AtAHAS is *ca.* 64,000 mol wt., pepsin is *ca.* 40,000. Molecular weight (x 10^{-3}) markers are indicated.

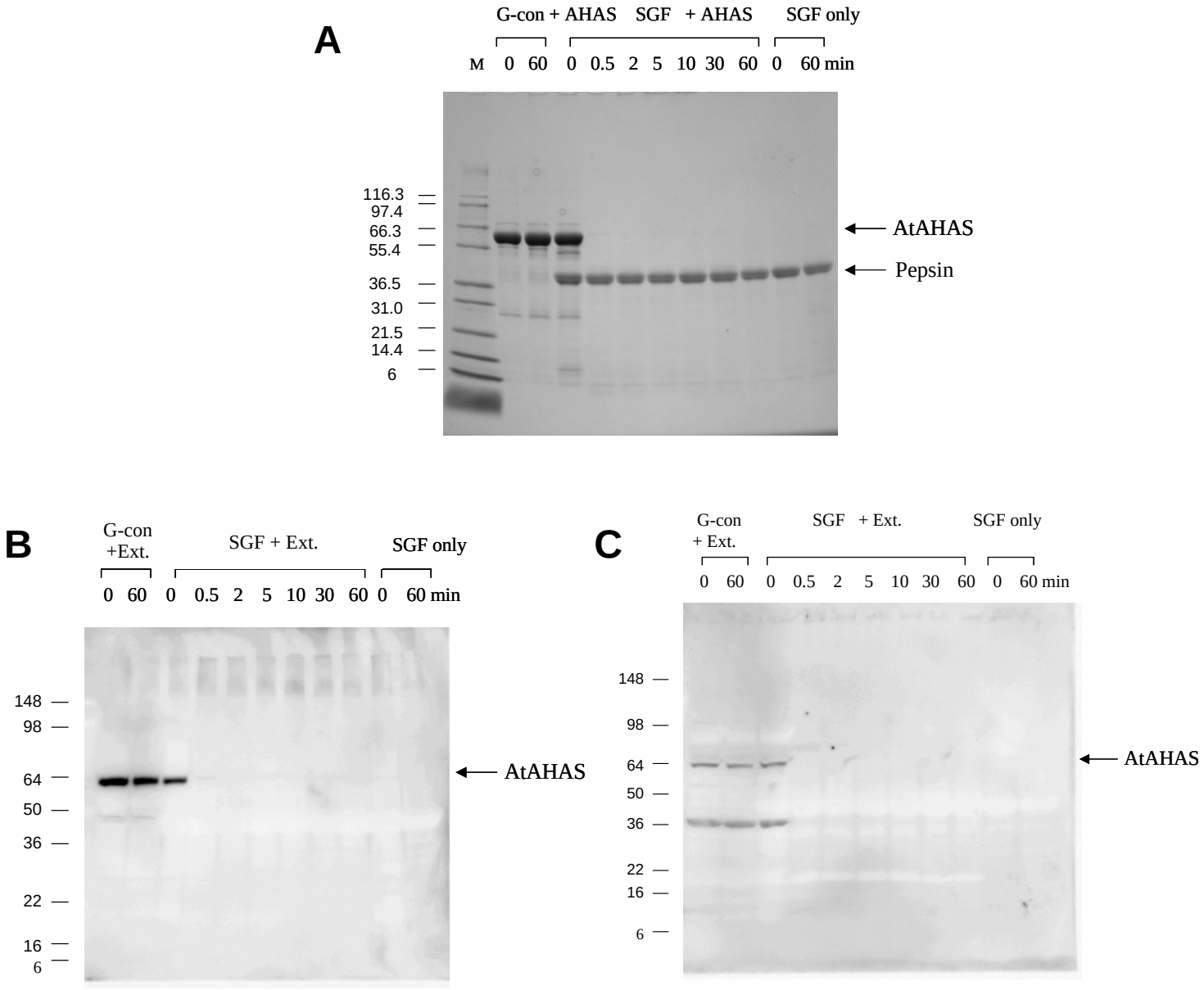
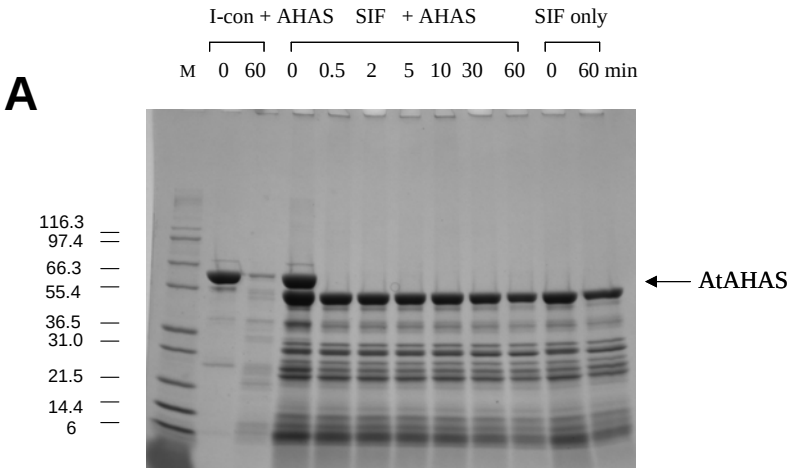
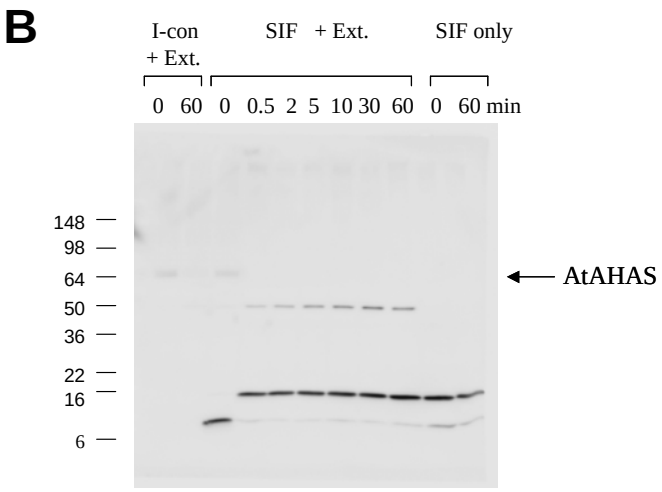


Figure B.4-2. Susceptibility of AtAHAS to digestion by simulated mammalian intestinal fluid (SIF). Incubation time at 37 ºC is indicated in minutes. SIF is simulated mammalian intestinal fluid containing pancreatin, I-con is SIF without pancreatin; SIF only indicates no AtAHAS sample was included. **Panel A**. Coomassie blue stained 4 - 20 % polyacrylamide gel containing digestion reactions carried out with the *E. coli*-produced AtAHAS protein. **Panel B**. Western blot analysis of digestion reactions carried out with leaf extract (Ext.) from CV127 soybean. **Panel C**. Western blot analysis of digestion reactions carried out with grain extract (Ext.) from CV127 soybean. AtAHAS is *ca.* 64,000 mol wt., and molecular weight (x 10^{-3}) markers are indicated.





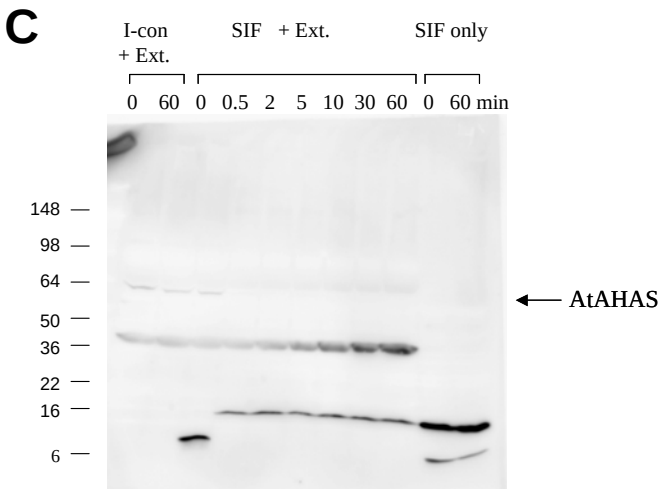
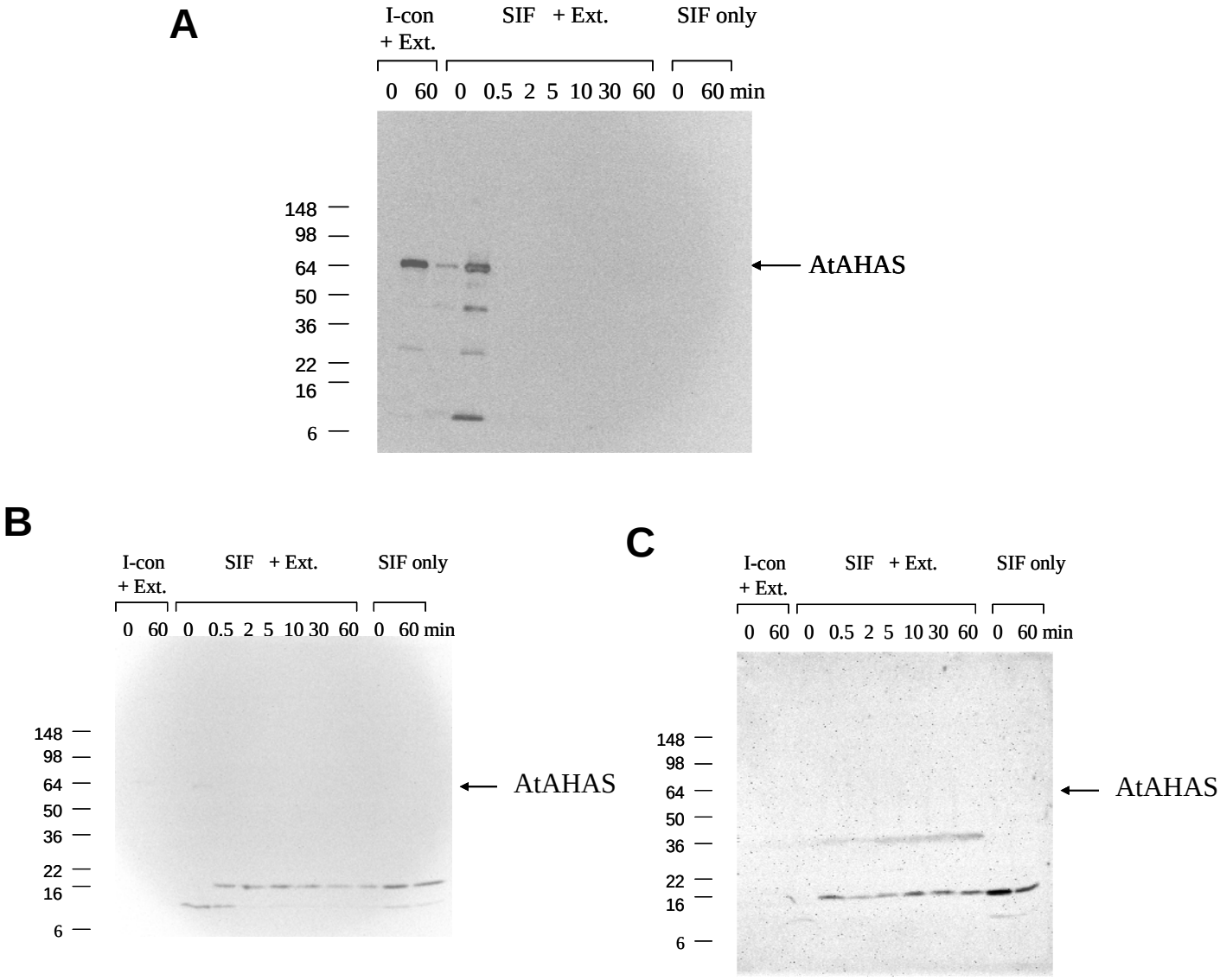


Figure B.4-3. Western blot analysis of simulated intestinal fluid (SIF) reactions for AtAHAS-0107, conventional soybean leaf and grain protein extracts. Incubation time at 37 ºC is indicated in minutes. SIF is simulated mammalian intestinal fluid containing pancreatin, I-con is SIF without pancreatin; SIF only indicates no AtAHAS sample was included. **Panel A**. Western blot analysis using a 4 - 20 % polyacrylamide gel containing the same digestion as in **Figure B.4-3** for AtAHAS-0107. **Panel B**. Western blot analysis of digestion reactions carried out with leaf extract from conventional, nontransgenic soybean leaves. **Panel C**. Western blot analysis of digestion reactions carried out with grain extract from conventional, non-transgenic soybeans. AHAS is *ca.* 64,000 mol wt., and molecular weight (x 10^{-3}) markers are indicated.



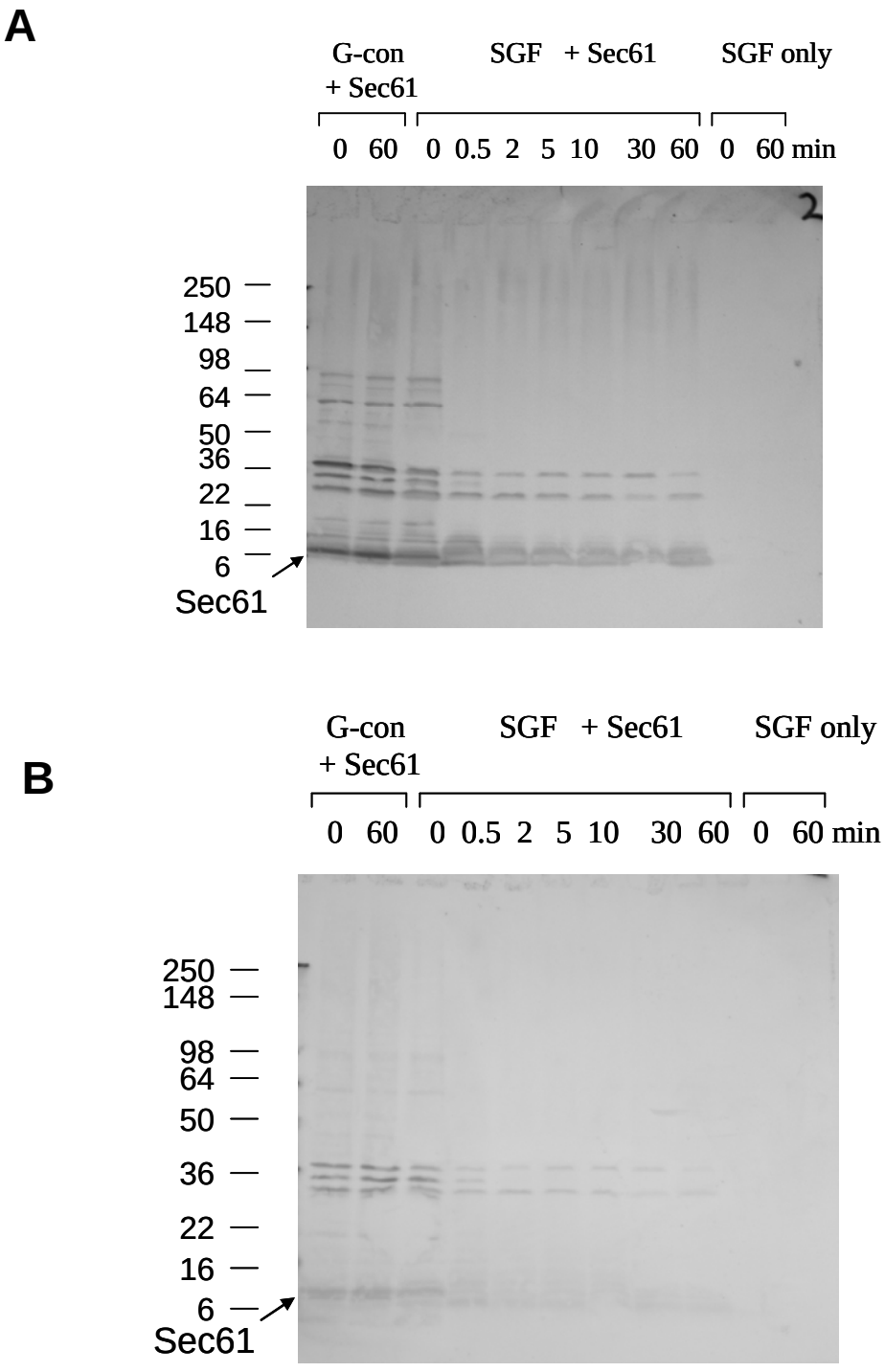
Digestive fate of the AtSEC61γ subunit protein in simulated gastric fluid
A study was conducted to determine if *E. coli*-produced AtSEC61γ subunit protein has the same susceptibility to digestion in simulated mammalian gastric fluid (SGF) as other dietary proteins with a history of safe use in foods.

