Application to FSANZ to Vary Food Standard 1.5.2 to Include the Double-Herbicide-Tolerant Soybean Event SYHT0H2

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Executive Summary

Bayer CropScience Pty Ltd seeks to vary FSANZ Standard 1.5.2 to allow the use of genetically modified soybean (*Glycine max* L. Merr.) derived from transformation event SYHT0H2 in the Australian and New Zealand food industries. Five food products are derived from soybean: whole soybeans, oil, meal, hulls and protein. Soybean oil is the primary food product consumed by humans in Australia, with the other products used either as food products or as components of animal feed.

SYHT0H2 soybean contains the transgene *avhppd-03* encoding an HPPD enzyme, designated AvHPPD-03, that is more than 99.7% identical in amino acid sequence to the native HPPD in common oat (*Avena sativa*). HPPD is a ubiquitous enzyme in the tyrosine catabolic pathway that is essential to plants, animals, and many microbes. In comparison with the native soybean HPPD, the HPPD isozyme from oat has lower binding affinity for HPPD-inhibiting herbicides, such as mesotrione, and confers tolerance to herbicide application rates that would otherwise injure soybean. SYHT0H2 soybean also contains the transgene *pat* derived from *Streptomyces viridochromogenes*, a ubiquitous soil microbe. The gene *pat* encodes phosphinothricin acetyltransferase (PAT), an enzyme that inactivates glufosinate-ammonium herbicide, an inhibitor of glutamine synthetase. Expression of *pat* confers a glufosinate-tolerance phenotype.

SYHT0H2 soybean was produced by transformation of immature soybean seed of variety 'Jack' using disarmed *Agrobacterium tumefaciens*. The region of the plasmid vector, pSYN15954, intended for insertion into the soybean genome consisted of three gene-expression cassettes: (1) the gene *avhppd-03* regulated by the figwort mosaic virus (FMV), cauliflower mosaic virus (CaMV) 35S, and tobacco mosaic virus (TMV) enhancer sequences, the synthetic minimal plant promoter sequence, and the nopaline synthase (NOS) terminator sequence, (2) the gene *pat-03-01* regulated by the CaMV 35S promoter sequence and NOS terminator sequence, and (3) the gene *pat-03-02* regulated by the Cestrum yellow leaf curling virus promoter (CMP) sequence, TMV enhancer sequence, and NOS terminator sequence. Both versions of *pat* (*pat-03-01* and *pat-03-02*) encode the identical PAT protein sequence.

Genetic characterization studies demonstrated that SYHT0H2 soybean contains, at a single locus within the soybean genome that is stably inherited, a single copy of *avhppd-03*, four copies of *pat*, a single copy of the *avhppd-03* enhancer complex sequence, two copies of the CaMV 35S promoter, two copies of the CMP promoter, two copies of the TMV enhancer, and five copies of the NOS terminator. It does not contain any extraneous DNA fragments of these functional elements elsewhere in the SYHT0H2 soybean genome, and it does not contain the FMV enhancer or plasmid backbone sequence from pSYN15954. Analyses comparing the soybean genomic sequence flanking the SYHT0H2 insert with sequences in public databases indicated that the inserted DNA does not disrupt any known endogenous soybean gene.

Analyses of seed and forage from several U.S. field testing sites demonstrate that SYHT0H2 soybean is nutritionally and compositionally similar to, and as safe and nutritious as, conventional soybean. The levels of endogenous allergens are not higher in SYHT0H2 soybean than in conventional soybean varieties. No deleterious effects of SYHT0H2 soybean on animal performance were observed in a study wherein rapidly growing broiler chickens were fed diets prepared with SYHT0H2 soybean meal or conventional soybean meal for 42 days.

Well-characterized modes of action, physicochemical properties, and results of safety studies demonstrate that the AvHPPD-03 and PAT proteins present in SYHT0H2 soybean present no risk of harm to humans or livestock that consume soybean products or to wildlife potentially exposed to SYHT0H2 soybean.

Part 1 General Information on the Application

1.1 Applicant Details

(a) Applicant's name

Bayer CropScience Pty Ltd
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Syngenta Seeds Pty Ltd
Contact person: [redacted]

(b) Company/organisation name

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(f) Nature of applicant's business

Bayer CropScience
Plant biotechnology, plant breeding, seed and trait research and development.

Syngenta Seeds
Plant biotechnology, plant breeding, seed and trait research and development.

(g) Details of other individuals, companies or organisations associated with the application.

SYHT0H2 soybean was co-developed by Bayer CropScience and Syngenta Crop Protection in the context of an agreement between Bayer CropScience AG and Syngenta Seeds Inc. This is a joint application from both Bayer CropScience and Syngenta Seeds.

Please note that the primary contact for inquiries regarding this report and the data contained herein should be addressed to Dr. Nina McCormick, as described in the applicant details in (a)-(e) above.

1.2 Purpose of the Application

This application, on behalf of Bayer CropScience Pty Ltd and Syngenta Seeds Pty Ltd, seeks to vary FSANZ Standard 1.5.2 to allow the use of genetically modified soybeans (*Glycine max* L.) derived from event SYHT0H2 in the Australian and New Zealand food industries.

Soybeans are cultivated for the production of seed which have a multitude of food, feed and industrial uses. Soybeans are one of the major sources of vegetable oil in the human diet. Soybeans are also a source of high protein meal for livestock.

Soybeans are cultivated primarily in the United States, Argentina, Brazil, China and India. Soybean varieties containing the SYHT0H2 event will be commercially cultivated in some of these countries. It is therefore anticipated that food products derived from soybean varieties containing the SYHT0H2 event will enter the Australian and New Zealand food supply via imports from countries of production.

1.3 Justification for the Application

The SYHT0H2 transformation event introduced two genes to the *G. max* genome. These genes confer two novel traits: tolerance to herbicides that inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD) and to the herbicide glufosinate-ammonium. Soybean varieties containing the SYHT0H2 event will be produced commercially in the major soybean producing countries of the world.

<u>Advantages of SYHT0H2 soybeans</u>
SYHT0H2 soybean will offer growers much-needed flexibility to use herbicides with two alternative modes of action in their weed management programs and will help mitigate and manage the evolution of herbicide resistance in weed populations.

<u>Food safety</u>
Information is provided in this application to support the safety of the AvHPPD-03 and PAT proteins expressed by the SYHT0H2 event.

<u>Costs and benefits, and impacts on trade</u>
Varying FSANZ Standard 1.5.2 to include commercial soybean varieties containing event SYHT0H2 is unlikely to have a detrimental impact on the Australian soybean industry. Despite being a small soybean producer, Australian soybean is sourced for food and feed products on the domestic market and also, culinary quality soybeans produced out of season are exported to the main northern hemisphere producers. Soybean food and feed ingredients are also obtained from imported soybean products, with the US a major source of imports. Once soybean varieties containing the SYHT0H2 event are launched for commercial production in the US as well as other parts of the world, food and feed products derived from soybean containing this event are likely to enter the domestic food and feed supply.