The method used in this study to determine digestive fate of the AtSEC61γ subunit protein in SGF were in accordance with procedures described in the United States Pharmacopoeia (2000), and are similar to the method described for digestion of the AtAHAS protein in SGF above (see also **Appendix 18**). The AtSEC61γ subunit protein was incubated for various times in the SGF, and the remaining protein was subjected to electrophoresis and visualized by western blot analysis. The *E. coli*-produced AtSEC61γ subunit protein was rapidly degraded in SGF (**Figure B.4-4A**). AtSEC61γ subunit protein (molecular weight *ca*. 7,000, the most intensely staining band in this size range) is readily detected in the time 0 sample and in the G-con samples. However, no full-length AtSEC61γ subunit protein is visible in the sample removed at 0.5 min after the initiation of the reaction. Some immunoreactive bands representing contaminating proteins of varying molecular weights (6,000, 10,000, 25,000, 36,000) were visible at all time points. Other immunoreactive protein bands at 20,000, 32,000, 63,000 and 98,000 molecular weights were also rapidly degraded similar to the AtSEC61γ subunit protein. The results shown in the blot pictured in **Figure B.4-4B** in which the immunoaffinity-purified rabbit anti-SEC61γ peptide was used, confirm the identity of the AtSEC61γ band and its rapid digestibility.

Results of the digestive fate study show that the AtSEC61γ subunit protein is highly digestible under the simulated digestion conditions, which is typical of most dietary proteins exposed to the proteases of the mammalian digestive tract. This result demonstrates that the AtSEC61γ subunit protein does not share one of the characteristics of some food allergens, resistance to digestive degradation. Furthermore, this result was as expected since the AtSEC61γ subunit protein is ubiquitous and highly conserved in eukaryotes, and is present in many plant species with a history of safe food use.

Collectively, results of the above studies demonstrate that in the unlikely event the AtSEC61γ subunit protein is expressed at extremely low levels (below the limit of detection of the western blot assay) in CV127 tissues, the AtSEC61γ subunit protein does not possess the attributes of known protein food allergens, and hence poses no risks for human and animal consumption.

Figure B.4-4. Susceptibility of AtSEC61γ subunit protein to digestion in simulated mammalian gastric fluid (SGF). Western blot analysis of digestion reactions carried out with AtSEC61γ subunit protein. Incubation time at 37°C is indicated in minutes. SGF is simulated mammalian gastric fluid containing pepsin, G-con is SGF without pepsin; and the lanes labelled "SGF only" contained SGF without AtSEC61γ subunit protein. AtSEC61γ subunit protein is *ca.* 7000 mol wt., pepsin is *ca.* 40,000 mol. wt. Molecular weight (x 10^{-3}) markers are indicated. **Panel A**. Protein A purified rabbit anti-SEC61γ peptide was used as the detection antibody. **Panel B**. Immunoaffinity purified rabbit anti-SEC61γ peptide was used as the detection antibody.



d) Specific serum screening where a newly expressed protein is derived from a source known to be allergenic or has sequence homology with a known allergen

Based on the results from the described studies in the previous sections it was concluded that neither AtAHAS nor AtSEC61γ subunit protein share any homology with known allergens nor are they derived from a source known to be allergenic. Therefore no specific serum screening is warranted in order to assess the potential allergenicity of these proteins.

Conclusion of the allergenicity assessment

The outcome of the allergenicity assessment (**Section B.4.**) supports the conclusion that CV127 soybean is not likely to be more allergenic than its commercial comparator for the following reasons: The source organism of the *csr1-2* gene that encodes the AtAHAS protein in CV127 soybean, *Arabidopsis thaliana*, is not known to cause allergic reactions in humans. AtAHAS expressed in CV127 soybean has amino acid sequence homologous to other AHAS proteins with a history of safe use in food and feed products. Neither AtAHAS nor AtSEC61γ subunit protein share any amino acid sequence homology to known protein food allergens. Both AtAHAS protein and AtSEC61γ subunit protein are rapidly degraded in simulated digestive fluids and AtAHAS is rapidly inactivated by temperatures above 60℃.

5. Compositional analyses of the GM food

a) The levels of key nutrients, toxicants and antinutrients in the GM food compared with the levels in an appropriate comparator (usually the non-GM counterpart). The statistical significance of any observed differences must be assessed in the context of the range of natural variations for that parameter to determine its biological significance

Study design

Compositional analyses were conducted on grain and forage as well as processed fractions to confirm that the levels of key nutrients and antinutrient in the grain and forage as well as processed fractions derived from CV127 soybean are comparable to those from the near-isogenic control soybean variety. As part of the safety studies several CV127 soybean generations were developed in successive steps of selfing and crossing. The null-segregant was uses as the isogenic comparator variety in the compositional studies, since it is considered most important and appropriate to use a near-isogenic line as a control and not rely on the comparison with the material that was originally used for the transformation. The null-segregant is as closely related to CV127 soybean as possible from a genetic point of view and only lacks the *csr1-2* gene. It was obtained from the same stage of breeding as the CV127 soybean line being evaluated. The breeding scheme for the generation of CV127 soybean is provided in **Figure A.3-6** (page 50). An overview on comparator lines used in the compositional studies is given in **Table B.5-1**. In addition, two conventional standard soybean varieties that are typical of soybean varieties commonly cultivated in Brazil, Monsoy 8001 and Coodetec 217, were included in these analyses to establish a range of natural variability for each analyte for soybeans grown in Brazil. Both of these reference varieties are typical of soybean varieties commonly cultivated in Brazil with a similar maturity classification as CV127 soybean. Any statistically significant differences between CV127 soybean and the near-isogenic control can be compared to the reference range to assess whether the differences are likely to be biologically relevant. Furthermore, results were compared to analytical data for soybean grain in the International Life Sciences Institute (ILSI) Crop Composition database (ILSI, 2006), both globally-derived values as well as data produced in Brazil, where available.

Table B.5-1. Material used for the comparative compositional analyses.

Analysis	CV127 Soybean Generation Used	Comparator Used
Compositional analysis (Grain) 2006/2007	F5 (CV127 line 127)	Isogenic control (F5 null) Monsoy 8001 Coodetec 217
Compositional analysis (Grain) 2007	F6 (CV127 line 127)	Isogenic control (F6 null) Monsoy 8001 Coodetec 217
Compositional analysis (Forage) 2007/2008	F7 (CV127 line 127)	Isogenic control (F7 null) Monsoy 8001 Coodetec 217

Grain samples of CV127 soybean, the near-isogenic control, and the conventional soybean varieties were harvested from soybeans grown in a complete randomized block design with four replicated plots at each of six field sites in Brazil during the 2006/2007 season and at each of four field sites in the 2007 short season. Forage tissues were harvested at the R2 growth stage from soybeans grown in a complete randomized block design with three replicated plots at each of six field sites in Brazil during the 2007/2008 growing season. All field trial locations for production of grain and forage for compositional analyses are presented in **Table B.5-2**. At all locations the plants were grown under standard agronomic practices. The field sites used in this study were located in regions of Brazil where soybeans are typically commercially cultivated.

Table B.5-2. Field trial locations in Brazil for grain and forage compositional analyses.

Location – State*	2006/2007 Season Grain	2007 Season Grain	2007/2008 Season Forage
Santo Antonio de Posse – SP	**x**		**x**
Londrina – PR/North	**x**		**x**
Uberaba – MG	**x**		**x**
Brasília – DF	**x**	**x**	**x**
Santo Antonio de Goiás – SG	**x**	**x**	**x**
Sete Lagoas-MG	**x**		**x**
Teresina - PI		**x**	
Vilhena - RO		**x**	

**SP, São Paulo; PR, Paraná; MG, Minas Gerais; DF, Distrito Federal; GO, Goiás; PI, Piauí; RO, Rondônia.

For grain production and analyses, treatments included CV127 soybean plants treated with imidazolinone herbicide (treatment presented as CV127 + imi), and the near-near-isogenic control variety and two other conventional standard soybean varieties commonly grown in Brazil (Monsoy 8001 and Coodetec 217, respectively presented

as Std 1 and Std 2 in the data tables) were treated with Bentazon + Acifluorfen-sodium (commercial name Volt). An additional treatment of CV127 treated with conventional herbicide Volt was included in these studies to confirm that imidazolinone herbicide application to CV127 does not have a significant effect on grain composition (treatment presented as CV127). The statistical analyses of the compositional data were conducted for the CV127 + imi, CV127 and near-isogenic control treatments, and data from the two conventional standard soybean reference varieties were used for comparative purposes to establish a range of natural variability for each analyte for soybeans grown in Brazil.

The land and crop management practices at each location, including the management of pests, pathogens, and weeds, were as recommended for each specific region in which the field trials were located. Insecticides, fungicides, and herbicides were applied at each location according to local recommendations and as needed to protect the plants from insect, fungal, and weed infestations. All agronomic practices used were those commonly recommended for soybean production in the region where the experiment was conducted.

The selection of components for analysis was based on an assessment of components significant to human an animal safety and nutrition. These components are consistent with the guidance contained in the OECD consensus document on Compositional Considerations for New Varieties of Soybean (OECD, 2001). Compositional analyses were carried out on the raw agricultural commodity grain, on forage and on processed soybean grain fractions. Generally, soybean is cultivated for the production of grain and seldom is it used as a forage crop. However, occasionally soybean is fed to animals (primarily dairy cattle) as forage.

A total of 70 components were analysed in the grain samples. Components analysed for grain included proximates (protein, fat, ash, carbohydrates, calories, total dietary fibre [TDF], and moisture), crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), amino acids, fatty acids (C14 - C22), minerals (calcium, iron, phosphorus, magnesium, and potassium), vitamins (α-, β-, γ-, δ-, and total tocopherols, vitamins E and B_1 and folic acid), isoflavones (daidzin, malonyl-daidzin, daidzein, glycitin, malonyl-glycitein, genistin, malonyl-genistin, and genistein), phospholipids (phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl inositol, and phosphatidyl choline), and antinutrients (phytate, raffinose, stachyose, lectin, urease and trypsin inhibitor). The methods used for these analyses as well as composition results for each individual field trial site for grain production are presented in **Appendix 19**.

Components analysed in forage samples included proximates (protein, fat, ash, and moisture), crude fibre, ADF, NDF, and carbohydrates and calories by calculation. The source of the forage samples and analytical methods are presented in **Appendix 20**.

In addition, grain from CV127 and the near-isogenic control were processed using standard soybean grain processing methods to produce toasted, defatted soybean meal,

protein isolate and concentrate, and refined oil. Components analysed in toasted soybean meal included proximates (moisture, ash, fat, protein, carbohydrates, and calories), fibre (crude, ADF and NDF), antinutrients (raffinose, stachyose, trypsin inhibitor, urease and phytic acid) and isoflavones. Untoasted defatted soybean meals were not analysed for composition because the nutrient composition is expected to be very similar to the toasted, defatted soybean meal samples. Also, the toasted soybean meal is used almost exclusively in animal feed, so it was the most relevant fraction for analysis to confirm nutritional value for animal feed. Proximates only were analysed for the protein isolate and concentrate fractions. The refined oil fractions were analysed for fatty acid composition. The methods of analysis and schematic diagram depicting the processing methods employed are described in **Appendix 15**.

Statistical analysis: for compositional analysis of grain, analysis of variance was carried out on the data using SAS Version 9.1 (SAS Institute Inc., Cary, NC) following the General Linear Model Procedure. With the exception of moisture content, all data were expressed on a dry weight basis for statistical analyses. Differences were assessed across location and by location. The model for across location:

y = variety + location + variety x location + block(location) + e
Random effects: block(location)
where y is the response variable (any analyte measured)

Means for varieties (averaged over locations) were compared using the Least Significant Difference (LSD) Procedure at significance levels p = 0.05.

The model for separate analyses by location:

y = variety + block + e
where e is the response error

Following the analysis by location, contrasts were carried out to compare each of the sprayed and unsprayed CV127 treatments (CV127 + imi and CV127 treated with Volt) with the near-isogenic control. Differences were considered statistically significant at the 0.05 significance level. For compositional analysis of forage samples, ANOVA was carried out as described above for grain. Contrasts were carried out to compare the near-isogenic control and each of the commercial standards with the treatment CV127 + imi. For compositional analysis of processed fractions from grain, analysis of variance was basically carried out as described above for grain and forage samples. Differences were only assessed across location and contrasts were carried out to compare each treatment. Differences were considered statistically significant at the 0.05 confidence level.

Grain composition

Proximates. Grain samples from the 2006/2007 and the 2007 field trial seasons were analysed for moisture, ash, protein, crude fat, total dietary fibre. The carbohydrate

composition and calorie values were calculated. The means and ranges of values for proximates in the CV127 soybean treatments (CV127 and CV127 + imi), the near-isogenic control, and the conventional standard soybean varieties in two growing seasons and across all locations by year in comparison with the means and ranges published in the ILSI Crop Composition Database are shown in **Table B.5-3**.

These data show that in both seasons there were no statistically significant differences in the ash, total dietary fibre, or carbohydrate content between either of the CV127 treatments and the near-isogenic control soybeans. In addition, in the 2007 season there was no significant difference for calories between grain from the near-isogenic control and the CV127 treatments.

Slight grain moisture differences were observed between both CV127 treatments and the control in the 2006/2007 season and between the CV127 + imi treatment in the 2007 season, but the differences were inconsistent, since grain moisture content of both CV127 treatments was lower than the control in the 2006/2007 season, but was at a higher level in the CV127 + imi treatment compared to the control in the 2007 season. Therefore, these differences were not considered biologically meaningful. Furthermore, the mean and range of grain moisture levels in the CV127 treatments were comparable to the mean and range levels in the two conventional standard soybean varieties cultivated in the same trials, and were within the range of moisture values reported for soybeans globally as well as for soybeans produced in Brazil in the ILSI Crop Composition Database. In the 2006/2007 season, grain from both CV127 soybean treatments were statistically significantly higher for fat and caloric content and significantly lower for protein content compared to grain from the near-isogenic control soybeans. In the 2007 season, there was no statistically significant difference in grain fat levels between the CV127 + imi treatment and the near-isogenic control, but the protein content of grain from CV127 + imi soybean was significantly higher than that for grain from the near-isogenic control. In contrast, fat level in grain of the CV127 treatment was slightly statistically significantly higher than in the grain of the near-isogenic control, but there were no statistically significant differences in grain protein content between the two treatments. There were no statistically significant differences in grain proximate composition in either growing season between the CV127 and CV127 + imi treatments, except for moisture, which in the 2007 season was slightly statistically significant higher in grain from the CV127 + imi treatment compared to CV127, showing that the imidazolinone herbicide application to CV127 did not have a significant effect on grain proximate composition. Overall, mean and range of values for proximates in both treatments of CV127 grain from both seasons were either within or comparable to the range of values for the conventional soybean varieties cultivated in the same trials as well as to the range reported for soybeans globally and for soybeans produced in Brazil. Collectively, these results show that proximate levels (moisture, ash, protein, fat, total dietary fibre, carbohydrate and calories) in both treatments of CV127 grain are equivalent to proximate levels in the control grain and comparable to and in the same range as grain proximate content of other conventional soybean varieties with a history of safe food and feed uses.

Fibres. Grain samples from the 2006/2007 and the 2007 field trial seasons were analysed for crude fibre, ADF and NDF. The means and ranges of values for crude fibre, ADF and NDF in CV127 soybean treatments (CV127 and CV127 + imi), the near-isogenic control and the conventional standard soybean varieties treatments in two field trial seasons and across all locations by year in comparison with the mean and ranges published in the ILSI Crop Composition Database are shown in **Table B.5-4**.

In both field trial seasons, there were no statistically significant differences in grain crude fibre content between either of the CV127 soybean treatments and the near-isogenic control. However, the ADF and NDF values in grain of both the CV127 soybean treatments were statistically significantly different (higher) than those in grain from the near-isogenic control in the 2006/2007 season. In contrast, in the 2007 season, there were no statistically significant differences in grain ADF content between either of the CV127 soybean treatments and the near-isogenic control, and no statistically significant difference in grain NDF content between the CV127 treatment and the near-isogenic control, but NDF levels in grain of the CV127 + imi treatment were slightly, but statistically significantly higher compared to levels in the near-isogenic control. However, the crude fibre, ADF and NDF mean and range of values obtained for both CV127 soybean treatments in the two seasons were either within or comparable to the mean and range of values of the conventional standard soybean varieties cultivated in the same trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil. These results show that grain fibre content of CV127 soybean, either treated with imidazolinone or conventional herbicide, is equivalent to the grain fibre content of the control and comparable to and in the same range as grain fibre content of other conventional soybean varieties with a history of safe food and feed uses. These results also confirm that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain fibre composition.

Amino Acids. Grain samples from the two field trial seasons were analysed for amino acid content. The means and ranges of values for all amino acids in the CV127 soybean treatments (CV127 and CV127 + imi), the near-isogenic control and the conventional soybean control treatments in two field trial seasons and across all locations by year in comparison with the mean and ranges published in the ILSI Crop Composition Database are shown in **Table B.5-5**.

For grain obtained from the 2006/2007 season, there were no statistically significant differences detected in amino acid content between the CV127 + imi treatment and the near-isogenic control soybeans. A similar result was observed in comparison of grain amino acid content of the CV127 treatment compared to the near-isogenic control, except for statistically significantly lower levels of proline and tyrosine in grain of CV127. In the grain harvested from the 2007 season, there were no statistically significant differences in grain amino acid content between the two CV127 soybean treatments and the near-isogenic control soybeans, except for the five

amino acids alanine, histidine, tyrosine, cysteine and methionine, with the differences in levels of the latter two amino acids being very small, and in addition valine in the case of the CV127 treatment only compared to the levels of this amino acid in the near-isogenic control. The mean and range of values for the grain amino acid content of both CV127 soybean treatments from both seasons were either within or comparable to the range of values of the conventional standard soybean varieties cultivated in the same trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil. These results demonstrate that the grain amino acid content of CV127 soybean, either treated with imidazolinone or conventional herbicide, is equivalent to the amino acid content of the control grain and is comparable to, and in the same range as, grain amino acid content of conventional soybean varieties with a history of safe food and feed uses. These results also confirm that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain amino acid composition.

Results of the amino acid analyses in grain also confirm that the amino acid mutation in the AtAHAS protein responsible for conferring imidazolinone herbicide tolerance in CV127 soybean has no impact on the feedback regulation of this enzyme by branched-chain amino acids. The AHAS enzyme catalyses the first common step in branched-chain amino acid biosynthesis (leucine, isoleucine and valine). The data presented in this section show there were no statistically significant differences in levels of the branched-chain amino acids between grain of the CV127 + imi soybean treatment and the control for either the 2006/2007 or 2007 field trial seasons. A similar result was observed for grain branched-chain amino acid content between the CV127 and the near-isogenic control soybean treatments, except for the 2007 field trial season only, where grain valine content was statistically significantly higher for CV127 compared to the near-isogenic control. However, the mean value for grain valine content of the CV127 soybean treatment from the 2007 field trial season was within the range of values of the conventional standard soybean varieties cultivated in the same trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil. Therefore, these data confirm that the mutation in the AtAHAS enzyme has no effect on feedback regulation of the enzyme by the branched-chain amino acids or the biosynthetic function of the enzyme.

Fatty Acids. Grain samples from the two field trial seasons were analysed for the complete spectrum of fatty acids levels (a total of 37 fatty acids). Multiple fatty acids were either not present or present at levels below the limit of quantitation for the assay, including butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), undecanoic acid (C11:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), palmitoleic acid (C16:1), heptadecenoic acid (C17:1), α-linolenic acid (C18:3), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), erucic acid (C22:1), and others. Statistical analyses were conducted only for those fatty acids which had quantifiable levels.

The most prevalent fatty acids in the grain from both seasons included myristic, palmitic, margaric, stearic, oleic, linoleic, linolenic, arachidic, and behenic acids. In addition, low levels of eicosenoic acid were detected in grain of the different soybean treatments from the 2006/2007 season, but were not detected in the grain from the 2007 season. The mean and range of values for fatty acids in grain from both CV127 soybean treatments, the near-isogenic control soybeans and the conventional standard soybean varieties in two field trial seasons and across all locations by year in comparison with the mean and ranges for fatty acids in soybean grain published in the ILSI Crop Composition Database are shown in **Table B.5-6**.

In grain from the 2006/2007 season there were no statistically significant differences in levels of myristic, stearic, arachidic, and eicosenoic acids between the grain from either of the CV127 soybean treatments and the near-isogenic control soybeans. Two fatty acids (palmitic and oleic) were significantly higher and two others (linoleic and linolenic) were significantly lower in grain from the CV127 soybean treatments compared to the near-isogenic control soybean. Also, levels of margaric acid were statistically significantly different between the CV127 soybean treatments and the near-isogenic control soybeans. In the case of behenic acid, there was no statistically significant difference in levels of this fatty acid between the CV127 + imi treatment and the near-isogenic control, but levels in CV127 were statistically significantly lower compared to the near-isogenic control.
In grain from the 2007 season, there were no statistically significant differences in levels of myristic, palmitic, margaric, arachidic and behenic fatty acids between the grain from either of the CV127 soybean treatments and the near-isogenic control soybeans. There were no statistically significant differences in levels of any of the measured fatty acids between grain of the CV127 treatment and the near-isogenic control, but statistically significant differences in levels of stearic, oleic, linoleic, and linolenic fatty acids were observed between grain of the CV127 + imi treatment and the near-isogenic control.
For all fatty acids, in both seasons the mean and range of grain fatty acid values for both CV127 soybean treatments were either within or comparable to the range of values of the conventional standard soybean varieties cultivated in the same trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil. These results demonstrate that the fatty acid content of CV127 grain, either treated with imidazolinone or conventional herbicide, is equivalent to the fatty acid content in control grain and is comparable to and in the same range as the grain fatty acid content of other conventional soybean varieties with a history of safe food and feed use. Although a few statistically significant differences in grain fatty acid content were observed between the two CV127 treatments, in general grain fatty acid content of the two treatments were equivalent, and shows that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain fatty acid composition.

Minerals. Soybeans are considered a significant source of potassium and magnesium and a source of bioavailable iron in the animal feed diet (Baker, 2000). Soybeans are also a source of phosphorus and calcium in the animal feed diet; however the

bioavailability of these minerals is typically limited by chelation in phytate complexes. Therefore, these five minerals, calcium, iron, magnesium, phosphorous and potassium were quantified in soybean grain harvested from the different treatments from two growing seasons. The mean and range of values for minerals in grain from both CV127 soybean treatments, the near-isogenic control soybeans and the conventional standard soybean varieties in two field trial seasons and across all locations per year in comparison with the mean and ranges for minerals in soybean grain published in the ILSI Crop Composition Database are shown in **Table B.5-7**.

In the 2006/2007 season, there were no statistically significant differences in grain phosphorus and potassium levels between the CV127 treatment and the near-isogenic control, and no statistically significant differences in grain content of calcium and phosphorus between the CV127 + imi treatment and the near-isogenic control. Levels of iron (lower) and magnesium (higher) in grain of both CV127 treatments were statistically significantly different from the near-isogenic control, and differences were observed between the CV127 treatment and the near-isogenic control for grain calcium content, and between the CV127 + imi treatment and the near-isogenic control for grain potassium content. All mineral mean values determined for grain from both CV127 soybean treatments in the 2007 season were statistically significantly different from the mean values obtained for grain from the near-isogenic control soybean, except for grain iron content of the CV127 + imi treatment compared to the near-isogenic control. However, for each of the five minerals measured, the mean and range of grain mineral values for both CV127 soybean treatments were either within or comparable to the range of values of the conventional soybean varieties cultivated in the same trials as well as to the range of values reported globally for soybeans**.** These results demonstrate that the mineral content in grain produced from CV127 soybean is comparable to, and in the same range as, the mineral content of grain of the control as well as other conventional soybean varieties with a history of safe food and feed use. Although a few statistically significant differences in grain mineral content were observed between the two CV127 treatments, in general grain mineral content of the two treatments were equivalent, and shows that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain mineral composition.

Vitamins. The tocopherols (α, β, γ, δ and total tocopherols) together with vitamins E, B1, folic acid, niacin, and riboflavin were analysed in the soybean grain samples obtained from trials conducted in two field trial seasons. Levels of niacin and riboflavin in the grain samples were below the level of detection for the assays and so these analytes are not included in the data table. The mean and range of values for vitamins in grain from both CV127 soybean treatments, the near-isogenic control soybeans and the conventional soybean control treatments in two field trial seasons and across all locations per year in comparison with the mean and ranges for vitamins in soybean grain published in the ILSI Crop Composition Database are shown in **Table B.5-8**.

The mean grain vitamin content of both CV127 treatments produced in the 2006/2007 season was found to be statistically significantly lower than that in the grain of the near-isogenic control for folic acid, γ-tocopherol and vitamin B1 and significantly higher for α-, β-, and δ-tocopherol, and vitamin E. The mean values for total tocopherols in the grain of both CV127 treatments and the near-isogenic control soybeans from the 2006/2007 season were not statistically significantly different. There were no statistically significant differences in the mean vitamin content of grain from either of the CV127 soybean treatments in the 2007 season compared to those from the near-isogenic control except for β- and δ-tocopherols and vitamin E for the CV127 treatment compared to the near-isogenic control, and α- and γ-tocopherols for the CV127 + imi treatment compared to the near-isogenic control. However, for each of the different vitamins measured, the mean and range of grain vitamin values for both CV127 soybean treatments were either within or comparable to the range of values of the conventional soybean varieties cultivated in the same field trials. Global ranges for typical vitamin values in soybean grain were only available for folic acid, α-tocopherol, and vitamin B1. The mean values of folic acid and α-tocopherol in grain from CV127 soybeans in both seasons were within the ranges reported globally for soybeans in the ILSI composition database. In the case of vitamin B1, the grain from both CV127 soybean treatments, the near-isogenic control as well as the conventional standard soybean varieties in both seasons had higher values compared to the typical global range of values for this vitamin, and this may be a characteristic of soybean germplasm adapted for tropical growing conditions in Brazil. Overall, these results demonstrate that vitamin levels in grain produced by CV127 soybean are comparable to and in the same range as vitamin levels in the grain of control as well as other conventional soybean varieties with a long history of safe food and feed use. There were few statistically significant differences in grain vitamin content observed between the two CV127 treatments which show that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain vitamin composition.

Isoflavones. Grain samples from two field trial seasons were analysed for their isoflavone composition, including daidzin, malonyl daidzin, daidzein, glycitin, malonylglycitin, glycitein, genistin, malonyl genistin, and genistein. The mean and range of values for the total isoflavones (total daidzein, total genistein, and total glycitein) in grain from both CV127 treatments, the near-isogenic control soybeans and the conventional standard soybean varieties in two field trial seasons and across all locations by year in comparison with the mean and ranges for isoflavones in soybean grain published in the ILSI Crop Composition Database are shown in **Table B.5-9**.

There was no statistically significant difference in the mean values for total glycitein between the grain from both CV127 treatments and the near-isogenic control soybeans in the 2006/2007 season. Similarly, there were no statistically significant differences in grain glycitein levels between the CV127 and near-isogenic control treatments in the 2007 season, but levels of this isoflavone were statistically significantly lower in grain of CV127 + imi compared to levels in the grain of the

near-isogenic control in the 2007 season. Levels of total daidzein and genistein were statistically significantly lower in grain of both CV127 treatments compared to levels in the near-isogenic control in both field trial seasons. However, for each of the isoflavones, the mean and range of isoflavone values for CV127 soybean grain were either within or comparable to the range of values of the conventional soybean varieties cultivated in the same field trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil. These results demonstrate that the isoflavone content in grain produced from CV127 soybean is comparable to, and in the same range as, the grain isoflavone content of the control as well as conventional soybean varieties with a history of safe food and feed use. There were no statistically significant differences in grain isoflavone content observed between the two CV127 treatments, which shows that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain isoflavone composition.

Phospholipids. Phospholipids are common contaminants during soybean oil processing, often referred to as gums in the solvent extracted oil. If not removed the gum material typically settles out and can cause significant losses in oil refining. Therefore, phospholipid levels were analysed in grain samples and the mean and range of values for phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl inositol and phosphatidyl choline in grain from both CV127 treatments, the near-isogenic control soybeans and the conventional standard soybean varieties in two field trial seasons and across all locations by year are shown in **Table B.5-10**.

Data were not available for these analytes in the ILSI Composition Database. In the 2006/2007 season, the mean values for all measured phospholipids in grain from both CV127 soybean treatments were statistically significantly lower compared to the corresponding values from the grain of the near-isogenic control. Conversely, in the 2007 season, there were no statistically significant differences in the mean phospholipid values of grain from both CV127 treatments and the near-isogenic control soybeans, except for levels of phosphatidic acid levels that were slightly but statistically significantly higher in grain of the CV127 + imi treatment compared to levels in grain of the near-isogenic control. Therefore, the differences observed from the first field season were not considered biologically meaningful since they were not consistently observed. Since phospholipid content of soybeans is not included in the ILSI Crop Composition Database, comparison of these values to this database was not possible. However, comparisons to mean and range of values for the conventional standard soybean varieties that were included in this study demonstrated that levels of phospholipids in grain from both CV127 soybean treatments were comparable to those in grain produced by conventional soybean varieties that are commonly cultivated in Brazil and that have a long history of safe food and feed use. Also, there was only one instance of a statistically significant difference in grain phospholipid content observed between the two CV127 treatments (phosphatidic acid in the 2007 season), which shows that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain phospholipid composition.

Antinutrients. Grain samples were analysed for antinutrient content, and the mean and range of values for phytic acid, raffinose, stachyose, lectins, urease and trypsin inhibitor in grain from both CV127 treatments, the near-isogenic control soybeans and the conventional standard soybean varieties in two field trial seasons and across all locations by year in comparison with the mean and ranges for antinutrients in soybean grain published in the ILSI Crop Composition Database are shown in **Table B.5-11.**

There were no statistically significant differences in the levels of phytic acid between grain from either of the CV127 treatments and the near-isogenic control soybeans in either field trial season, and mean and range of phytic acid values for CV127 grain were either within or comparable to the range of values for the conventional standard soybean varieties included in this study. However, the mean and range of phytic acid values for both CV127 treatments, the near-isogenic control and the conventional standard soybean varieties values were lower than the range reported for soybeans in the global category of the ILSI Crop Composition Database, and this most likely reflects a germplasm characteristic of soybean varieties adapted for tropical cultivation conditions in Brazil.

The mean values for grain raffinose content for both CV127 soybean treatments produced in the 2006/2007 season were slightly, but statistically significantly higher than the grain content of the near-isogenic control variety. In contrast, in the 2007 season, the grain raffinose content of the CV127 treatment was statistically significantly lower compared to levels for the near-isogenic control, and there was no statistically significant difference in grain raffinose levels between the CV127 + imi treatment and the near-isogenic control. Therefore the difference in grain raffinose levels observed between the two CV127 treatments and the near-isogenic control in the 2006/2007 season was not considered biologically meaningful since it was not observed consistently. Although the mean values for raffinose in both CV127 treatments and the near-isogenic control soybeans in grain from both seasons were higher than the range for raffinose in soybeans cultivated globally (ILSI, 2006), the mean and range of raffinose levels in both CV127 treatments and the near-isogenic control were comparable to the mean and range of raffinose values obtained for the two conventional standard soybean varieties included in the field trials (**Table B.5-11**). Therefore the difference in raffinose levels in grain of the two CV127 treatments, the near-isogenic control and the conventional standard soybean varieties from the range of raffinose levels that are reported for soybeans in the global category of the ILSI Crop Composition Database most likely reflects a characteristic of soybean varieties adapted for tropical cultivation conditions in Brazil.

The mean values for stachyose content in grain of both CV127 soybean treatments in both field trial seasons were slightly, but statistically significantly lower than the mean values for the grain from the near-isogenic control. Although the mean and range of values for stachyose in grain from both CV127 treatments and the near-isogenic control soybeans in both seasons were higher than the range for stachyose in soybeans reported in the global category of the ILSI Crop Composition Database, these mean and range of values were comparable to the mean and range of stachyose

values obtained for the two conventional standard soybean varieties included in the field trials (**Table B.5-11**). Again, this suggests that higher stachyose levels observed in grain of CV127, the near-isogenic control and the conventional standard soybean varieties compared to levels reported for soybeans in the global category of the ILSI Crop Composition Database is the result of a characteristic of soybean varieties adapted for tropical cultivation conditions in Brazil.

The mean values for lectins in the 2006/2007 season, and urease and trypsin inhibitor in both seasons for both CV127 treatments were not statistically significantly different from levels of the same analytes in the near-isogenic control soybeans (**Table B.5-11**). The mean values for lectin levels in grain from both CV127 soybean treatments from the 2007 season were statistically significantly lower than the values obtained from the grain of the near-isogenic control. The mean and range of values for lectins in both CV127 treatments were either within or comparable to the range of values of the conventional standard soybean varieties cultivated in the same field trials as well as to the range of values reported globally for soybeans and for soybeans produced in Brazil in the ILSI Composition Database. The mean and range of urease values in grain of both CV127 treatments and the near-isogenic control soybeans were comparable to the mean and range of urease values obtained for the two conventional standard soybean varieties. In the case of trypsin inhibitor, the mean and range of values in grain of both CV127 treatments and the near-isogenic control soybeans were comparable to the mean and range of trypsin inhibitor values obtained for the two conventional standard soybean varieties, but values were below the range reported in the global category of the ILSI Crop Composition Database, and again this most likely is a characteristic of soybean varieties adapted for cultivation under tropical conditions in Brazil.

Overall, comparing antinutrient levels in both CV127 soybean treatments to levels in the near-isogenic control and the conventional standard Brazilian commercial varieties, these results show that antinutrient levels in grain produced by CV127 soybeans are comparable to and in the same range as antinutrient levels in grain of the control as well as conventional soybean varieties cultivated in Brazil that have a long history of safe food and feed uses. The instances where grain antinutrient levels of CV127, the near-isogenic control and the conventional standard soybean varieties differed from the range of levels reported in the global category of the ILSI Crop Composition Database are most likely due to characteristics of soybean varieties adapted for cultivation under tropical growing conditions in Brazil. Also, there was only one instance of a statistically significant difference in grain content of an antinutrient observed between the two CV127 treatments (raffinose in the 2006/2007 season), which shows that imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain antinutrient composition.