If the soybean event SYHT0H2 is not incorporated into the FSANZ Standards, this could have wide ranging impacts on the price of food and feed products containing the ingredients derived from soybean. These would arise from the need to source varieties that do not contain the SYHT0H2 event. These products may attract a premium price that must be met by the manufacturer, with those costs eventually passed on to the consumer. This would be compounded by the costs of segregating SYHT0H2 soybean products, where trading partners are willing to comply with this requirement. Other factors to consider include disruptions to the food supply, and the significant costs of recalling food products if the SYHT0H2 event were to be distributed in the local food and feed supply.

Varying the FSANZ Standards to include SYHT0H2 will contribute to maintaining stable food prices, consumer choice in the marketplace, and decreased production costs for transgenic soybean varieties in the longer term. The potential trade implications of not including soybean event SYHT0H2 in the FSANZ Standards are significant. Segregating SYHT0H2 soybean products from other soybean products has compliance and identification requirements that are difficult and costly to meet. The US is the major trading partner of Australia, and approved transgenic crops are considered to be substantially equivalent to conventional crops. Therefore, in the US, there are no intentions of segregating or labelling transgenic crops or their products. Products containing event SYHT0H2 imported into Australia from the US, or other trading partners with similar treatments of transgenic crops, may need to be removed from sale. This could expose Australia to disputes with trading partners at the World Trade Organisation.

1.4 Assessment Procedure

We consider that the appropriate assessment for this application is the General Procedure. Event SYHT0H2 expresses two novel proteins as a result of genetic modification. The HPPD and PAT proteins have been assessed for their safety by FSANZ previously.

1.5 Confidential Commercial Information

Parts of the Bayer CropScience and Syngenta reports provided in Appendices 1 (Volume 2), 3 (Volume 2) and 5 (Volume 2) contain confidential commercial information. A formal request for this information to be treated as such has been submitted to FSANZ.

1.6 Exclusive Capturable Commercial Benefit (ECCB)

The application is not expected to confer an ECCB upon Bayer CropScience or Syngenta since soybean varieties containing the SYHT0H2 event will not be commercially propagated in Australia.

1.7 International and Other Standards

The Bayer CropScience and Syngenta reports and studies included in the information supporting this application have been conducted according to international standards. In the safety assessment of biotechnology products, Bayer CropScience and Syngenta refer primarily to the *Codex Alimentarius* Commission Foods Derived from Modern Biotechnology (CAC, 2009), and the relevant Codex Standard is:

Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. CAC/GL 45-2003.

Other guidelines and recommendations are also considered including those of the World Health Organisation (WHO), the United Nations Food and Agriculture Organisation (FAO), the Organisation for Economic Cooperation and Development (OECD), the United States Food and Drug Administration (US-FDA), the United States Environment Protection Agency (US-EPA), and the European Food Safety Agency (EFSA) (see CAC, 2009; EFSA, 2006; FAO/WHO, 2001; OECD, 2001a, 2001b; US-EPA, 2002; US-FDA, 2006).

1.8 Statutory Declaration

Included in the application cover letter to FSANZ.

1.9 Checklist for Standards Related to New Foods

APPLICATION REQUIREMENT CHECKLIST	SECTION IN THIS APPLICATION	PAGE NUMBER
General Requirements (Application Handbook section 3.1)		
Form of application		
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Purpose of the application	1.2	12
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Foods Produced Using Gene Technology (Application Handbook section 3.5.1)		
Nature and identity of the GM food	2.1	15
History of use of host and donor organisms	2.2	16
Nature of genetic modification	2.3	27
Labelling information on GM food	2.4	60
Antibiotic resistance marker genes (of used)	3.1	62
Characterisation of novel protein(s)/substances	3.2	63
Toxicity of novel protein(s)/substances	3.3	80
Potential allergenicity of novel protein(s)	3.4	88
Compositional analysis of GM food	3.5	89
Nutritional impact of GM food	4.1	103
Animal feeding studies (if available)	4.2	103

Part 2 Technical Information on the Genetically Modified Food

2.1 Nature and Identity of the Genetically Modified Food

(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

The bioengineered food is soybean (*Glycine max*) and the cultivar that was transformed to produce the bioengineered food was soybean variety 'Jack'.

SYHT0H2 contains the gene *avhppd-03* derived from *Avena sativa* (common oat) that encodes the enzyme p-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) and the gene *pat* from *Streptomyces viridochromogenes* that encodes phosphinothricin acetyltransferase (PAT).

SYHT0H2 soybean is tolerant to herbicides that inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD), such as mesotrione, and tolerant to applications of the herbicide glufosinate-ammonium. The isozyme AvHPPD-03 encoded by gene *avhppd*-03 has lower binding affinity to mesotrione than does native soybean HPPD. When expressed in soybean, *avhppd-03* conveys pre- and post-emergence tolerance to mesotrione. The gene *pat* encodes the phosphinothricin acetyltransferase enzyme (PAT) which, when produced in plants, acetylates L-phosphinothricin, the active form of glufosinate-ammonium herbicide, resulting in post-emergence tolerance.

HPPD is an enzyme in the tyrosine catabolic pathway which leads to the production of tocochromanols, the family of vitamin E isoforms. Over-expression of *avhppd-03* in soybean was expected, but not intended, to have a small effect on vitamin E production.

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

The designation of the transformant is event SYHT0H2 soybean (hereafter SYHT0H2 soybean), which has been assigned the OECD Unique Identifier SYN-ØØØH2-5.

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

This is unknown as this application is related to a commodity crop rather than a specific food or feed additive.

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

The major soybean commodity products are seeds, oil, and meal. Unprocessed soybeans are not suitable for food and their use for animal feed remains limited because they contain anti-nutritional factors such as trypsin inhibitors and lectins. Heat processing inactivates these factors. In addition to whole soybean oil for human consumption, refined soybean oil has many other technical and industrial applications. Glycerol, fatty acids, sterols, and lecithin are all derived from soybean oil. Whole soybeans are utilized to produce soy sprouts, baked soybeans, roasted soybeans, full fat soy flour and the traditional soy foods (*e.g.*, miso, soy milk, soy sauce, and tofu). Soy protein isolate is used as a source of amino acids in the production of infant food formula and other food products. Soybean meal is rich in essential amino acids, particularly lysine and tryptophan, which are required supplements in animal diets for optimum growth and health. Soybean meal is used in diets for poultry, swine, dairy cattle, beef cattle, and pets. Food and feed products derived from SYHT0H2 soybean are not materially different from food and feed commodities derived from conventional

soybean and hence, there are no expected differences in uses of SYHT0H2 soybean-derived food and feed products.