Table B.5-3. Proximate composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons compared with global and Brazilian ranges of soybean grain proximate composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Proximates										
Moisture %	**10.1**a* (9.2 - 10.9)	**9.3**b (8.7 - 10.2)	**9.4**b (8.8 - 9.9)	**9.8** (9.4-10.5)	**7.6**a* (7.1 - 8.2)	**7.8**a (7.4 - 8.2)	**7.9**b (7.6 - 8.2)	**7.7** (7.0-8.2)	**10.1** (4.7 - 34.4)	**9.8** (7.6 - 11.2)
Ash g/100 g dw	**5.0**a (4.6 - 5.3)	**5.0**a (4.6 - 5.5)	**4.9**a (4.5 - 5.4)	**4.9** (4.5-5.3)	**5.2**a (4.8 - 5.7)	**5.2**a (4.8 - 5.7)	**5.1**a (4.7 - 5.6)	**5.2** (4.9 - 5.5)	**5.32** (3.89 - 6.99)	**5.00** (4.58 - 5.47)
Protein g/100 g dw	**40.3**a (38.1 - 42.2)	**39.2**b (37.0 - 42.0)	**39.4**b (37.3 - 41.9)	**37.6** (36.4-39.6)	**39.2**a (36.4-41.6)	**39.2**ab (37.0-41.5)	**39.7**b (36.7-41.6)	**39.2** (36.8-42.0)	**39.47** (33.19-45.48)	**40.15** (37.19-44.85)
Fat g/100 g dw	**21.7**b (20.0 - 23.3)	**22.6**a (20.3 - 24.6)	**22.7**a (20.1 - 24.2)	**22.8** (20.2-24.8)	**20.2**a (17.7-23.8)	**20.7**b (18.8-24.7)	**20.5**ab (18.9-24.1)	**20.6** (16.9-25.1)	**16.68** (8.10-23.56)	**18.85** (14.44-23.56)
Total Dietary Fibre (g/100 g dw)	**24.90**a (21.93 - 26.90)	**24.55**a (21.61 - 26.64)	**24.70**a (22.13 - 28.11)	**25.43** (22.29-28.03)	**24.54**a (18.94-28.01)	**24.73**a (22.62-26.55)	**24.66**a (21.37-28.43)	**25.0** (21.9-27.3)	NA^	NA
Carbohydrate[1] g/100 g dw	**33.1**a (31.6 - 34.4)	**33.2**a (31.1 - 34.1)	**32.9**a (31.9 - 34.9)	**34.7** (27.39-42.03)	**35.4**a (25.2-44.9)	**34.8**a (27.9-39.9)	**34.7**a (24.2-43.3)	**35.0** (26.7-39.8)	**38.2** (29.6 - 50.2)	**36.0** (29.6 - 41.6)
Calories kcal/100 g dw	**390**b (379 - 402)	**395**a (389 - 407)	**395**a (384 - 405)	**393** (377-400)	**382**a (362-403)	**377**a (281-409)	**383**a (365-413)	**382** (374-395)	NA	NA

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$

^NA = not available

[1]Carbohydrates including total dietary fibre.

Table B.5-4.Fibre composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain fibre composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Fibre										
Crude Fibre g/100 g dw	**8.5**a* (6.9-11.0)	**8.3**a (6.9-11.1)	**8.1**a (6.4-11.3)	**8.2** (6.7-9.3)	**7.9**a* (6.7-10.6)	**8.0**a (7.1-9.7)	**8.2**a (6.2-14.7)	**8.2** (7.2-12.1)	**7.81** (4.12-13.87)	**8.46** (6.42-10.93)
ADF g/100 g dw	**11.39**b (8.84-14.95)	**13.14**a (9.75-15.92)	**13.76**a (10.96-19.00)	**11.76** (9.32-14.43)	**10.25**ab (8.53-12.05)	**9.66**a (8.09-11.19)	**10.49**b (7.35-13.40)	**11.11** (8.89-12.59)	**11.97** (7.81-18.61)	**11.34** (7.81-16.39)
NDF g/100 g dw	**14.98**b (12.04-17.32)	**17.46**a (12.72-20.55)	**17.52**a (14.06-20.01)	**14.85** (10.63-16.92)	**14.08**a (11.32-15.93)	**14.24**a (11.86-16.91)	**15.13**b (12.53-18.17)	**14.11** (12.26-16.18)	**12.33** (8.53-21.25)	**12.39** (8.53-21.25)

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

Table B.5-5.Amino acid composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain amino acid composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 08	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Amino Acids										
Alanine g/100 g dw	**1.64**a* (1.45 – 1.92)	**1.59**a (1.33 – 1.89)	**1.63**a (1.27 – 1.76)	**1.56** (1.39 – 1.68)	**1.51**a* (1.29 – 1.71)	**1.58**b (1.33 – 1.90)	**1.62**b (1.38 – 1.86)	**1.58** (1.41 – 1.79)	**1.72** (1.51 – 2.10)	**1.73** (1.62 – 1.85)
Arginine g/100 g dw	**3.03**a (2.64 – 3.09)	**2.91**a (2.35 – 3.51)	**3.01**a (2.36 – 3.33)	**2.71** (2.34 – 2.97)	**2.84**a (2.37 – 3.34)	**2.77**a (2.44 – 3.22)	**2.84**a (2.53 – 3.15)	**2.95** (1.49– 3.45)	**2.84** (2.29 – 3.40)	**2.93** (2.59 – 3.28)
Aspartate g/100 g dw	**4.64**a (4.02 – 5.37)	**4.46**a (3.67 – 5.24)	**4.61**a (3.66 – 5.17)	**4.32** (3.94 – 4.72)	**4.17**a (3.50 – 4.66)	**4.15**a (3.32 – 4.68)	**4.29**a (3.85 – 4.65)	**4.45** (3.70 – 5.06)	**4.49** (3.81 – 5.12)	**4.59** (4.23 – 5.12)
Cysteine g/100 g dw	**0.54**a (0.51 – 0.58)	**0.52**b (0.48 – 0.56)	**0.53**b (0.50 – 0.56)	**0.52** (0.49 – 0.56)	**0.51**b (0.43 – 0.55)	**0.52**a (0.43 – 0.56)	**0.52**a (0.46 – 0.56)	**0.52** (0.44 – 0.59)	**0.59** (0.37 – 0.81)	**0.57** (0.50 – 0.81)
Glutamate g/100 g dw	**7.61**a (6.59 – 8.92)	**7.30**a (6.10 – 8.46)	**7.54**a (5.83 – 8.45)	**6.98** (6.16 – 7.68)	**6.74**a (5.72 – 7.41)	**6.66**a (5.49 – 7.52)	**6.78**a (6.17 – 7.46)	**7.18** (5.90 – 8.55)	**7.09** (5.84 – 8.20)	**7.29** (6.58 – 8.09)
Glycine g/100 g dw	**1.65**a (1.45 – 1.87)	**1.62**a (1.33 – 1.94)	**1.65**a (1.32 – 1.81)	**1.56** (1.41 – 1.71)	**1.50**a (1.28 – 1.68)	**1.47**a (1.24 – 1.69)	**1.50**a (1.34 – 1.83)	**1.56** (1.35 – 1.72)	**1.69** (1.46 – 2.00)	**1.70** (1.56 – 1.82)
Histidine g/100 g dw	**0.87**a (0.78 – 1.03)	**0.83**a (0.69 – 1.04)	**0.85**a (0.67 – 0.97)	**0.79** (0.72 – 0.86)	**1.11**a (0.80 – 1.32)	**1.45**b (0.93 –2.11)	**1.37**b (0.91 – 2.13)	**1.10** (0.73 – 1.34)	**1.04** (0.88 – 1.18)	**1.06** (0.98 – 1.18)
Isoleucine g/100 g dw	**1.61**a (1.42 – 1.94)	**1.56**a (1.28 – 1.88)	**1.61**a (1.24 – 1.75)	**1.54** (1.38 – 1.71)	**1.46**a (1.22 – 1.62)	**1.41**a (1.10 – 1.83)	**1.43**a (1.08 – 1.97)	**1.50** (1.35 – 1.65)	**1.81** (1.54 – 2.08)	**1.85** (1.59 – 2.04)
Leucine g/100 g dw	**2.89**a (2.51 – 3.40)	**2.78**a (2.31 – 3.36)	**2.87**a (2.24 – 3.16)	**2.80** (2.45 – 3.65)	**2.56**a (2.19 – 2.86)	**2.60**a (2.30 – 2.86)	**2.61**a (2.44 – 2.85)	**2.73** (2.39 – 3.15)	**3.04** (2.59 – 3.62)	**3.07** (2.81 – 3.38)

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

Table B.5-5. Continued

Analyte (unit)	2006/2007 SEASON Isoline	CV127 N = 24	CV127 + imi	Conv. Stds. N = 12	2007 SEASON Isoline	CV127 N = 15	CV127 + imi	Conv. Stds. N = 8	Global N = 80 – 323	Brazilian N = 69
					Mean (range)					
Amino Acids										
Lysine g/100 g dw	**2.48**a* (2.12 – 2.85)	**2.40**a (2.00 – 2.93)	**2.46**a (1.93 – 2.68)	**2.33** (2.08 – 2.54)	**2.24**a (1.97 – 2.47)	**2.29**a (2.12 – 2.76)	**2.27**a (1.99 – 2.62)	**2.32** (2.03 – 2.58)	**2.56** (2.29 – 2.84)	**2.58** (2.42 – 2.82)
Methionine g/100 g dw	**0.63**a (0.60 – 0.67)	**0.62**b (0.59 – 0.68)	**0.62**b (0.59 – 0.64)	**0.60** (0.55 – 0.65)	**0.60**b (0.53 – 0.66)	**0.61**a (0.51 – 0.66)	**0.62**a (0.58 – 0.66)	**0.60** (0.53 – 0.69)	**0.55** (0.43 – 0.68)	**0.55** (0.50 – 0.68)
Phenylalanine g/100 g dw	**1.99**a (1.77 – 2.37)	**1.91**a (1.59 – 2.29)	**1.98**a (1.52 – 2.19)	**1.87** (1.64 – 2.09)	**1.76**a* (1.52 – 1.94)	**1.80**a (1.55 – 2.16)	**1.78**a (1.65 – 1.90)	**1.87** (1.64 – 2.12)	**1.98** (1.63 – 2.35)	**2.06** (1.82 – 2.24)
Proline g/100 g dw	**1.98**a (1.74 – 2.32)	**1.86**b (1.56 – 2.31)	**1.91**ab (1.49 – 2.12)	**1.82** (1.58 – 2.03)	**1.74**a (1.50 – 1.95)	**1.77**a (1.56 – 1.99)	**1.73**a (1.11 – 1.89)	**1.86** (1.60 – 2.05)	**2.00** (1.69 – 2.28)	**2.06** (1.86 – 2.28)
Serine g/100 g dw	**2.09**a (1.83 – 2.39)	**2.02**a (1.7 – 2.41)	**2.07**a (1.63 – 2.29)	**1.96** (1.78 – 2.16)	**1.82**a (1.54 – 2.05)	**1.81**a (1.56 – 1.99)	**1.83**a (1.65 – 1.97)	**1.92** (1.67 – 2.21)	**2.02** (1.11 – 2.48)	**2.17** (1.96 – 2.48)
Threonine g/100 g dw	**1.56**a (1.34 – 1.80)	**1.50**a (1.25 – 1.81)	**1.55**a (1.25 – 1.72)	**1.48** (1.36 – 1.55)	**1.37**a (1.21 – 1.50)	**1.35**a (1.14 – 1.62)	**1.37**a (1.17 – 1.67)	**1.40** (1.23 – 1.55)	**1.47** (1.14 – 1.86)	**1.40** (1.28 – 1.52)
Tryptophan g/100 g dw	**0.73**a (0.59 – 0.84)	**0.72**a (0.60 – 0.92)	**0.76**a (0.60 – 1.03)	**0.77** (0.57 – 1.16)	**0.64**a (0.53 – 0.74)	**0.65**a (0.50 – 0.79)	**0.67**a (0.51 – 0.84)	**0.65** (0.56 – 0.73)	**0.43** (0.36 – 0.50)	**0.44** (0.38 – 0.49)
Tyrosine g/100 g dw	**1.34**a (1.17 – 1.55)	**1.27**b (1.08 – 1.50)	**1.31**ab (1.06 – 1.42)	**1.27** (1.12 – 1.39)	**1.18**a (0.98 – 1.33)	**1.13**b (1.01 – 1.33)	**1.14**b (1.02 – 1.38)	**1.25** (1.14 – 1.45)	**1.32** (1.02 – 1.61)	**1.38** (1.27 – 1.56)
Valine g/100 g dw	**1.66**a (1.36 – 1.98)	**1.60**a (1.34 – 1.94)	**1.64**a (1.26 – 1.79)	**1.58** (1.40 – 1.67)	**1.56**a (1.35 – 1.83)	**1.64**b (1.49 – 1.84)	**1.62**ab (1.41 – 1.79)	**1.62** (1.31 – 1.87)	**1.91** (1.60 – 2.20)	**1.91** (1.63 – 2.08)

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

Table B.5-6. Fatty acid composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain fatty acid Composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Fatty Acids										
Myristic 14:0 %Total FA	**<0.09**a* (nd – 0.10)	**<0.09**a (nd – 0.10)	**<0.09**a (nd – 0.10)	**0.10** N = 7 (0.09 – 0.11)	**0.11**a*N = 5 (0.09 - 0.12)	**0.11**a N = 13 (0.9 - 0.12)	**0.11**a N = 7 (0.10 - 0.11)	**0.09** N = 4 (0.06 - 0.12)	NA^	NA
Palmitic 16:0 % Total FA	**9.77**b (9.12 – 10.44)	**10.00**a (9.23 – 10.42)	**9.98**a (9.35 – 10.59)	**10.17** (9.08 – 11.16)	**10.32**a (9.67 – 10.82)	**10.24**a (9.85 – 10.93)	**10.18**a (9.75 – 10.64)	**9.77** (8.31 – 10.67)	**11.12** (9.55 – 15.77)	**11.27** (10.28 – 12.73)
Margaric 17:0 % Total FA	**<0.10**a (nd¥ – 0.11)	**<0.10**b (nd – 0.11)	**<0.10**b (nd – 0.11)	**<0.10** (nd – 0.11)	**0.11**a (0.09 – 0.12)	**0.11**a (0.09 – 0.12)	**0.11**a (0.09 – 0.11)	**0.10** (0.06 – 0.12)	**0.114** (0.085 – 0.146)	**0.106** (0.086 – 0.116)
Stearic 18:0 % Total FA	**3.39**a (2.84 – 3.87)	**3.38**a (2.93 – 3.86)	**3.30**a (2.77 – 3.61)	**3.46** (2.97 – 3.92)	**4.04**b (3.14 – 5.05)	**4.13**ab (3.45 – 4.98)	**4.21**a (3.50 – 4.80)	**3.99** (3.04 – 5.01)	**4.01** (2.70 – 5.88)	**3.95** (2.70 – 5.52)
Oleic 18:1 % Total FA	**20.07**c (18.47 – 21.02)	**21.44**b (20.34 – 22.74)	**22.07**a (20.10 – 25.76)	**20.06** (18.54 – 21.38)	**24.38**b (21.72– 33.15)	**25.64**b (22.81 – 32.45)	**27.79**a (22.65 – 43.63)	**23.39** (20.13 – 28.06)	**20.7** (14.3 – 32.2)	**22.6** (18.7 – 28.9)
Linoleic 18:2 % Total FA	**45.87**b (44.01 – 47.68)	**45.42**a (43.05 – 46.70)	**45.00**a (42.60 – 46.62)	**53.54** (52.36 – 54.40)	**48.86**a (42.54 – 50.83)	**47.72**a (43.25 – 50.50)	**45.65**b (31.97 – 49.57)	**50.10** (46.78 – 52.80)	**53.3** (42.3 – 58.8)	**52.6** (48.2 – 55.5)
Linolenic 18:3 % Total FA	**5.65**a (5.05 – 6.10)	**5.21**b (4.80 – 5.67)	**5.10**c (4.59 – 5.72)	**7.23** (6.31 – 8.15)	**6.62**a (4.20 – 8.11)	**6.47**ab (3.92 – 8.25)	**6.32**b (3.42 – 8.12)	**7.06** (4.76 – 8.52)	**8.34** (3.00 – 12.52)	**7.06** (5.92 – 8.18)
Arachidic 20:0 % Total FA	**0.37**a (0.32 – 0.44)	**0.35**a (0.24 – 0.44)	**0.34**a (0.26 – 0.39)	**0.31** (0.26 – 0.40)	**0.39**a (0.32 – 0.49)	**0.42**a (0.39 – 0.46)	**0.42**a (0.27 – 0.55)	**0.33** (0.26 – 0.48)	**0.32** (0.16 – 0.48)	**0.37** (0.28 – 0.48)
Eicosenoic 20:1 % Total FA	**0.13**a (0.09 – 0.19)	**0.12**a (0.08 – 0.20)	**0.13**a (0.08 – 0.18)	**0.14** (0.09 – 0.20)	Not detected	Not detected	Not detected	Not detected	**0.20** (0.14 – 0.35)	**0.22** (0.17 – 0.28)
Behenic 22:0 % Total FA	**0.46**a (0.37 – 0.52)	**0.42b** (0.34 – 0.46)	**0.43**ab (0.39 – 0.53)	**0.40** (0.37 – 0.50)	**0.51**a (0.40 – 0.75)	**0.49**a (0.40 – 0.63)	**0.50**a (0.38 – 0.80)	**0.44** (0.37 – 0.51)	**0.40** (0.28 – 0.60)	**0.45** (0.37 – 0.57)

*Numbers followed by the same letter are not statistically significantly different at p<0.05.