2.2 History and Use of the Host and Donor Organisms

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

(i) Common and scientific names and taxonomic classification;

The taxonomic classifications of the organisms from which the genetic elements of event SYHT0H2 are derived are presented below in Table 1.

Glycine max
Soybean (*Glycine max* [L.] Merrill) is a dicotyledenous annual legume originating from Northeast Asia that has been an important source of protein and oil for thousands of years. Soybean is cultivated widely around the world, with the largest production in the United States, Brazil, Argentina, China, and India (Wilcox 2004).

The biology of soybean has been well characterized by many authors. The Organisation for Economic Co-operation and Development (OECD) Consensus Document on the Biology of *Glycine max* (OECD 2000) contains a general description of soybean as a crop plant and its taxonomy, centre of origin and diversity, identification, reproductive biology, crosses, and ecology.
The recipient organism for the transformation that produced Event SYHT0H2 was the soybean cultivar 'Jack' (Reg. No. 265, Plant Introduction No. 540556), which was developed at the Illinois Agricultural Experiment Station (Nickell *et al.* 1990). It was released for use in 1989 because of its resistance to soybean cyst nematode and higher yield than cultivars of similar maturity. 'Jack' is classified as Group II maturity (relative maturity 2.9) and in the U.S. is best adapted to geographic regions between 40º and 42º north latitude. 'Jack' has white flowers, grey pubescence, brown pods, and seeds with dull yellow coat and yellow hila. 'Jack' is easily transformable and commonly used for genetic engineering of new soybean lines.

The transformation plasmid pSYN15954 was used to produce SYHT0H2 soybean by *A. tumefaciens*–mediated transformation of immature soybean seed. The DNA region between the left and right borders of the transformation plasmid included gene-expression cassettes for *avhppd-03*, *pat-03-01*, and *pat-03-02*. The *avhppd-03* expression cassette consisted of the *avhppd-03* coding region regulated by a synthetic minimal plant (SMP) promoter, figwort mosaic virus (FMV) enhancer, CaMV 35S enhancer (35S enhancer), tobacco mosaic virus (TMV) enhancer, and nopaline synthase (NOS) polyadenylation terminator sequence. The *pat-03-01* expression cassette consisted of the *pat-03-01* coding region regulated by a CaMV 35S promoter (35S promoter) and NOS terminator sequence. The *pat-03-02* expression cassette consisted of the *pat-03-02* coding region regulated by a Cestrum yellow leaf curling virus promoter (CMP), TMV enhancer, and NOS terminator sequence. Table 1 describes the elements contained within the transformation plasmid pSYN15954.

Table 1 Taxonomy of the donor organisms from which the genetic elements of the SYNHYOH2 event are derived

GENETIC ELEMENT	DONOR ORGANISM TAXONOMY							
	Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Scientific Name**	**Common Name**
Plant Genome								
Genomic DNA	Plantae	Magnoliophyta	Magnoliopsida	Fabales	Fabaceae	Glycine	*Glycine max* (L.) Merr.	soy bean
Gene Construct								
avhppd-03 cassette								
FMV enhancer	-	-	-	-	Caulimoviridae	*Caulimovirus*	Figwort mosaic virus	-
35S enhancer	-	-	-	-	Caulimoviridae	*Caulimovirus*	Cauliflower mosaic virus	-
SMP promoter	-	-	-	-	Caulimoviridae	*Caulimovirus*	Cestrum yellow leaf curling virus	-
TMV enhancer	-	-	-	-	Virgaviridae	*Tobamovirus*	Tobacco mosaic virus	-
avhppd-03	Plantae	Magnoliophyta	Liliopsida	Cyperales	Poaceae	*Avena*	*Avena sativa*	oats
NOS terminator	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	*Agrobacterium tumefaciens*	-
pat-03-01-cassette								
35S promoter	-	-	-	-	Caulimoviridae	*Caulimovirus*	Cauliflower mosaic virus	-
pat-03-01	Bacteria	Actinobacteria	Actinomycetes	Actinomycetales	Streptomycetaceae	*Streptomyces*	*Streptomyces viridochromogenes*	-
NOS terminator	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	*Agrobacterium tumefaciens*	-
pat-03-02 cassette								
CMP promoter	-	-	-	-	Caulimoviridae	*Caulimovirus*	Cestrum yellow leaf curling promoter	-
TMV enhancer	-	-	-	-	Virgaviridae	*Tobamovirus*	Tobacco mosaic virus	-
pat-03-02	Bacteria	Actinobacteria	Actinomycetes	Actinomycetales	Streptomycetaceae	*Streptomyces*	*Streptomyces viridochromogenes*	-

NOS terminator	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	*Agrobacterium tumefaciens*	-
Border Region								
Left border	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	Agrobacterium tumefaciens	-
Plasmid backbone								
spec	Bacteria	Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	*Escherichia coli*	-
virG	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	Agrobacterium tumefaciens	-
repA	Bacteria	Proteobacteria	Gamma Proteobacteria	Pseudomonadales	Pseudomonadadaceae	*Pseudomonas*	Pseudomonas aeruginosa	-
VS1 ori	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	Agrobacterium tumefaciens	-
ColE1 ori	Bacteria	Proteobacteria	Gamma Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	*Escherichia coli*	-
Border Region								
Right border	Bacteria	Proteobacteria	Alpha Proteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	Agrobacterium tumefaciens	-

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

Anti-nutrients in Soybean

There are a several compounds in legumes, and therefore also in soybeans, which are not favourable for human or animal nutrition. These anti-nutritional factors include phytic acid, raffinose and stachyose, protease inhibitors, and hemagglutinins (lectins). Intact antinutrients are present in raw soybean. Soybeans are typically processed to reduce the levels of antinutrients and make to make the product palatable and safe for food and feed (see section 2.2.b.iv).

Phytic acid

In most plant tissues, large portions of phosphorus are present in form of phytic acid (1,2,3,4,5,6-hexakis (dihydrogen phosphate) myo-inositol). Phytic acid is regarded as the primary storage form of phosphorus and inositol in almost all seeds. During seed germination, phytin, the calcium-magnesium salt of phytic acid, is hydrolysed by the enzyme phytase and serves as a source of inorganic phosphorus and cations for the emerging seedling. The term phytate is used for the mono to dodeca anion of phytic acid (Ravindran *et al.* 1994; Maga, 1982).

Two-thirds of the phosphorus in soybeans is bound as phytate and unless freed is mostly unavailable to animals (Liener, 1994). Considerably more phosphorus is available to ruminants since rumen microbes produce phytase, a phytic acid degrading enzyme, that breaks down phytate and releases phosphorus. Phytic acid also chelates mineral nutrients including calcium, magnesium, potassium, iron, and zinc, rendering them unavailable to monogastric animals consuming the beans. In fact, phytic acid chelation of zinc present in corn-soybean meal diets used for growing swine requires supplements of zinc to avoid a parakeratosis (OECD, 2001c). It is becoming common for feed formulators to add phytase to swine and poultry diets to release phytin-bound phosphorus, so that the amount of this mineral added to the diet can be decreased, potentially reducing excess phosphorus in the environment. Phytic acid also impacts on protein bioavailability and enzyme activity since it is a strong anion and it can interfere with the polar side groups of proteins leading to complexion of nutritional proteins or changes in the molecular conformation of enzymes (Fretzdorff and Brümmer, 1992).

Phytic acid contents reported for soybean seeds are 1.0 - 1.5% (Liener, 1994). However, higher values, up to 2.74% have also been reported (Douglas, 1996).

Raffinose and stachyose

The low molecular weight carbohydrates, stachyose and raffinose, are present in defatted toasted soybean meal, as well as in raw soybeans. Raffinose is a trisaccharide containing galactose, glucose and fructose. Stachyose is a tetrasaccharide built of two galactose; one glucose and one fructose molecule. Stachyose and raffinose are considered anti-nutrients, because they remain unhydrolysed in the small intestine of monogastric animals and humans due to a lack of galactosidase and hence are not absorbed. They then pass into the large intestine where microbial fermentation converts them to CO_2, the main components of flatus (Vaidehi and Kadam, 1989). into concentrate or isolate reduces or removes these oligosaccharides.

Protease inhibitors

Protease inhibitors are anti-nutritional compounds present in soybeans, cereals and potatoes. Two types of protease inhibitors are present in soybeans: the Kunitz inhibitor and the Bowman-Birk inhibitor. Trypsin inhibitors are proteins with molecular weights between 6 - 46 kDalton, which form inactive complexes with the proteinase trypsin. The Kunitz inhibitor and the Bowman-Birk inhibitors are active against trypsin, while the latter is also active against

chymotrypsin (Liener, 1994). These protease inhibitors interfere with the digestion of proteins resulting in decreased animal growth. The activity of these inhibitors is destroyed when the bean or meal is toasted or heated during processing.

Lectins

Lectins are proteins that bind to carbohydrate-containing molecules. Lectins in raw soybeans can inhibit growth and cause death in animals and it is expected that similar effects would occur in humans (Liener, 1994). The ability of lectins to act as hemagglutinins that cause blood clotting is the basis for most quantitative analytical methods. Soybean lectin is sometimes referred to as soybean hemagglutinin. Lectins are rapidly degraded upon heating but are quite resistant to dry heat.

Isoflavones

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic, anti-estrogenic, and hypocholesterolemic effects, in mammalian species. These compounds have been implicated in adversely affecting reproduction in animals fed diets containing large amounts of soybean meal (Shutt, 1976). However, it is not universally accepted that isoflavones are anti-nutrients as they have also been reported to have beneficial anti-carcinogenic effects (Messina and Messina, 1991).

The isoflavones in soybeans and soy products have three basic types: daidzein, genistein, and glycitein. Each of these three isomers, known as aglucones or free forms, can also exist in three conjugate forms: glucoside, acetylglucoside, or malonylglucoside. Therefore, in total there are twelve isomers of isoflavones in soybeans. The isoflavone content of soybeans is greatly influenced by many factors including variety, growing locations and environmental conditions. In literature reports on isoflavone contents of soybeans, the specific substances investigated, the analytical methods and the reporting conventions have differed widely (Douglas, 1996). Isoflavones are heat stable and not destroyed by toasting of soybean meal (Liener, 1994).

Allergies to Soybeans

Several soybean food allergies have been recorded in most countries of the world (Ballmer-Weber and Vieths 2008). Clinical reactions are similar to those observed with other major food allergens (Besler *et al*., 2000). In the absence of epidemiological data, the estimated prevalence of soybean allergies could be 0.5% in the general population (Sicherer and Sampson, 2006; Ballmer-Weber and Vieths 2008). Due to the widespread use of soybean derivatives in the food and beverage industry, soybean allergens, when present, are often considered hidden allergens. Therefore, labelling regulations (e.g. from Codex, US Food and Drug Administration, European Union) incorporate soybean as part of the major allergenic food lists that should be labelled (Codex 1999; EU, 2000, 2003; US-FDA, 2004).

Saline extracts of soybeans have been reported to contain several antigenic proteins that stimulate the rabbit systemic immune system after injection and/or orally sensitise guinea pigs, calves, pigs, and humans. The presence of these allergenic proteins in the diet of sensitive individuals can cause severe adverse reactions in the gastrointestinal tract. The allergenic effect is most often attributed to the globulin fraction of soybean proteins that comprise about 85% of total protein. When compared to soybean seeds, sprouts exhibit a similar ability to bind IgE from soy-allergic individuals. A number of immunological or immunochemical tests have been developed to examine allergenic proteins usually based on sera from sensitive subjects (OECD, 2001c).

Many soybean allergenic proteins have been identified, characterized and recorded in multiple allergen databases. AllergenOnline (www.allergenonline.org; update in February 2012), from the Food Allergy Research and Resource Program (FARPP) program, contains the greatest number of allergen sequences (see Table 2) and provides a robust resource for searching potential similarities

with other proteins. Proteins such as glycinin consist of subunits which are named separately in terms of their sequence designation in the allergenonline.org database, yet are all considered as the 'Gly m 6' protein for which there is the supporting clinical evidence for allergy (Table 2). These allergens belong to five major protein families: beta-conglycinin, glycinin, Kunitz trypsin inhibitor, Bd 28K, and Bd 30K. These families have conserved structural features in relation with their endogenous biological activity, which explains the wide immunochemical cross-recognition observed among members of the legume family (Ballmer-Weber and Vieths 2008).

Table 2 Food Soybean Allergens

FAMILIES OF PROTEINS	PROTEIN NAMES	GI NUMBERS
Beta-conglycinin (7S-cupin, 7S-globulin, vicilin, Gly m 5)	Beta-conglycinin, alpha chain [Precursor] Beta-conglycinin-alpha subunit Beta-conglycinin storage protein CG4 beta-conglycinin	18536 169927 169929 256427
Bd 30K (Cysteine thiol-protease C1)	34 kDa maturing seed vacuolar thiol protease precursor Gly m Bd 30K P34 probable thiol protease [Precursor]	3097321 1199563 129353
Bd 28K (7S-cupin)	Gly m Bd 28K	12697782
Glycinin G1 (11S-globulin, legumin, Gly m 6)	Glycinin G1 [Precursor] Glycinin G1	18635 18615
Glycinin G2 (11S-globulin, legumin, Gly m 6)	Glycinin G2 [Precursor] Glycinin G2	18637 18609
Glycinin G3 (11S-globulin, legumin, Gly m 6)	Glycinin G3 [Precursor]	18639
Glycinin G4 (11S-globulin, legumin, Gly m 6)	Glycinin G4 [Precursor] Glycinin G4 Glycinin G4 A5A4B3 subunit	732706 18641 806556
Glycinin G5 (11S-globulin, legumin, Gly m 6)	Glycinin G5 [Precursor] Glycinin G5 Gy5 protein	169971 169969 736002
Kunitz trypsin inhibitor (Kunitz-legume)	Kunitz trypsin inhibitor Kti Kunitz trypsin inhibitor KTi1 Kunitz trypsin inhibitor Kti2 Trypsin inhibitor subtype A Trypsin inhibitor subtype B ..... Kunitz trypsin inhibitor	256429 256635 256636 18770 18772 510515