^NA = not available; ¥nd = not detectable

Table B.5-7. Mineral composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain mineral composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Minerals										
Calcium mg/100 g dw	**268**b* (221 – 330)	**277**a (231 – 327)	**266**b (214 – 318)	**254** (205 – 313)	**246**a* (172 – 359)	**274**b (190 – 427)	**286**b (198 – 484)	**234** (167 – 329)	**217** (117 – 307)	NA^
Iron mg/100 g dw	**8.50**a (6.01 – 10.43)	**7.89**b (5.56 – 10.91)	**7.75**b (5.79 – 10.48)	**8.57** (6.54 – 10.35)	**9.16**a (7.98 – 10.90)	**9.88**b (8.23 – 13.00)	**9.15**a (7.80 – 11.40)	**10.16** (7.93 – 14.13)	**7.81** (5.54 – 10.95)	NA
Magnesium mg/100 g dw	**246**b (204 – 266)	**266**a (218 – 326)	**266**a (227 – 304)	**269** (225 – 308)	**238**a (211 – 266)	**277**b (258 – 309)	**287**c (268 – 309)	**255** (223 – 293)	**264** (219 – 313)	NA
Phosphorus mg/100 g dw	**687**a (541 – 834)	**677**a (515 – 791)	**667**a (546 – 760)	**670** (527 – 875)	**768**a (655 – 845)	**717**b (654 – 784)	**732**b (580 – 871)	**745** (614 – 808)	**715** (507 – 935)	NA
Potassium mg/100 g dw	**1928**a (1782 – 2071)	**1897**ab (1720 – 2164)	**1881**b (1703 – 2069)	**1961** (1772 – 2103)	**1744**a (1593 – 2061)	**1877**b (1713 – 2079)	**1864**b (1599 – 2021)	**1758** (1595 – 2033)	**2061** (1868 – 2316)	NA

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$

^NA = not available.

Table B.5-8. Vitamin composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an Imidazolinone Herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain vitamin composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Vitamins										
Folic Acid µg/100 g dw	**330**a* (216 – 456)	**267**b (201 – 327)	**270**b (183 – 338)	**291** (205 – 403)	**365**a* (249 – 462)	**363**a (302 – 460)	**374**a (320 – 464)	**323** (261 – 438)	**360** (240 – 470)	NA^
α-tocopherol mg/100 g dw	**3.04b** (2.33 – 4.44)	**3.44**a (2.86 – 4.86)	**3.49**a (2.43 – 4.69)	**3.22** (2.45 – 4.61)	**3.21**a (1.74 – 8.17)	**3.53**ab (1.96 – 6.94)	**3.67**b (1.96 – 9.23)	**3.54** (2.07 – 7.51)	**1.91** (0.19 – 6.17)	**3.44** (1.36 – 6.17)
β-tocopherol mg/100 g dw	**0.60**b (0.20 – 1.01)	**0.90**a (0.58 – 1.32)	**0.90**a (0.58 – 1.22)	**0.70** (0.12 – 1.08)	**0.84**a (0.54 – 1.15)	**1.04**b (0.73 – 1.49)	**0.96**ab (0.68 – 1.37)	**0.88** (0.53 – 1.34)	NA	NA
γ-tocopherol mg/100 g dw	**16.51**a (11.62 – 20.83)	**15.83**b (12.50 – 17.69)	**15.81**b (12.31- 18.84)	**16.28** (12.68 – 20.88)	**16.41**a (13.79 – 21.63)	**16.06**a (13.91 – 21.05)	**14.96**b (12.28 – 18.46)	**15.68** (11.65 – 22.27)	NA	NA
δ-tocopherol mg/100 g dw	**6.18**b (4.41 -7.53)	**6.49**a (4.85 – 8.15)	**6.56**a (4.9 – 7.99)	**6.09** (5.31 – 7.42)	**7.65**a (4.52 – 9.72)	**8.68**b (4.67 – 11.72)	**8.15**ab (3.31 – 11.35)	**6.99** (5.16 – 8.94)	NA	NA
Total tocopherol mg/100 g dw	**26.19**a (19.26 – 31.54)	**26.57**a (21.59 – 29.61)	**26.75**a (21.95 – 31.12)	**26.32** (21.68 – 31.03)	**28.12**ab (25.02 – 34.43)	**29.31**a (26.72 – 35.38)	**27.41**b (23.38 – 31.40)	**27.09** (21.56 – 33.23)	NA	NA
Vitamin B1 mg/100 g dw	**0.65**a (0.44 – 0.86)	**0.51**b (0.40 – 0.67)	**0.52**b (0.34 – 0.78)	**0.58** (0.42 – 0.71)	**0.52**a (0.34 – 0.80)	**0.55**a (0.35 – 0.72)	**0.55**a (0.28 – 0.75)	**0.48** (0.31 – 0.71)	**0.20** (0.10 – 0.25)	NA
Vitamin E mg/100 g dw	**5.51**b (4.11 – 7.21)	**5.88**a (5.05 – 7.30)	**5.91**a (4.68 – 7.16)	**5.70** (4.95 - 7.05)	**5.75**a (3.94 – 10.79)	**6.08**b (4.35 – 10.09)	**6.05**ab (4.35 – 11.21)	**5.98** (4.64 – 9.92)	NA	NA

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$; ^NA = not available

Table B.5-9. Isoflavone composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain isoflavone composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Isoflavones										
Total Daidzein mg/100 g dw	**72.2**b* (48.4 – 106.6)	**52.8**a (41.2 – 67.7)	**52.4**a (41.4 – 72.4)	**81.3** (66.2 – 96.2)	**114.6**a* (19.7 – 237.8)	**77.9**b (22.6 – 162.2)	**79.0**b (14.7 – 161.0)	**100.2** (30.8 – 186.4)	**86.3** (6.0 – 245.4)	**51.0** (6.0 – 112.9)
Total Genistein mg/100 g dw	**101.7**b (57.1 – 153.4)	**86.1**a (56.9 – 138.0)	**83.4**a (60.2 – 121.0)	**134.5** (102.8 – 166.6)	**144.5**a (26.1 – 270.1)	**115.9**b (26.8 – 232.4)	**114.6**b (11.8 – 246.8)	**144.6** (54.3 – 255.1)	**97.9** (14.4 – 283.7)	**65.2** (14.4 – 135.7)
Total Glycitein mg/100 g dw	**22.3**a (14.9 – 31.4)	**21.7**a (16.6 – 27.5)	**21.7**a (14.6 – 30.2)	**49.2** (36.3 – 75.4)	**19.0**a (7.3 – 30.2)	**17.5**ab (11.5 – 25.7)	**16.5**b (7.4 – 23.9)	**35.3** (27.9 – 43.0)	**16.1** (1.5 – 31.0)	**13.3** (1.5 – 26.4)

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

Table B.5-10. Phospholipid composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain phospholipid composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Phospholipids										
Phosphatidyl ethanolamine mg/g fat	**101.9**a* (89.7 – 127.9)	**92.6**b (51.0 – 110.3)	**90.9**b (57.9 – 108.9)	**98.2** (79.4 – 113.7)	**109.4**a* (65.7 – 156.8)	**109.2**a (61.7 – 155.5)	**106.0**a (52.1 – 139.5)	**100.5** (51.1 – 133.7)	NA^	NA
Phosphatidic acid mg/g fat	**4.0**a (1.8 – 6.9)	**2.8**b (0.8 – 4.6)	**2.9**b (1.0 – 4.7)	**4.1** (2.1 – 7.5)	**2.1**a (0.7 – 6.7)	**2.1**a (0.6 – 6.1)	**2.6**b (0.5 – 9.9)	**3.0** (0.8 – 7.2)	NA	NA
Phosphatidyl inositol mg/g fat	**11.8**a (10.1 – 14.2)	**9.8**b (6.8 – 11.0)	**9.6**b (6.1 – 11.2)	**12.3** (11.4 – 13.5)	**10.4**a (8.6 – 14.8)	**10.0**a (8.1 – 13.2)	**9.7**a (8.0 – 11.0)	**10.8** (8.1 – 12.4)	NA	NA
Phosphatidyl choline mg/g fat	**29.3**a (24.9 – 38.5)	**27.0**b (14.9- 34.1)	**26.9**b (15.9 – 32.3)	**30.7** (25.6 – 38.2)	**32.9**a (20.6 – 45.0)	**32.2**a (19.8 – 39.4)	**32.5**a (17.5 – 40.2)	**33.4** (17.0 – 42.0)	NA	NA

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.
^NA = not available

Table B.5-11. Antinutrient composition of grain derived from the near-isogenic control (isoline), CV127 soybean treated with a conventional herbicide (CV127), CV127 treated with an imidazolinone herbicide (CV127 + imi) and two conventional standard soybean varieties grown in the 2006/2007 and 2007 seasons and compared with global and Brazilian ranges of soybean grain antinutrient composition values from the ILSI Crop Composition Database. The mean values for the conventional soybean comparator varieties, Monsoy 8001 and Coodetec 217, were combined to provide a single mean value (Conv. Stds.) for each analyte. Values that are statistically significantly different are shaded in grey.

	2006/2007 SEASON				2007 SEASON					
Analyte (unit)	**Isoline**	**CV127** N = 24	**CV127 + imi**	**Conv. Stds.** N = 12	**Isoline**	**CV127** N = 15	**CV127 + imi**	**Conv. Stds.** N = 8	**Global** N = 80 – 323	**Brazilian** N = 69
	Mean (range)									
Antinutrients										
Phytic Acid mg/g dw	**2.95**a* (1.43 – 6.09)	**2.75**a (1.25 – 4.52)	**2.54**a (0.71 – 5.27)	**2.89** (1.47 – 7.39)	**3.36**a* (1.89 – 6.00)	**3.96**a (2.57 – 6.22)	**3.81**a (2.63 – 4.77)	**4.06** (2.37 – 5.44)	**11.21** (6.34 – 19.60)	NA
Raffinose g/100 g dw	**1.1**c (0.9 – 1.5)	**1.4**a (1.0 – 1.8)	**1.3**b (1.0 – 1.7)	**1.1** (0.8 – 1.6)	**1.30**a (1.00 – 1.50)	**1.2**b (0.9 – 1.3)	**1.2**ab (0.9 – 1.7)	**1.3** (1.1 – 1.4)	**0.355** (0.212 – 0.661)	NA
Stachyose g/100 g dw	**3.7**a (3.0 – 4.2)	**3.6**b (2.9 – 4.0)	**3.6**b (3.1 – 4.1)	**3.8** (3.1 – 4.6)	**4.0**a (3.0 – 4.6)	**3.7**b (3.0 – 4.2)	**3.6**b (2.4 – 4.1)	**4.1** (3.1 – 4.8)	**2.19** (1.21 – 3.50)	NA
Lectins HU^1/mg dw	**2.24**a (1.35 – 3.35)	**2.23**a (1.43 – 3.70)	**2.20**a (1.27 – 3.49)	**2.03** (1.38 – 3.53)	**1.70**a (0.85 – 3.43)	**0.84**b (0.17 – 1.71)	**0.92**b (0.11 – 3.43)	**1.65** (0.67 – 2.67)	**1.718** (0.105 – 9.038)	**0.815** (0.299 – 1.892)
Urease ΔpH	**1.93**a (1.27 – 2.12)	**1.85**a (0.67 – 2.18)	**1.91**a (0.90 – 2.21)	**1.93** (1.43 – 2.16)	**1.53**a (0.28 – 2.06)	**1.57**a (0.41 – 2.00)	**1.58**a (0.49 – 2.03)	**1.63** (0.27 – 2.02)	NA^	NA
Trypsin Inhibitor TIU/mg dw	**12.29**a (6.03 – 16.4)	**12.38**a (8.69 – 16.58)	**12.01**a (9.14 – 15.13)	**11.45** (5.03 – 19.64)	**13.16**a (8.48 – 17.97)	**13.72**a (8.16 – 18.20)	**13.80**a (7.82 – 18.03)	**14.02** (9.84 – 16.76)	**48.33** (19.59 – 118.68)	NA

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

^NA = not available

[1] Hemagglutinating Units

Forage composition

Soybean has many uses in animal and human nutrition. The main soybean product fed to animals is the defatted/toasted soybean meal (OECD, 2001). However, other components of the soybean plant, including the forage, are also fed to a limited extent to animals, primarily to cattle. Soybean forage is typically harvested between the time the plants reach the sixth node stage to the beginning of pod formation. Forage of CV127 soybeans was produced from plants grown in field trials at six field trial locations in Brazil during the summer of 2007/2008. At each field location, the above ground portion of three plants from three replicate plots was harvested when the plants were at the R2 growth stage. The purpose of this study was to determine if forage of CV127, treated with an imidazolinone herbicide, is substantially equivalent in composition to forage from the near-isogenic control and other conventional standard soybean varieties, and that CV127 forage is appropriate for use in animal feed. The results of the analysis are presented in **Appendix 20**.

Proximates and fibres. The proximate and fibre composition results calculated and statistically analysed across all locations for forage samples of CV127 soybean treated with imidazolinone herbicide are compared in **Table B.5-12** to those of the near-isogenic control variety and the two commercial standard varieties treated with conventional herbicide. There were no statistically significant differences in levels of moisture, ash, fat, protein, carbohydrates, calories, crude fibre, ADF and NDF in forage samples produced from CV127 soybean compared to levels of these analytes in the near-isogenic control variety. In addition, comparison of the forage composition of CV127 soybean and the near-isogenic control variety with that of the two conventional standard soybean varieties demonstrated that there were no statistically significant differences among these treatments for ash, fat, calories, ADF or NDF. Where small differences in analyte values were observed between CV127 and the conventional standard soybean varieties, this was attributed to germplasm differences, since the same differences were observed between the near-isogenic control and the conventional standard varieties.

The results of this study confirm that forage derived from CV127 soybean contains the same level of nutrients as the control and similar levels as conventional standard soybean varieties that are currently cultivated. These results further support the conclusion that CV127 soybean is compositionally equivalent and as nutritious as conventional varieties with a long history of safe use in animal feed.

Table B.5-12. Proximate and fibre composition of soybean forage, comparing CV127 soybean, the near-isogenic control and two conventional soybean varieties (Std 1 and Std 2) across six locations in Brazil in the 2007/2008 season.

Analyte/Unit	Isoline	CV127	Std 1	Std 2
	N = 18**			
Proximates	Mean (range)			
Moisture g/100 g fw	**81.4** ab* (79.0 – 84.3)	**81.1** b (78.4 – 85.3)	**82.3** a (78.4 – 85.3)	**81.4** a (73.1 – 84.2)
Ash g/100 g dw	**8.5** c (7.1 – 10.3)	**8.6** bc (7.0 – 10.7)	**9.0** a (6.4 – 11.2)	**9.0** ab (6.8 – 10.5)
Fat g/100 g dw	**2.5** a (1.7 – 3.5)	**2.6** a (1.6 – 3.4)	**2.6** a (1.9 – 3.5)	**2.5** a (1.7 – 4.1)
Protein g/100 g dw	**17.7** b (15.1 – 19.5)	**17.3** b (15.3 – 19.5)	**19.0** a (15.9 – 23.1)	**19.1** a (17.0 – 22.4)
Carbohydrates g/100 g dw	**71.0** a (66.9 – 73.1)	**71.6** a (66.7 – 75.6)	**69.5** b (62.4 – 74.9)	**69.6** b (64.8 – 73.5)
Calories kcal/100 g dw	**378** a (367 – 386)	**379** a (371 – 387)	**377** a (368 – 385)	**378** a (372 – 387)
Fibre				
Crude Fibre g/100 g dw	**29.8** a (27.5 – 33.2)	**29.8** a (27.9 – 32.7)	**28.8** b (25.1 – 32.3)	**29.6** a (27.0 – 33.1)
ADF g/100 g dw	**36.57** a (31.79 – 42.81)	**36.41** a (33.39 – 42.16)	**35.68** a (28.94 – 41.82)	**36.00** a (30.43 – 44.58)
NDF g/100 g dw	**45.28** a (39.71 – 50.90)	**45.36** a (40.07 – 52.85)	**44.45** a (39.33 – 50.67)	**44.68** a (38.93 – 52.28)

*Numbers followed by the same letter are not statistically significantly different at $p<0.05$.