Source: www.allergenonline.org; accessed 23rd of October 2009)

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

(i) Any relevant phenotypic information;

Cultivated soybean, *Glycine max* (L.) Merr, is grown as a commercial crop in over 35 countries. Today the major producers of soybeans are the United States, China, Democratic People's Republic of Korea and Republic of Korea, Argentina and Brazil. Soybean is one of the oldest cultivated crops, native to North and Central China. The first recording of soybeans was in a series of books known as Pen Ts'ao Kong Mu written by the emperor Sheng Nung in the year 2838 B.C., in which the various plants of China are described. Historical and geographical evidence suggests that soybeans were first domesticated in the eastern half of China between the 17th and 11th century B.C. (OECD, 2000). Domestication occurred over many centuries and was highlighted during the Shang Dynasty about 1700-1100 B.C. During the period of strong emperors, soybean remained only in China. In later centuries, increased trading and emigration brought soybean germplasm to other areas of Southeast and South-central Asia, which became the secondary centre of soybean germplasm. These events occurred during the 1st through the 15 - 16th century A.D. (Hymowitz *et al*., 1981). Soybeans were first introduced into the United States, now a major producer, in 1765 (OECD, 2000), and became

established as an oilseed crop by the late 1920s. By World War II soybeans attained major commercial importance, and in the present day soybeans belong to the four principal oilseed crops in the US (soybean, cottonseed, peanuts and sunflowers) (Hui, 1992).

Soybean is grown primarily for the production of seed, has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use. A major food use is purified oil, utilised in margarines, shortenings and cooking and salad oils. Other food products include tofu, soya sauce, simulated milk and meat products. Soybean meal is also used as a high protein supplement in feed rations for livestock. Industrial uses of soybeans range from the production of yeasts and antibodies to the manufacture of soaps and disinfectants (OECD, 2000).

Glycine max is an established agricultural field crop that has been grown for millennia as a source of food and feed, and has a long history of safe use. Cultivated soybean is an erect, bushy herbaceous annual that can reach a height of 1.5 metres. Amongst the cultivated soybean varieties there are three types of growth habit: determinate, semi-determinate and indeterminate (Bernard and Weiss, 1973). Determinate growth is characterised by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both axillary and terminal racemes. Determinate genotypes are primarily grown in the southern US (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). Semideterminate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period. No cultivated soybean varieties are frost tolerant and they are unable to survive freezing winter conditions (OECD, 2001c).

Cultivated soybeans are characterised by primary leaves that are unifoliate, opposite and ovate; secondary leaves that are trifoliolate and alternate; and compound leaves with four or more leaflets. Soybean has a nodulated root system consisting of a taproot from which the lateral root system emerges. The plants of most cultivars are covered with fine trichomes, but glabrous types also exist. The papilionaceous 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 (OECD, 2001c).

Soybean is a quantitative short day plant. Consequently, photoperiodism and temperature response is important in determining areas of cultivar adaptation. Seed will germinate when the soil temperature reaches 10°C and will emerge in a 5 - 7 day period under favourable conditions. In new areas of soybean production an inoculation with *Bradyrhizobium japonicum* will be necessary for optimum efficiency of the nodulated root system. Soybeans do not yield well on acid soils and the addition of limestone may be required. Soybeans are often rotated with such crops as corn, winter wheat, spring cereals and dry beans (OECD, 2001c).

(ii) How the organism is typically propagated for food use;

Soybean is considered a self-pollinated species that is propagated commercially for food use by seed. Artificial hybridisation is used to breed commercial cultivars. The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation, and cross pollination is usually less than one percent (Caviness, 1966). A soybean plant can produce as many as 400 pods, with two to twenty pods at a single node. Each pod contains one to five seeds. Neither the seedpod, nor the seed, has morphological characteristics that would encourage animal transportation (OECD, 2000).

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

The two primary products of soybeans used in food and feed, oil and meal respectively, are derived from the bean or seed. The various food (and feed) uses of these products are detailed above in Sections 2.1(d) and 2.2(a)(iii).

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

Three basic methods are used to process soybeans for use as food as feed: solvent extraction, hydraulic extraction and expeller extraction. Almost all soybean oil is extracted from the seed using the solvent process. Prior to processing, seeds are cleaned, cracked to loosen the seed coat or hulls, dehulled and then conditioned to 10 - 11% moisture. The conditioned meats are then flaked and extracted with hexane to remove the oil. Hexane and oil in the miscella are separated by evaporation and the hexane is recovered. Residual hexane in the flakes is removed by steam treatment in a desolventiser-toaster. The heat treatment inactivates antinutritional factors, such as trypsin inhibitors and lectins, in the raw flakes and increases protein digestibility. A metric ton of soybeans yields about 180 kg oil and 790 kg meal. (Hui, 1992). Figure 1 below shows the solvent extraction process.

Soybean oil
Soybean oil is the most valuable of the soybean products and is consumed almost entirely (more than 95%) as food. Food-grade soybean oil is used as salad and cooking oil, shortenings and margarines. For non-food uses, soybean oil is converted into alkyd resins for protective coatings, plasticisers, dimer acids, surfactants and a number of other products (Hui, 1992). To be suitable for human consumption, the extracted oil must undergo further processing, which is referred to as refining. Figure 2 below shows the oil refining procedure.

Soybean meal
Most soybean meal obtained via processing is used as a protein supplement in animal feeds. Only in the last 30 years have appreciable amounts been converted into products for human consumption, and these have been almost exclusively derived from defatted soybean flakes (Hui, 1992).

Soybean meal normally contains 41 – 50% protein, depending on the amount of hull removed. Because of their high protein content, protein meals are essential ingredients of poultry and livestock feeds. Soybean meal is often blended with corn meal in animal feeds because the two protein sources complement each other; soy supplies the lysine and corn the methionine necessary to provide a balanced ration at relatively low cost (Hui, 1992).

Soybean hulls
The hull is the tough protective covering of the seed which must be removed before the oil can be extracted. The primary use for soybean hulls is animal feed. Hulls are routinely removed during crushing of soybeans but are returned to the processing stream to be added to the meal fraction. Hulls are withheld from the meal only if their inclusion would cause the product to exceed the limit of allowable fibre. Excess hulls may be sold as feedstuffs or discarded as waste.