**N = 17 for Std 1 and Std 2 for ADF and NDF

Composition of processed fractions of grain

A grain processing study was conducted to confirm that the grain processing characteristics as well as composition of the processed fractions from CV127 soybean are equivalent to those of the near-isogenic control variety and other conventional standard soybean varieties commonly cultivated in Brazil, and thereby also confirm that processed fractions from CV127 soybean are appropriate for use in soybean-derived food and feed products. The methods used to produce and process the different fractions were standard methods that are representative of those currently used to commercially process soybean grain. A schematic diagram that presents the methods used to produce the processed soybean fractions is presented in **Appendix 15.**

The nutrient composition of the toasted, defatted soybean meal, protein isolate and concentrate and the refined oil samples were analysed. Since the only difference between the toasted and untoasted defatted soybean meals is the toasting or heating treatment used to produce the former, the nutrient composition of these fractions are expected to be very similar and so only the toasted, defatted soybean meal samples were analysed for composition. Also, the toasted soybean meal is used almost exclusively in animal feed compared to the untoasted meal, so the toasted soybean meal was the most relevant fraction for analysis to confirm nutritional value for animal feed. The analyses conducted included proximates (moisture, ash, fat, protein, carbohydrates, and calories), fibre (crude, acid detergent [ADF] and neutral detergent [NDF]), antinutrients (raffinose, stachyose, trypsin inhibitor, urease and phytic acid) and isoflavones for the toasted soybean meal and proximates only for the protein isolate and concentrate fractions. The refined oil fractions were analysed for fatty acid composition. The methods of analysis are presented in **Appendix 15** of this submission. Results are reported on a dry weight basis unless otherwise noted. In this study, statistical analyses of the compositional data included all treatments: CV127 + imi, near-isogenic control, and the two conventional standard soybean reference varieties. Statistical analysis methods were as described for compositional analyses of the grain.

<u>Toasted defatted soybean meal</u>

Proximates: The mean proximate and fibre values of the toasted defatted soybean meals across all field trial locations are presented in **Table B.5-13**. Except for moisture, there were no statistically significant differences in proximate or fibre composition in toasted defatted soybean meal derived from CV127 soybean compared to toasted defatted soybean meal derived from grain of the near-isogenic control. In the case of moisture, the value obtained for the soybean meal from CV127 soybean was significantly lower than the value for meal from the near-isogenic control, but it was not significantly different from the same values for meal produced from one of the two conventional standard soybean varieties. Therefore, the difference in moisture content of the toasted soybean meal between CV127 and the near-isogenic control is most likely due to slight variations in toasting conditions between treatments. In addition, there were no statistically significant differences in levels of proximates and the different fibre fractions in toasted meal between CV127 and either one or both of the two conventional standard soybean varieties in this study, except for protein,

carbohydrates and ADF. These differences were attributed to germplasm differences between the varieties, since similar differences were observed between the near-isogenic control and the two conventional standard soybean varieties. Finally, all proximate mean values for the toasted, defatted soybean meal from CV127 soybeans, except for carbohydrates, were within the ranges of those nutrients reported in the literature (**Table B.5-13**). Levels of carbohydrates in the toasted meal of all four soybean treatments in this study were slightly higher than the range reported for this nutrient class in the literature, suggesting that this may be a characteristic of soybean varieties adapted for cultivation under the tropical agricultural conditions of Brazil. Collectively, these data show that proximate and fibre levels in toasted soybean meal derived from CV127 soybean grain is equivalent to levels in the meal derived from the near-isogenic control as well as other conventional standard soybean varieties commonly cultivated in Brazil.

Antinutrients: The mean values for antinutrients of the toasted defatted soybean meals across all locations are presented in **Table B.5-14**. Except for trypsin inhibitor, the antinutrient values obtained for toasted defatted soybean meal produced from grain of CV127 soybean were not significantly different from the values obtained for the soybean meal from the near-isogenic control soybean. In addition, there were no statistically significant differences in levels of antinutrients in the toasted meal fraction between CV127 and either one or both of the two conventional standard soybean varieties in this study, except for the trypsin inhibitor. The mean value for trypsin inhibitor from meal of CV127 soybean was statistically significantly higher than the values obtained for the meal from the near-isogenic control and the two conventional standard soybean varieties but, it was below the lower end of the range reported for this antinutrient in the literature for other soybean varieties (**Table B.5-14**). However, the mean values of the other antinutrients present in toasted soybean meal determined for CV127 soybean were within the ranges for these antinutrients reported in the literature.

Isoflavones: The mean values for isoflavones in toasted defatted soybean meals were determined and the values for total isoflavones were calculated by adding the values for daidzin, malonyl daidzin, acetyl daidzin and daidzein for total daidzein; glycitin, malonyl glycitin, acetyl glycitin, and glycitein for total glycitein; and genistin, malonyl genistin, acetyl genistin, and genistein for total genistein. The mean values for total isoflavones in the toasted defatted soybean meals across all locations are presented in **Table B.5-15**. A comparison of the mean total isoflavone values determined for CV127 soybean meal with those from the near-isogenic control show that for total glycitein, there was no significant difference between the mean values for meal from CV127 and the near-isogenic control, but levels of this isoflavone were lower in both CV127 and the near-isogenic control compared to the two conventional standard soybean varieties, suggesting that this difference is due to varietal differences. For total daidzein and total genistein, the values for CV127 soybean meal were significantly lower than the values obtained for the near-isogenic control and the two conventional soybean varieties included in this study. These results were consistent with differences in levels of these isoflavones measured in grain of the

treatments (**Table B.5-9**). Levels of total daidzein and genistein were statistically significantly lower in grain of CV127 compared to levels in grain of the near-isogenic control. However, mean levels of these isoflavones in CV127 were within the range of values reported for soybean varieties globally as well as for varieties cultivated in Brazil (ILSI Composition Database, **Table B.5-9**). Therefore, it is expected that levels of these isoflavones in toasted meal of CV127 would similarly be within the range of values for toasted meal of other soybean varieties both globally and in Brazil.

Protein isolate and protein concentrate

Proximates: The mean proximate values for both protein isolate and protein concentrate fractions for all treatments across locations are presented in **Tables B.5-16 and B.5-17**, respectively. For the proximate values of the protein isolate fraction, the only analyte that was statistically significantly different between CV127 soybean and the near-isogenic control was ash where the value for CV127 was significantly lower compared to the near-isogenic control, but it was not different from either of the two conventional standard soybean varieties (**Table B.5-16**). Comparison of the mean proximate values obtained from the protein isolate fraction produced from CV127 soybean with the ranges of these nutrients reported for protein isolate from soybean in the literature demonstrated that, except for the mean fat values for the protein isolate, the mean values from CV127 soybean were either within or comparable to the ranges reported for these nutrients in the literature (**Table B.5-16**). For all four treatments, the mean value for fat in the protein isolate was above the range for fat that is reported for soybean protein isolates in the literature, which suggests that the higher levels of fat observed in the protein isolate from the four treatments in this study is a characteristic of soybean varieties adapted for cultivation in Brazil. In the case of the proximate values for protein concentrate, there were no statistically significant differences in any of the proximate analytes between protein concentrate prepared from grain of CV127 soybean and the near-isogenic control variety as well as the two conventional soybean varieties, with the one exception of ash in Standard variety 1 (**Table B.5-17**). Furthermore, mean values for ash and protein in CV127 protein concentrate were either within or comparable to the range of values reported in the literature for protein concentrate from other soybean varieties. Levels of fat and carbohydrates in the protein concentrate fraction for all treatments were outside the range reported in the literature for this soybean protein fraction. Therefore these differences from the literature range are most likely a characteristic common to soybean varieties adapted for cultivation in Brazil.

Refined oil

Fatty acids: The mean values for fatty acids in the refined oil fractions of the different soybean treatments across all field locations are presented in **Table B.5-18**. Myristic acid (14:0) was detected in the processed oil fractions, but was present below the level of quantification and is not included in the table. Comparison of the mean fatty acid values of the refined oil from grain of CV127 soybean with those from grain derived from the near-isogenic control variety showed no statistically significant differences in fatty acid content between the treatments, except for higher oleic acid content and significantly lower content for linoleic, linolenic, and behenic acids in CV127 oil

compared to that of the near-isogenic control. Furthermore, comparison of the mean fatty acid values obtained from the refined oil produced from grain of CV127 soybean with the range of values reported in the literature for soybean oil from different soybean varieties demonstrated that, with the exception of behenic acid, all mean values for fatty acids from the refined oil of CV127 soybean were within the ranges for fatty acid content of soybean oil reported in the literature (**Table B.5-18**). The mean values obtained for behenic acid from both CV127 and the near-isogenic control soybean were slightly higher than the range reported in the literature. There were statistically significant differences in fatty acid content of the oil between the two conventional standard comparator soybean varieties in this study compared to both CV127 and the near-isogenic control, suggesting that these differences in fatty acid content of the oil reflected differences between soybean varieties.

The results generated by these analyses of the processed soybean fractions produced from CV127 soybean, the near-isogenic control, and two conventional standard varieties support the conclusion that the nutrient and antinutrient composition of the processed fractions from CV127 soybean are within the same range or comparable to the composition of similar processed soybean fractions produced from grain of the near-isogenic control and two conventional standard soybean comparator varieties. Also for most analytes the values for CV127 soybean were either within or comparable to the literature reference range of values reported for these soybean processed fractions. Therefore, composition of the toasted meal, protein isolate and concentrate, and refined oil fractions of CV127 soybean are equivalent to composition of these same processed fractions derived from the near-isogenic control and other conventional soybean varieties, and confirms that processed fractions from CV127 soybean are appropriate for use in human foods as well as animal feeds.

Table B.5-13. Defatted toasted meal proximate and fibre composition.
Comparison of the means and ranges for the proximate and fibre content of the defatted toasted meal processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The defatted toasted meal was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte unit	Isoline	CV127	Comm. Std. 1	Comm. Std. 2	Literature Range
	N = 4				
Proximates	**Mean** (range)				
Moisture g/100 g fw	**4.3**a* (3.8 - 4.8)	**3.4**b (3.2 - 3.8)	**3.9**ab (3.6 - 4.1)	**4.5**a (3.6 - 5.5)	NA^
Ash g/100 g dw	**6.3**ab (6.0 - 6.6)	**6.3**ab (6.1 - 6.6)	**6.2**b (5.8 - 6.5)	**6.3**ab (5.8 - 6.6)	**5.5 - 6.5**[1]
Fat g/100 g dw	**1.2**a (0.4 - 1.9)	**1.2**a (0.9 - 1.9)	**1.0**a (0.6 - 1.4)	**1.2**a (0.7 - 2.4)	**0.5 - 2.40**[2]
Protein g/100 g dw	**51.1**a (49.8 - 53.0)	**50.6**a (48.8 - 51.4)	**48.2**b (46.4 - 49.3)	**48.1**b (47.3 - 49.1)	**44 - 61.4**[3]
Carbohydrates‡ g/100 g dw	**41.5**b (40.1 - 42.1)	**41.9**b (41.3 - 42.7)	**44.6**a (43.4 - 45.7)	**44.4**a (43.8 - 45.4)	**32.0 - 38.0**[4]
Calories kcal/100 g dw	**381**a (377 - 384)	**381**a (380 - 383)	**381**a (378 - 382)	**381**a (377 - 389)	NA
Fibre					
Crude Fibre g/100 g dw	**10.1**a (9.5 - 10.7)	**10**a (9.7 - 10.2)	**10.5**a (9.9 - 11.3)	**10.5**a (8.9 - 12.4)	NA
ADF g/100 g dw	**8.41**bc (7.87 - 8.94)	**7.74**c (7.31 - 7.92)	**9.44**a (8.72 - 9.88)	**9.11**ab (8.41 - 9.83)	NA
NDF g/100 g dw	**16.49**ab (15.01 - 17.26)	**14.86**b (13.17 - 17.17)	**16.4**ab (15.43 - 17.50)	**18.01**a (16.50 - 20.46)	NA

*Values in the same row followed by the same letter are not significantly different at $p<0.05$.
‡Carbohydrates including total dietary fibre.
[1]Fulmer (1988), Orthoefer (1978); [2]Han *et al.* (1991), Orthoefer (1978); [3]Orthoefer (1978), Smith and Circle (1972); [4]Waggle and Kolar (1979)
^Not available

Table B.5-14. Defatted toasted meal antinutrient composition.
Comparison of the means and ranges for the antinutrient content of the defatted toasted meal processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The defatted toasted meal was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte (unit)	Isoline	CV127	Conv. Std. 1	Conv. Std. 2	Literature Range
	N = 4				
Antinutrients	Mean (range)				
Raffinose g/100 g dw	**1.9**ab* (1.6 – 2.3)	**1.9**b (1.5 – 2.2)	**2.1**a (1.7 – 2.5)	**1.8**b (1.5 – 2.1)	**1.0 – 2.0**[1]
Stachyose g/100 g dw	**5.1**ab (4.4 – 5.9)	**5.0**ab (4.5 – 5.4)	**5.4**a (5.0 – 5.8)	**4.8**ab (4.0 – 5.5)	**4.0 – 5.3**[2]
Trypsin inhibitor TIU/mg	**1.24**b (0.84 – 1.56)	**2.03**a (1.69 – 2.56)	**1.16**b (0.52 – 1.64)	**1.16**b (1.01 – 1.23)	**3.8 – 17.9**[3]
Urease Δ pH	**0.02**a (0.01 – 0.04)	**0.04**a (0.02 – 0.06)	**0.05**a (0.02 – 0.09)	**0.04**a (0.03 – 0.05)	**0.05 – 0.20**[4]
Phytic Acid mg/g dw	**4.32**ab (4.0 – 4.8)	**4.07**b (2.9 – 5.0)	**3.78**b (3.3 – 4.7)	**4.51**ab (3.6 – 5.2)	**1.3 – 4.1**[5]

*Values in the same row followed by the same letter are not significantly different at p<0.05
[1]Rackis (1974); [2]Coon *et al.* (1988), Kuo *et al.* (1988), Rackis (1974); [3]Anderson and Wolf (1995), Rackis (1974); [4]Lee and Garlich (1992); [5]Anderson and Wolf (1995), Mohamed *et al.* (1991).

Table B.5-15. Defatted Toasted Meal Isoflavone Composition.
Comparison of the means and ranges for the isoflavone content of the defatted toasted meal processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The defatted toasted meal was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte (unit)	Isoline	CV127	Conv. Std. 1	Conv. Std. 2
	N = 4			
Isoflavones	Mean (range)			
Total Daidzein mg/100 g dw	**99.0**a* (85.1 – 123.6)	**74.8**b (67.4 – 82.7)	**103.3**a (95.2 – 118.5)	**100.6**a (87.3 – 123.6)
Total Glycitein mg/100 g dw	**25.1**b (24.1 – 26.5)	**22.9**b (20.9 – 27.9)	**48.3**a (42.8 – 52.4)	**52.4**a (45.9 – 57.9)
Total Genistein mg/100 g dw	**133.5**b (114.5 – 167.9)	**115.7**c (97.7 – 132.6)	**166.8**a (149.7 – 199.1)	**161.7**a (138.7 – 203.4)

*Values in the same row followed by the same letter are not significantly different at p<0.05

Table B.5-16. Protein isolate proximate composition.
Comparison of the means and ranges for the proximate content of the protein isolate processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The protein isolate fraction was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte (unit)	Isoline	CV127	Conv. Std. 1	Conv. Std. 2	Literature Range
	N = 4				
Proximates	Mean (range)				
Ash g/100 g dw	**3.3**a* (2.9 – 4.0)	**2.6**b (2.2 – 3.2)	**3.2**ab (2.7 – 3.8)	**2.6**ab (1.9 – 3.1)	**2.3 - 7.6**[1]
Fat g/100 g dw	**5.4**a (4.1 – 6.6)	**7.0**a (5.8 – 9.0)	**5.2**a (3.9 – 6.1)	**5.6**a (4.6 – 6.9)	**0.1 - 2.5**[2]
Protein g/100 g dw	**90.4**ab (89.0 – 91.0)	**90.4**ab (88.1 – 92.7)	**89.6**b (88.4 – 91.1)	**91.5**a (90.6 – 92.5)	**85.2 - 92.0**[3]
Carbohydrates ‡ g/100 g dw	**0.9**ab (0.1 – 1.8)	**0.7**ab (0.0 – 2.1)	**2.1**a (1.0 – 3.5)	**0.8**ab (0.0 – 1.7)	**0.3 - 0.6**[4]
Calories kcal/100 g dw	**414**a (407 – 417)	**428**a (417 – 436)	**414**a (408 – 418)	**420**a (411 – 427)	NA^

*Values in the same row followed by the same letter are not significantly different at $p<0.05$
‡Carbohydrates including total dietary fibre.
[1]Smith and Circle (1972), Wolf (1983); [2]Horan (1974), Wolf (1983); [3]Torun (1979), Waggle and Kolar (1979); [4]Waggle and Kolar (1979), Wolf (1983)
^Not available

Table B.5-17. Protein Concentrate Proximate Composition.
Comparison of the means and ranges for the proximate content of the protein concentrate processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The protein concentrate fraction was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte (unit)	Isoline	CV127	Conv. Std. 1	Conv. Std. 2	Literature Range
	N = 4				
	Mean (range)				
Ash g/100 g dw	**4.0**ab* (3.6 – 4.1)	**4.4**a (4.9 – 4.0)	**3.7**b (3.2 – 4.4)	**4.0**ab (3.8 – 4.2)	**4.7 – 6.5**[1]
Fat g/100 g dw	**5.1**a (3.7 – 7.4)	**6.2**a (4.5 – 9.2)	**5.1**a (3.7 – 6.8)	**4.4**a (3.8 – 5.7)	**0.9 – 2.0**[2]
Protein g/100 g dw	**79.5**a (76.7 – 81.1)	**78.2**a (70.9 – 85.6)	**80.9**a (77.1 – 82.8)	**78.2**a (77.0 – 79.3)	**66.2 – 78.1**[1]
Carbohydrates‡ g/100 g dw	**11.5**a (7.4 – 14.2)	**11.4**a (1.0 – 18.6)	**10.4**a (7.3 – 13.5)	**13.3**a (11.5 – 14.9)	**17.1 – 25.0**[3]
Calories kcal/100 g dw	**410**a (403 – 421)	**414**a (406 – 429)	**411**a (404 – 421)	**406**a (402 – 412)	NA^

*Values in the same row followed by the same letter are not significantly different at $p<0.05$
‡Carbohydrates including total dietary fibre.
[1]Bookwalter (1978), Smith and Circle (1972); [2]O'Dell (1979), Wolf (1983); [3]Mattil (1974), Smith and Circle (1972)
^Not available

Table B.5-18. Refined Oil Fatty Acid Composition.
Comparison of the means and ranges for the fatty acid content of the refined oil processed fraction of the isoline control, CV127 and conventional standard soybean varieties Monsoy 8001(Std 1) and Coodetec 217 (Std 2). The refined oil fraction was generated from grain produced from each treatment grown in four separate field trial locations in Brazil during the 2006/2007 growing season.