Soybean protein products
Three classes of protein products are derived from soybeans: defatted flours and grits, protein concentrates and protein isolates. Flours and grits (containing 40 – 50% protein) are made by grinding and sieving flakes. Concentrates (containing about 70% protein) are prepared by extracting and removing the soluble sugars from the defatted flakes by leaching with dilute acid at pH 4.5 or leaching with aqueous ethanol. Isolated soy proteins are obtained by extracting the soluble proteins with water at pH 8-9, precipitating at pH 4.5, centrifuging the resulting protein curd, washing,

redispersing in water, and finally spray drying. Flours and concentrates are further processed into textured products that are used as meat extenders and substitutes. Protein isolate is used primarily as adhesives for clays used in coating of paper and paperboard to render surfaces suitable for printing (Hui, 1992).

Soy Lecithin - Phospholipids

Soybean has the highest phospholipid content of the common oilseeds. Crude lecithin is obtained by degumming the crude soybean oil. This process involves mixing the crude oil with about 2% water at a temperature of 60 – 80°C. The mixture is then centrifuged to separate the lecithin emulsion which is vacuum dried in a thin film evaporator to a water content of 0.2 - 0.8%. Crude lecithin consists of 45 – 60% phosphatides and 30 – 35% triglycerides, the remaining 5 – 10% are free fatty acids, carbohydrates, glycolipids, sterols, and tocopherols (Pardun, 1989). Soy lecithin is used as ingredient in margarine, chocolate, icecream and baked goods. Its non-food applications are in cosmetics, pharmaceuticals and as additives in technical products.

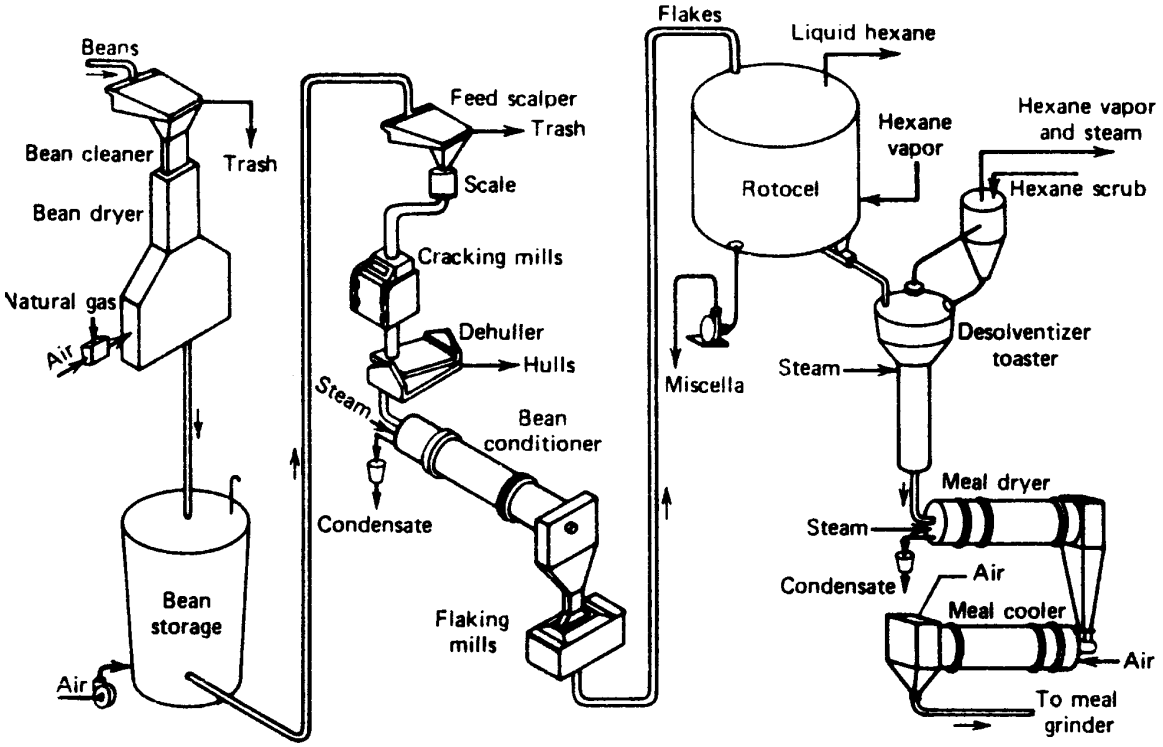
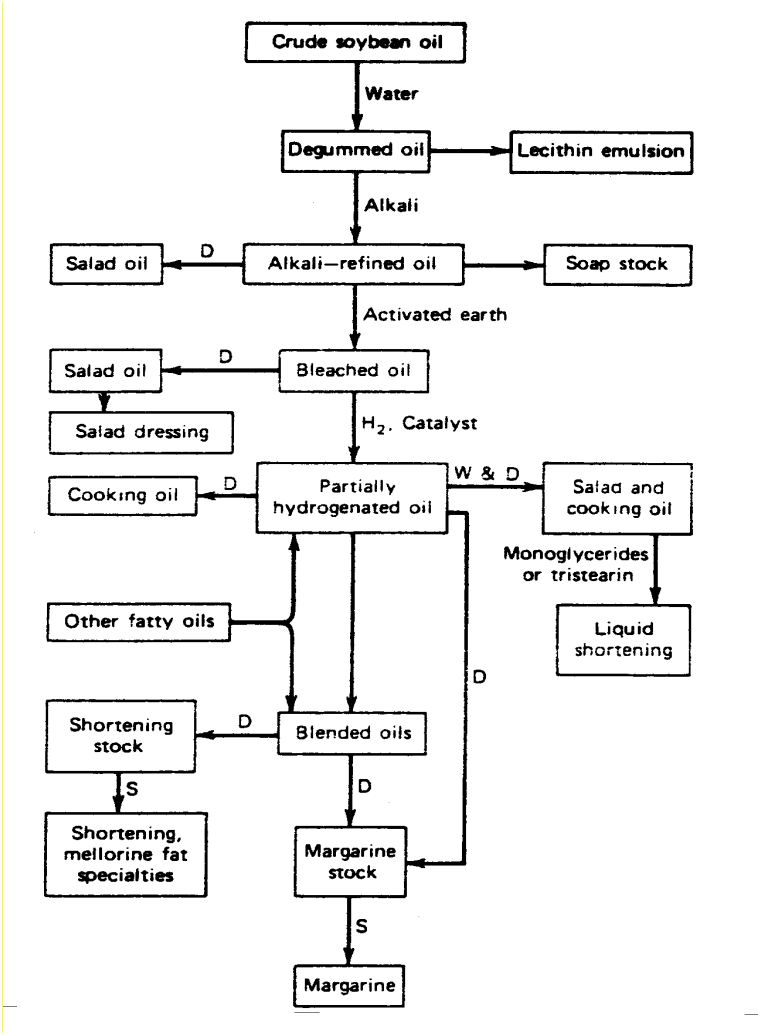


Figure 1 Processing of soybean into oil and meal by solvent extraction, courtesy of Dravo Corp. (Hui, 1992).