Analyte (g/100 g oil)	Isoline	CV127	Conv. Std. 1	Conv. Std. 2	Literature Values[1]
	N = 4				
	Mean (range)				
Palmitic 16:0	**10.33**ab* (9.66 – 10.90)	**9.94**b (9.61 – 10.42)	**10.38**a (9.66 – 10.76)	**9.19**c (8.94 – 9.32)	**7 - 12**
Stearic 18:0	**3.84**a (3.54 – 4.21)	**3.75**a (3.25 – 4.21)	**3.67**a (3.44 – 3.97)	**3.36**b (3.15 – 3.59)	**2 – 5.5**
Oleic 18:1	**21.96**c (21.03 – 22.66)	**24.28**a (22.56 – 25.62)	**18.88**d (17.78 – 19.74)	**21.24**c (20.65 – 21.94)	**20 – 50**
Linoleic 18:2	**51.52**b (50.95 – 52.29)	**50.45**c (49.43 – 51.67)	**53.66**a (53.20 – 54.06)	**53.99**a (53.58 – 54.30)	**35 – 60**
Linolenic 18:3	**6.19**b (5.93 – 6.41)	**5.64**c (5.26 – 6.02)	**7.75**a (7.27 – 8.27)	**6.49**b (6.31 – 6.64)	**2 – 13**
Arachidic 20:0	**0.42**a (0.38 – 0.48)	**0.42**a (0.38 – 0.48)	**0.35**b (0.29 – 0.38)	**0.29**c (0.29)	**0.2 – 1.0**
Eicosenoic 20:1	**0.23**a (0.14 – 0.29)	**0.22**a (0.19 – 0.29)	**0.18**a (0.14 – 0.19)	**0.19**a (0.19)	**<1.0**
Behenic 22:0	**0.60**a (0.57 – 0.67)	**0.54**b (0.48 – 0.57)	**0.48**c (0.43 – 0.53)	**0.46**c (0.43 – 0.48)	**<0.5**
Tetracosanoic 24:0	**0.19**a (0.19)	**0.19**a (0.19)	**0.13**b (0.10 – 0.19)	**0.19**a (0.19)	NA^

*Values in the same row followed by the same letter are not significantly different at $p<0.05$
[1]Pryde (1990)
^Not available

Conclusions

The results of the compositional analyses conducted with grain produced in Brazil in two different growing seasons (2006/2007 and 2007) demonstrate that the introduction of the *csr1-2* gene into the soybean genome does not impact the nutritional composition of grain produced by CV127 soybean. In summary, results of these analyses demonstrate that grain from CV127 soybean is compositionally equivalent to, and as nutritious as, grain from the near-isogenic control soybean as well as other conventional soybean varieties typically cultivated in Brazil. Minor differences in amounts of individual nutrient and antinutrient constituents that were detected between the grain of CV127 soybean and that of the conventional parental near-isogenic control soybean are likely due to the natural genetic heterogeneity that exists between these two varieties. Also, differences in grain nutrient and antinutrient content of CV127, the near-isogenic control and the conventional standard soybean varieties included in the field tests compared to those reported in the global category of the ILSI Crop Composition Database most likely reflect characteristics of soybean varieties bred and developed for production under tropical growing conditions in Brazil. Finally, levels of nutrients and antinutrients in grain of the two CV127 soybean treatments were equivalent between the two treatments; therefore imidazolinone herbicide application to CV127 soybean does not have a significant effect on grain nutrient and antinutrient composition.

b) The levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator

The AHAS enzyme catalyses the first common step in branched-chain amino acid biosynthesis (leucine, isoleucine and valine). The data presented in **Section B.5.a)** show that there were no statistically significant differences in levels of these amino acids between grain of CV127 soybean and the near-isogenic control. Therefore, the results of the amino acid analyses in grain also confirm that the amino acid mutation in the AtAHAS protein responsible for conferring imidazolinone herbicide tolerance in CV127 soybean has no impact on the feedback regulation of this enzyme by branched-chain amino acids.

Furthermore, there was no evidence of changes to other plant metabolic pathways in CV127 soybean as a result of the modification to the AHAS enzyme, based on grain composition evaluations. Each of the measured grain composition parameters provides an assessment of the cumulative result of numerous biochemical pathways in the plant. Results of these evaluations showed that levels of nutrients and antinutrients in grain of the imidazolinone-tolerant CV127 soybean are comparable to levels in the control and other conventional soybean varieties. Therefore, grain produced from CV127 soybean is compositionally equivalent to that from the control as well as other commercial soybean varieties. These data confirm that the modification to the AtAHAS enzyme in CV127 soybean specifically confers tolerance to imidazolinone herbicides and has no effect on the branched-chain amino acid biosynthetic pathway or other metabolic pathways in the soybean plant.

No secondary effects were anticipated and none have been observed or identified. No novel constituents from the intentional modification to CV127 soybean have been identified and none were anticipated.

c) The levels of any naturally occurring allergenic proteins in the GM food compared with the levels in an appropriate comparator. Particular attention must be paid to those foods that are required to be declared when present as an ingredient, and where significant alterations to protein content could be reasonably anticipated

In order to demonstrate that there are no significant changes in the levels of known food allergens that are typically present in soybean a comparative assessment between CV127 soybean and the parental variety was performed. Soybeans are well known to be a significant source of dietary allergens. The Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (AllergenOnline database version 8.00; http://www.allergenonline.com/) lists 33 allergenic proteins for soybean grain. These include the β-conglycinin family of proteins, the glycinin family, the trypsin inhibitor family and other miscellaneous allergens. The major allergens are also among the major proteins in soybean grain. In a food safety assessment of a genetically modified crop, it is important to demonstrate that there are no significant changes in the levels

of known food allergens that are typically present in the crop, due to genetic disturbances resulting from the insertion of the transgene into the genome of the crop. Several publications describing proteomic studies (two dimensional [2D] gel electrophoresis separation of proteins with identification of individual proteins using mass spectral analysis) in soybeans are available in the literature (Herman *et al.*, 2003; Hajduch *et al.*, 2005; Natarajan *et al.*, 2006a, 2006b, and 2007). Hajduch *et al.* (2005) have also published an interactive web database containing clickable 2D-protein maps of proteins from soybean seeds with multiple annotated spots (accessible at http://oilseedproteomics.missouri.edu/). The availability of this data renders 2D-polyacrylamide gel electrophoresis a valid tool to examine and compare the content of different soybean allergens in CV127 and control soybean lines. Therefore a proteomic study was conducted using protein extracts from soybean seed obtained from CV127 soybean and a control variety Conquista (the parental soybean variety). This allowed the major allergenic proteins in soybean to be compared between these two soybean varieties that differ only by the introduced *csr1-2* gene cassette.

This study focused on both the major allergens, including the glycinins, and the major lectin, soybean agglutinin. Two other major proteins, lipoxygenase and sucrose binding protein were monitored as controls. The complete methods of this study are presented in **Appendix 21**. Briefly, protein extracts of the soybean grain samples were subjected to 2D-PAGE and the proteins were visualized by silver staining. Image analysis and comparison of the relative quantities of identical proteins among the grain samples was conducted using the ImageMaster platinum software package from GE Healthcare (version 5.0.0.0).

From each of the two soybean treatments, three replicate analytical gels were prepared. The gels had protein patterns that were very similar to the published gels of proteins from soybean grain. Thus several protein spots could be identified as allergens simply by means of pattern comparison of the gels with the internet database as well as with the literature (**Figure B.5-1**). The gel images of protein extracts from the grain of Conquista and CV127 soybean show a high similarity (>90 %) to each other (**Figures B.5-2** and **B.5-3**).

The amount of each protein in CV127 soybean and the parental variety Conquista was determined by image analysis of the gels for the major soybean allergens (Kunitz trypsin inhibitor, dehydrin-like protein, β-conglycinin alpha subunit, glycinin A5A4D3 subunit precursor, glycinin precursor [as a chain], glycinin precursor, [spot-shaped], β-conglycinin alpha prime subunit), soybean lectin (agglutinin) and two nonallergens (sucrose binding protein and lipoxygenase). Most of the allergens show up in the gels as multiple protein spots, as many of them are heteromeric, differentially glycosylated or partly deaminated. The staining intensities of the corresponding protein spots were summed up to calculate the total protein amount of each allergen and the ratios of the proteins in grain of CV127 soybean to those in grain from Conquista were calculated (**Table B.5-19**). Protein staining intensity ratios approaching the value one indicate little change in the amount of a protein between CV127 soybean and the control variety Conquista. The variation in relative intensity

of the allergenic proteins between CV127 soybean and Conquista was low, ranging from 0.9 to 1.5. Also, the average difference from the value of 1.0 for all protein staining ratios for the CV127 to Conquista comparison was 0.29. The variation in the relative intensity of the nonallergenic control proteins, lipoxygenase and sucrose binding protein, was higher with values of 0.5 and 2.0, representing a two-fold difference below and above the perfect ratio of 1.0, respectively. The results indicate that within the inherent variability of the method of analysis, there were no significant differences between the allergen contents in grain from Conquista and CV127 soybeans (**Table B.5-19**). These results are consistent with those from the grain composition study in which the amount of trypsin inhibitor was measured as an antinutrient. The amount of trypsin inhibitor in the grain of CV127 soybean and the near-isogenic control variety was not significantly different in grain produced in two different growing seasons (**Section B.5.a), Table B.5-11**).

These results demonstrate that there is no significant change in the levels of the major allergenic proteins in the grain of CV127 soybean compared to grain from Conquista, a closely related conventional soybean variety that is commonly cultivated in Brazil. These results provide further support of the conclusion that the grain from CV127 soybean is as safe and as nutritious as grain from conventional soybean varieties that have a long history of safe use as food and feed ingredients.

Figure B.5-1. Example of a silver stained 2D-PAGE gel (pH 4 - 7) of a protein extract of grain from soybean variety Conquista grown in the U.S. The major soybean protein allergens and two nonallergen control proteins (lipoxygenase and sucrose binding protein) are indicated.

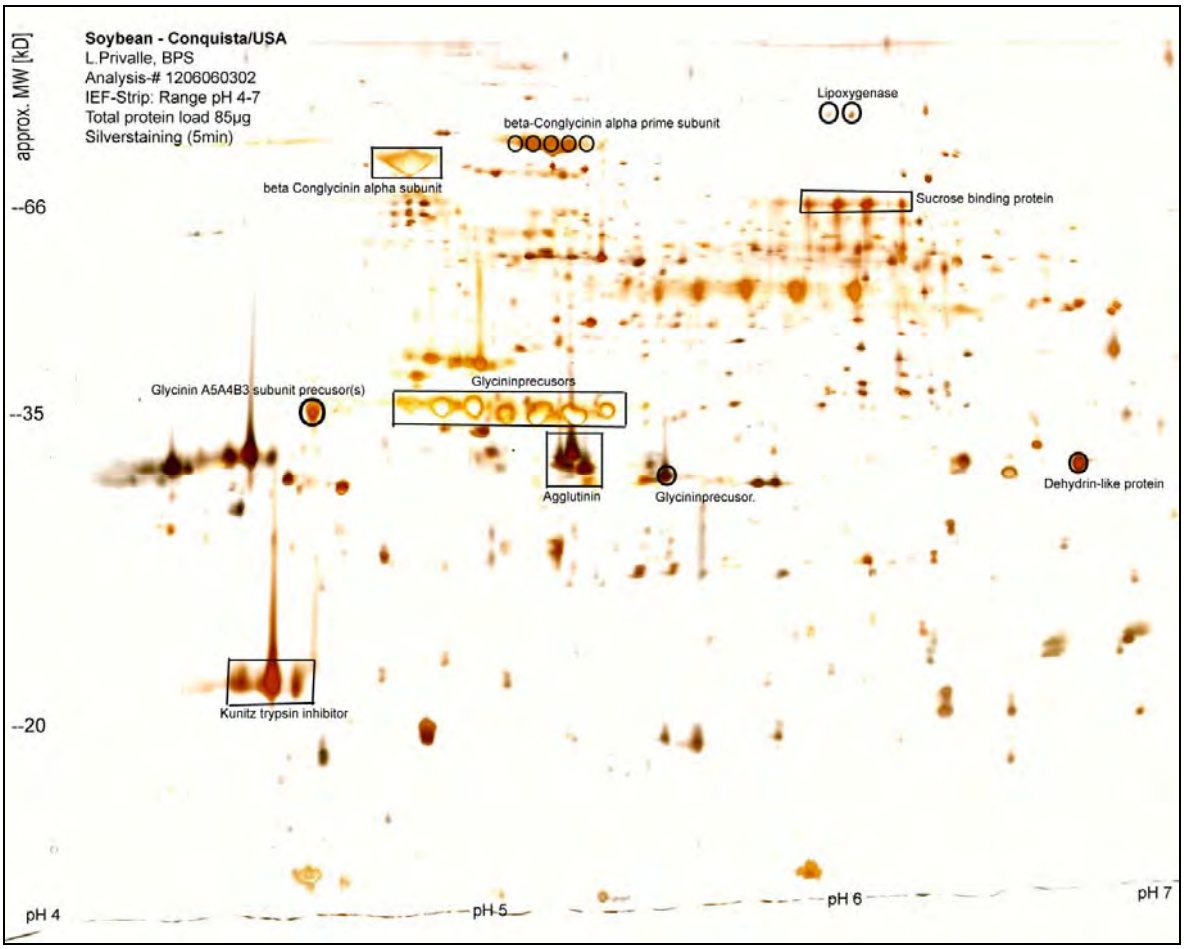


Figure B.5-2. Example of a silver stained 2D-PAGE gel (pH 4 - 7) of a protein extract of grain from soybean variety Conquista grown in Brazil.

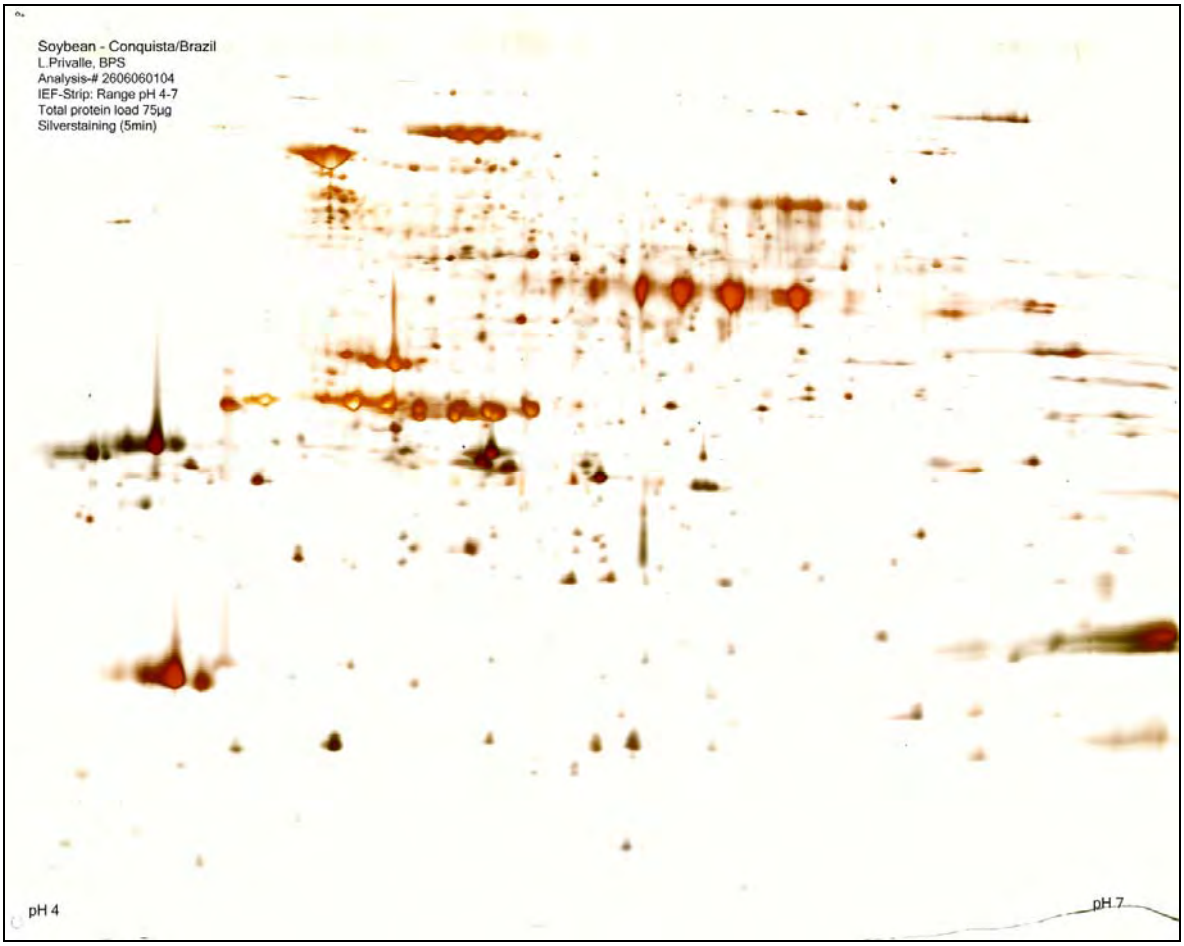


Figure B.5-3. Example of a silver stained 2D-PAGE gel (pH 4 - 7) of a protein extract of grain from CV127 soybean variety grown in Brazil.

Table B.5-19. The ratios of known allergenic proteins from grain of CV127 soybean and the parental soybean variety Conquista. Proteins were quantified by image analysis and integration of corresponding protein spots in silver stained 2D-gels. The protein ratios were calculated from the averages of the quantification of proteins in two gels.

	Protein Identity	**CV127/Conquista**
Allergens	Agglutinin	0.9
	Kunitz Trypsin inhibitor	1.5
	Dehydrin-like protein	1.1
	ß-Conglycinin alpha subunit	1.0
	Glycinin A5A4D3 subunit precursor	1.5
	Glycinin precursor (as a chain)	1.2
	Glycinin precursor (spot-shaped)	1.0
	ß-Conglycinin alpha prime subunit	1.0
	Total Glycinin (including precursors and subunits)	1.1
Non-allergens	Sucrose Binding protein	2.0
	Lipoxygenase	0.5

C. Information related to the nutritional impact of the genetically-modified food

1. Data to allow the nutritional impact of compositional changes in the food to be assessed

Calculation of Exposure Margins for Consumption of AtAHAS Protein in Food and Feed Derived from CV127 Soybean

An important aspect of an assessment of food and feed safety of a transgenic crop is the dietary exposure that is expected for the newly expressed protein. In Western societies such as in the U.S., Australia, Canada and Europe, soybeans do not constitute a significant portion of the diet. In these regions, soybeans are typically processed into oil and soybean meal. Soybean oil is used for cooking and to produce edible food products such as margarine, salad dressings, etc. However, soybean oil does not contain significant amounts of protein and so soybean oil will not be considered as a potential dietary source for the AtAHAS protein in CV127 soybeans. Soybean meal is primarily used as an animal feed ingredient and is also not a significant source of protein in human diets in Western societies. Americans, Australians, Canadians, and Europeans as a whole consume very little soy protein. Based on data from a dietary survey conducted by the UN Food and Agriculture Organization (FAO, 2003), the per capita consumption of soybean protein in these regions is less than 1 gram per day (**Table C.1-1**).

In the Asia-Pacific countries, soybeans constitute a more significant part of the human diet compared to societies in the West. In the Asia-Pacific countries, soybeans are used to produce a variety of foods, including non-fermented soy foods such as tofu, soymilk, and soy sprouts, and fermented soy foods such as soy sauce, miso, tempeh, and natto. As a result, the database from the FAO shows that the per capita consumption of soybean protein in Asian-Pacific societies ranges from 3 to nearly 10 gram per day (**Table C.1-1**).

Amount of AtAHAS protein in CV127 soybean grain

The amount of AHAS protein present in the grain of CV127 soybeans was examined and the results of this examination are presented in **Section B.2.c)**. AHAS levels were determined for grain that was produced by CV127 and the control variety at four replicated plots at six different field trial locations in Brazil during the 2006/2007 and 2007 growing seasons. In these studies, an enzyme-linked immunosorbent assay (ELISA) was used to measure the total AHAS protein in the grain. This assay measures total AHAS, including both the native soybean AHAS and the AtAHAS. For the purpose of estimating the dietary exposure of humans to AtAHAS from CV127 soybeans, it will be assumed that AtAHAS comprises the total amount of AHAS measured in the grain. For all grain samples analysed by ELISA in these studies, low levels of AHAS that were below the LOQ for AHAS in grain (<13ng/g) were detected. For the purposes of estimating the dietary exposure to AtAHAS, it will be assumed that grain from CV127 soybean uniformly contains 15 ng AtAHAS/g.

Other assumptions

It has been estimated that on average 10gram of soy foods are consumed for every 1gram of soy protein in the diet (Messina *et al.*, 2006). The estimated per capita consumption of soybean protein in different countries was obtained from the UN FAO food/stat database (FAO, 2003). For the purposes of estimating dietary exposure to the AtAHAS protein, the most conservative exposure scenario is considered in which it is assumed that 100 % of the soybeans consumed by humans are derived from CV127 soybeans. It is highly unlikely that CV127 soybeans would constitute such a high level of the total soybeans consumed since other soybean varieties will be cultivated and mixed in the commodity stream with CV127 soybeans.

Based on the above assumptions, the daily per capita consumption of AtAHAS protein for different countries was calculated based on the following formula:

Average daily per capita consumption of AtAHAS =

Average daily per capita consumption of soy protein (g/day) x 10g soy food/g soy protein x 15 ng AtAHAS/g

Using the above formula, the estimated maximum average daily consumption of AtAHAS protein in different countries and the estimated maximum dietary exposure presented as ng AtAHAS per day and as g AtAHAS consumed per kg body weight are presented in **Table C.1-1**.

Conclusions on human safety

The maximum estimated dietary exposure to AtAHAS protein from CV127 soybeans ranges from 3 ng per day in the U.S. to 1440 ng per day in South Korea. Assuming an average human body weight of 60 kg, the maximum amount of AtAHAS consumed expressed on a per-kg of body weight basis is presented in **Table C.1-1**. The highest consumption is in South Korea with an estimated maximum dietary exposure to AtAHAS equal to approximately 1440 ng/day that is equivalent to about 2.4×10^{-8} g AtAHAS per kg body weight. This represents a very low dietary exposure to the AtAHAS protein. Relative to the NOEL for AtAHAS established by the acute oral toxicity study (**Section B.3.c)**) of greater than 2620 mg per kg of body weight, this exposure level represents a safety factor of approximately 1×10^{8} fold relative to the NOEL for the AtAHAS protein. In Australia, where human consumption of soybean derived products is far less, the safety-fold factor is approximately 4.2×10^{10} relative to the NOEL.

It should be noted that since the biological activity of the AtAHAS protein is destroyed after 2 minutes at 75°C and 10 minutes at 60°C and since the preparation of most soy foods involves heating or some form of cooking, it is unlikely that any active AtAHAS protein would remain in soy foods prepared from CV127 soybeans. Based on the very large dietary safety factor relative to the NOEL for AtAHAS, it is concluded that CV127 soybeans and food or feed products containing ingredients derived from them are safe for consumption by humans and animals.

It should be considered, though, that processing reduces the levels of AHAS protein from the very low levels that are detectable but not quantifiable in soybean grain. The lack of stability of the AtAHAS protein in processed fractions of CV127 grain was discussed earlier (**Section B.5.a)**). This demonstrates that the imidazolinone-tolerance trait of CV127 has no significant impact on the nutrient composition.

Table C.1-1. Estimated daily dietary exposure to the AtAHAS protein in different countries.

Country	Average Consumption of Soy Protein (g/day)[1]	Estimated Maximum Dietary Exposure to AtAHAS Protein (ng/day)	Estimated Maximum Dietary Exposure to AtAHAS Protein (10^{-10}g/kg body weight)[2]
Australia	0.1	15	2.5
Brazil	2.0	300	50
Canada	0.5	75	12.5
China	3.4	510	85
Europe	0.2	30	5
Japan	8.7	1304	216
South Korea	9.6	1440	240
United States	0.02	3	0.5

[1]From FAO, 2003
[2]Assuming an average human body weight of 60 kg

2. Data from an animal feeding study, if available

Soybeans are used primarily to produce oil and high protein soybean meal. Soybean meal is the predominant protein component of animal feeds with 97 % of soybean meal being used in animal feeds and accounting for nearly 65 % of the world's protein in animal feed. In order to assess the wholesomeness of soybean meal from CV127 soybeans and to compare it with that of soybean meals from conventional soybean varieties, a 42-day feeding study with rapidly growing broiler chickens was conducted. The broiler chicken consumes high amounts of feed per unit of body weight and is a well recognized model animal for assessing the wholesomeness of feed ingredients (ILSI, 2003).

In order to produce the grain for this feeding study, CV127 soybeans and three conventional soybean varieties, including Conquista, Monsoy 8001, and Coodetec 217 were grown at a field location near Santo Antonio de Posse, Brazil during the 2006/2007 growing season. Conquista is the conventional variety that was originally transformed with the *csr1-2* gene encoding an imidazolinone-tolerant AHAS enzyme and it is closely related genetically to CV127 soybean. The soybeans were cultivated according to standard agricultural practices for soybean production in Brazil. The generation of CV127 soybean used as a grain source in this feeding study was designated the F9 generation of imidazolinone-tolerant CV127 Line 603, and is presented in the breeding history of CV127 in **Section A.3.**, **Figure A.3-6** (page 50). The CV127 plants were treated with 70 g imazapyr per ha, while the conventional

soybean varieties Conquista, Monsoy 8001, and Coodetec 217 were treated with the conventional soybean herbicide Volt.

Grain from each treatment was harvested from the field trial and was processed separately to toasted soybean meal using a pilot-scale processing method**.** First, the soybeans were cleaned of impurities such as dust, stones, branches and weed seeds using manual separation and sieves. The soybeans were flaked in an expeller type press with a 40 kg per hour processing capacity. Approximately 5 % of the oil was removed during this preparation. The flaked soybean material was extracted in a batch type extractor, using indirect steam to heat the n-hexane solvent to 45 - 50 ºC. In the production of the defatted and toasted soybean meal, the extractors were heated with indirect steam for 20 minutes in order to evaporate the solvent in the meal. Subsequently, direct steam (30 psi) was applied for 30 minutes through an orifice plate with a 2.5 mm aperture. The residual solvent was removed by treating with direct steam for 30 minutes under vacuum (250 mm Hg). To finish the process, the meal was subjected to a pressure of 0.2 kg/cm^2 for 10 minutes. The meal moisture was reduced to less than 12 % (w/w) in a flash dryer with a temperature of 200 - 250 ºC for 54 seconds.

The identity of test and control soybean grain used to produce the meal as well as the soybean meal itself was confirmed using an event specific PCR assay for the detection of the *csr1-2* gene in CV127. These analyses were conducted by GeneScan, Brazil. Of the four grain and four related soybean meal samples, only the CV127 soybean grain and soybean meal tested positive by PCR, thereby verifying the identity of these samples and demonstrating that there was no cross-contamination of the conventional soybean grain or meal with grain or meal from CV127 soybean. In addition, the grain samples and the corresponding soybean meal samples were tested for the presence of relevant mycotoxins, including aflatoxins B1, B2, G1, and G2, zearalenone, and ochratoxin A by the Instituto de Tecnologia de Alimentos (ITAL). None of these mycotoxins were detected in any of the soybean grain or meal samples. The grain samples were also tested by Bioensaios (Viamão, Brazil) for the presence of residues of pesticides that were used in the cultivation of the soybeans. For all pesticides tested, residues were either not detectable or were well below the levels of concern for broiler performance studies.

The poultry feeding experiments were conducted by the Embrapa Suínos e Aves (Embrapa Swine and Poultry) at their animal feeding facilities in Concórdia, Brazil. Details of the study are presented in **Appendix 22**. Preliminary experiments were conducted with feeds containing the soybean meals from CV127 soybean and the three conventional soybean varieties to determine the apparent metabolisable energy corrected for nitrogen and the true digestibility values of the amino acids. The results of these experiments were used to produce nutritionally balanced feeds in the feeding study to assess the wholesomeness of soybean meal from CV127 soybeans. Five hundred and seventy-six broiler chickens of the lineage AgRoss508, half male and half female, were used in the study. The birds were organized randomly in blocks by body weight with 12 replications (6 males and 6 females) with 12 broilers per

replication for a total of 144 broilers per treatment. Feeds were formulated for each of the four different treatments that included the soybean meal and all feeds were formulated to be isoenergetic and isoproteic. The experiment was divided into four phases, the initial (1 to 10 days of age), growth (11 to 28 days), and two final stages (29 to 35 and 36 to 42 days, respectively) and the feed was balanced for each phase to meet the changing nutritional requirements of the animals. The feeds contained varying amounts of soybean meal that were approximately 40 % of the feed in the initial stage and declining to approximately 30 % in the final stage. During the study the initial weight, body weight, weight gain, feed intake, and feed conversion were assessed at the end of each experimental stage (at 10, 28, 35, and 42 days of age). Statistical analysis of the resulting data was performed using the Dunnett Test (SAS software, 2003) to compare the results from the animals fed feed containing soybean meal from CV127 soybean with those from the animals fed feed containing the soybean meals from the conventional soybean varieties.

Analysis of the resulting data demonstrated that there was no interaction between the different feed treatments and the sex of the animals, so the statistical analysis was conducted without segregation of the sexes. The results demonstrated that there were no statistically significant differences ($p>0.05$) in body weight, weight gain, feed intake or feed conversion between animals fed feed containing soybean meal from CV127 soybeans and those fed feeds containing soybean meal from the conventional varieties Conquista and Monsoy 8001 (**Table C.2-1**). Animals fed diets containing soybean meal from Coodetec 217 soybeans had significantly lower body weights and weight gain in all growth stages compared to animals fed diets containing soybean meal from CV127 soybean. Based on the results of this study, it can be concluded that soybean meal derived from CV127 soybean is nutritionally comparable to soybean meals derived from conventional soybean varieties that are cultivated commercially. This study also confirms that meal produced from CV127 soybean grain is appropriate for use in animal feed.

Table C.2-1. Performance of broilers fed CV127, Conquista, Monsoy 8001 or Coodetec 217 soybean meal. Measurements Included Corporal Weight (CW), Weight Gain (WG), Feed Intake (FI), and Feed Conversion (FC) with their Respective Average Standard Errors for Broiler Chickens during the Periods Studied.

Performance	Treatments			
	T1	T2	T3	T4
	CV127	Conquista	Monsoy 8001	Coodetec 217
Initial weight (g)	44.44 ± 0.05	44.40 ± 0.05	44.40 ± 0.05	44.43 ± 0.05
Period from 1 to 10 days of age				
CW (g)	289 ± 4	295 ± 4	292 ± 4	273 ± 4 *
WG (g)	244 ± 4	251 ± 4	247 ± 4	228 ± 4 *
FI (g)	272 ± 3	273 ± 3	271 ± 3	258 ± 3*
FC	1.11 ± 0.01	1.09 ± 0.01	1.10 ± 0.01	1.13 ± 0.01
Period from 1 to 28 days of age				
CW (g)	1480 ± 10	1503 ± 10	1507 ± 10	1443 ± 10 *
WG (g)	1436 ± 10	1459 ± 10	1463 ± 10	1398 ± 10 *
FI (g)	1955 ± 15	1983 ± 15	1970 ± 15	1915 ± 15
FC	1.36 ± 0.01	1.36 ± 0.01	1.35 ± 0.01	1.37 ± 0.01
Period from 1 to 35 days of age				
CW (g)	2068 ± 18	2106 ± 18	2101 ± 18	2004 ± 18 *
WG (g)	2024 ± 18	2062 ± 18	2057 ± 18	1959 ± 18 *
FI (g)	3023 ± 18	3055 ± 18	3037 ± 18	2975 ± 18
FC	1.50 ± 0.01	1.49 ± 0.01	1.48 ± 0.01	1.52 ± 0.01
Period from 1 to 42 days of age				
CW (g)	2620 ± 15	2644 ± 15	2666 ± 15	2567 ± 15 *
WG (g)	2576 ± 15	2600 ± 15	2621 ± 15	2522 ± 15 *
FI (g)	4183 ± 22	4187 ± 22	4210 ± 22	4125 ± 22
FC	1.63 ± 0.01	1.62 ± 0.01	1.61 ± 0.01	1.64 ± 0.01

*****Averages with an asterisk are significantly different ($p<0.05$) as compared with the CV127 soybean meal treatment.

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