D = Deodorization, W = Winterisation, S = Solidification

Figure 2 Soybean oil refinement and edible soybean oil products, courtesy of the American Soybean Association and the American Oil Chemists' Society (Hui, 1992).

(v) *The significance to the diet in Australia and New Zealand of food derived from the host organism.*

Table 3 below details the import and export statistics for soybeans and process commodities for Australia and New Zealand.

Table 3 Soybean import and export statistics for Australia and New Zealand

	2004		2005		2006		2007		2008		2009		2010	
Commodity	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**
Australia														
Cake of Soybeans	226,490	1,333	320,081	6	349,587	31	226,490	1,333	505,766	71	467,919	416	471,846	200
Soya Sauce	8,536	84	6,644	173	7,820	121	8,536	84	10225	102	9846	55	10801	72
Soybean oil	13,397	1,730*	12,153	2,376	12,826	1,736	13,397	1,730*	22889	1697	21392	3262	26547	1852
Soybeans	9,412	7,540	594*	7,096	74,264	3,189	9,412	7,540	1121	1477	1368	6706	655	2466
New Zealand														
Cake of Soybeans	64838	0	59834	0	68738	2	64838	0	137996	80	99764	840	128627	0
Soya Sauce	1733	26	1655	22	1526	30	1733	26	1958	15	2153	31	3048	30
Soybean oil	22479	71	19281	46	17226	76	22479	71	20823	337	13343	400	15684	335
Soybeans	807	7	749	3	730	3	807	7	1018	6	1346	5	1181	3

* Unofficial figure

	2004		2005		2006		2007		2008		2009		2010	
Commodity	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**	**Import**	**Export**
Australia														
Cake of Soybeans	73,706	4	78,721	30	57,817	485	73,706	4	188737	54	187945	277	170206	205
Soya Sauce	10,336	310	11,774	238	13,806	95	10,336	310	19054	295	19409	183	22679	342
Soybean oil	6,540	1,531	7,933	1,164	9,220	1,320	6,540	1,531	30648	2624	20311	4203	29442	2428
Soybeans	367	2,701	18,793	1,386	3,136	3,931	367	2,701	1209	1376	1404	4385	944	2125
New Zealand														
Cake of Soybeans	15649	0**	19587	1	22258	0	15649	0**	64480	50	43909	477	55021	0
Soya Sauce	2120	64	1918	117	2220	105	2120	64	3624	39	3725	64	4323	74
Soybean oil	10295	53	11088	105	16089	98	10295	53	30471	647	14350	625	17793	630
Soybeans	349	2	385	2	490	7	349	2	956	6	1478	8	1179	5

** FAO estimate

FAOSTAT accessed 7th November 2012

2.3 The Nature of the Genetic Modification

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

Transformation of soybean to produce herbicide-tolerant soybean plants was accomplished through the use of immature seed of variety 'Jack' via *Agrobacterium tumefaciens*–mediated transformation (Hwang *et al.* 2008, Que *et al.* 2008). By this method, genetic elements within the left and right border regions of the transformation plasmid are transferred and integrated into the genome of the target plant cell, while genetic elements outside these border regions generally are not transferred.

Maturing soybean pods were harvested from greenhouse-grown plants, sterilized with diluted bleach solution, and rinsed with sterile water. Immature seeds were then excised from the seed pods, sterilized, and rinsed briefly with sterile water. The explants were prepared from sterilized immature seeds as described in Hwang *et al.* (2008), infected with *A. tumefaciens* strain EHA101 harboring the transformation binary plasmid pSYN15954, and allowed to incubate for 30 to 210 minutes. Excess *A. tumefaciens* suspension was then removed by aspiration, and the explants were moved to plates containing a non-selective co-culture medium. The explants were co-cultured with the remaining *A. tumefaciens* at 23°C for four days in the dark. The explants were then transferred to regeneration medium supplemented with an antibiotic mixture to kill *A. tumefaciens,* consisting of ticarcillin, cefotaxime, and vancomycin (75 mg/l each), and incubated in the dark for seven days. The explants were then transferred to cell-culture medium containing glufosinate-ammonium (6 to 8 mg/l) and the antibiotic mixture. The gene *pat* was used as a selectable marker during the transformation process. The glufosinate-ammonium selection concentration was kept low enough to allow for optimal shoot growth.

The regenerated plantlets were tested for the presence of the genes *pat* and *avhppd-03* and for the absence of the spectinomycin resistance gene *(spec)* present on the transformation plasmid backbone by real-time polymerase chain reaction (PCR) analysis (Ingham *et al.* 2001). This screen allowed for the selection of transformation events that carried the transferred deoxyribonucleic acid (T-DNA) and were free of plasmid backbone DNA. Plants positive for *avhppd-03* and *pat* and negative for *spec* were transferred to the greenhouse for seed setting.

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

The only intermediate organism used prior to transformation was *Escherichia coli*. Strains of *E. coli* are natural residents of the normal intestinal microbial flora of humans and animals. Standard *E. coli* strains used in laboratory techniques are non-pathogenic (Mühldorfer and Hacker, 1994).

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

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

The transformation plasmid pSYN15954 was used to produce SYHT0H2 soybean by *A. tumefaciens*–mediated transformation of immature soybean seed. The DNA region between the left and right borders of the transformation plasmid included gene-expression cassettes for *avhppd-03*, *pat-03-01*, and *pat-03-02*. The *avhppd-03* expression cassette consisted of the *avhppd-03* coding region regulated by a synthetic minimal plant (SMP) promoter, figwort mosaic virus (FMV) enhancer, CaMV 35S enhancer (35S enhancer), tobacco mosaic virus (TMV) enhancer, and nopaline synthase (NOS) polyadenylation

terminator sequence. The *pat-03-01* expression cassette consisted of the *pat-03-01* coding region regulated by a CaMV 35S promoter (35S promoter) and NOS terminator sequence. The *pat-03-02* expression cassette consisted of the *pat-03-02* coding region regulated by a Cestrum yellow leaf curling virus promoter (CMP), TMV enhancer, and NOS terminator sequence. Each genetic element in the transformation plasmid is described in Table 4.

Table 4 Genetic elements comprising the pSYN15954 vector used in soybean event SYHT0H2

Genetic element	Size (bp)	Position	Description
***avhppd-03* cassette**			
Intervening sequence	282	26 to 307	Noncoding intervening sequence with restriction sites used for cloning.
FMV enhancer	194	308 to 501	Figwort mosaic virus transcriptional enhancer region (similar to Accession No. X06166.1 [NCBI 2012]), which increases gene expression (Maiti *et al.* 1997).
Intervening sequence	6	502 to 507	Noncoding intervening sequence with restriction sites used for cloning.
35S enhancer	293	508 to 800	Cauliflower mosaic virus 35S transcriptional enhancer region (Ow *et al.* 1987).
Intervening sequence	20	801 to 820	Noncoding intervening sequence with restriction sites used for cloning.
SMP promoter	39	821 to 859	Synthetic minimal plant promoter including the TATA box, an adenine-rich sequence involved in transcription initiation, from the Cestrum yellow leaf curling virus promoter (Stavolone *et al.* 2003b), linked to a sequence taken from the region that is 3′ to the TATA box of the 35S promoter (Ow *et al.* 1987).
Intervening sequence	5	860 to 864	Noncoding intervening sequence with restriction sites used for cloning.
TMV enhancer	68	865 to 932	The 5′ non-coding leader sequence (called omega) from tobacco mosaic virus (Gallie *et al.* 1987), which functions as a translational enhancer in plants (Gallie 2002).
Intervening sequence	3	933 to 935	Noncoding intervening sequence with restriction sites used for cloning.
avhppd-03	1320	936 to 2255	The gene *avhppd-03*, derived from oat and codon optimized for enhanced expression, which encodes the enzyme AvHPPD-03. This enzyme catalyzes the formation of homogentisic acid, the aromatic precursor of plastoquinone and vitamin E biosynthesis (Matringe *et al.* 2005). In comparison with the native soybean HPPD, AvHPPD-03 has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in plant cells confers a tolerance to HPPD-inhibitor herbicides such as mesotrione.
Intervening sequence	16	2256 to 2271	Noncoding intervening sequence with restriction sites used for cloning.

Genetic element	Size (bp)	Position	Description
NOS terminator	253	2272 to 2524	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Accession No. V00087.1 [NCBI 2012]). Provides a polyadenylation site (Depicker *et al.* 1982).
Intervening sequence	8	2525 to 2532	Noncoding intervening sequence with restriction sites used for cloning.
***pat-03-01* cassette**			
35S promoter	521	2533 to 3053	Promoter region of cauliflower mosaic virus (Ow *et al.* 1987).
Intervening sequence	24	3054 to 3077	Noncoding intervening sequence with restriction sites used for cloning.
pat-03-01	552	3078 to 3629	*Streptomyces viridochromogenes* strain Tü494 gene, which encodes the selectable marker PAT. The native coding sequence (Wohlleben *et al.* 1988) was codon-optimized for enhanced expression. The synthetic gene *pat-03-01* was obtained from AgrEvo, Germany. PAT confers resistance to herbicides containing glufosinate-ammonium (phosphinothricin).
Intervening sequence	33	3630 to 3662	Intervening sequence with restriction sites used for cloning.
NOS terminator	253	3663 to 3915	Terminator sequence from the NOS gene of *A. tumefaciens* (Accession No. V00087.1 [NCBI 2012]). Provides a polyadenylation site (Depicker *et al.* 1982).
Intervening sequence	8	3916 to 3923	Noncoding intervening sequence with restriction sites used for cloning.
***pat-03-02* cassette**			
CMP promoter	654	3924 to 4577	Promoter and leader sequence from the Cestrum yellow leaf curling virus, similar to Accession No. AF364175.3 (NCBI 2012) (Stavolone *et al.* 2003a).
Intervening sequence	5	4578 to 4582	Noncoding intervening sequence with restriction sites used for cloning.
TMV enhancer	68	4583 to 4650	The 5′ noncoding leader sequence (called omega) from tobacco mosaic virus (Gallie *et al.* 1987), which functions as a translational enhancer in plants (Gallie 2002).
Intervening sequence	10	4651 to 4660	Noncoding intervening sequence with restriction sites used for cloning.
pat-03-02	552	4661 to 5212	*S. viridochromogenes* strain Tü494 gene, which encodes the selectable marker PAT. The native coding sequence (Wohlleben *et al.* 1988) was codon-optimized for enhanced expression and altered to remove restriction sites. PAT confers resistance to herbicides containing glufosinate-ammonium (phosphinothricin).
Intervening sequence	28	5213 to 5240	Noncoding intervening sequence with restriction sites used for cloning.
NOS terminator	253	5241 to 5493	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Accession No. V00087.1 [NCBI 2012]). Provides a polyadenylation site (Depicker *et al.* 1982).

Genetic element	Size (bp)	Position	Description
Intervening sequence	77	5494 to 5570	Noncoding intervening sequence with restriction sites used for cloning.
Border region			
Left border	25	5571 to 5595	Left border region of T-DNA from the *A. tumefaciens* nopaline Ti-plasmid (Accession No. J01825.1 [NCBI 2012]). Short direct repeat that flanks the T-DNA and is required for transfer of the T-DNA into the plant cell (Zambryski *et al.* 1982).
Plasmid backbone			
Intervening sequence	349	5596 to 5944	Noncoding intervening sequence with restriction sites used for cloning.
spec	789	5945 to 6733	The aminoglycoside adenylyltransferase gene (aadA) from *E. coli* transposon Tn7 (similar to Accession No. X03043.1 [NCBI 2012]). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling *et al.* 1985).
Intervening sequence	299	6734 to 7032	Noncoding intervening sequence with restriction sites used for cloning.
virG	726	7033 to 7758	The VirGN54D gene (*virG*) from pAD1289 (similar to Accession No. AF242881.1 [NCBI 2012]). The coding sequence was changed to have a N54D amino acid substitution that results in a constitutive *virG* phenotype. The gene *virG* is part of the two-component regulatory system for the virulence regulon in *A. tumefaciens* (Hansen *et al.* 1994).
Intervening sequence	29	7759 to 7787	Noncoding intervening sequence with restriction sites used for cloning.
repA	1074	7788 to 8861	Gene encoding the plasmid pVS1 replication protein from *Pseudomonas aeruginosa* (similar to Accession No. AF133831.1 [NCBI 2012]), which is part of the minimal pVS1 replicon that is functional in Gram-negative plant-associated bacteria (Heeb *et al.* 2000).
Intervening sequence	42	8862 to 8903	Noncoding intervening sequence with restriction sites used for cloning.
VS1 ori	405	8904 to 9308	Consensus sequence for the origin of replication (ori) and partitioning region from plasmid pVS1 of *P. aeruginosa* (Accession No. U10487.1 [NCBI 2012]). Serves as origin of replication in *A. tumefaciens* host (Itoh *et al.* 1984).
Intervening sequence	677	9309 to 9985	Noncoding intervening sequence with restriction sites used for cloning.
ColE1 ori	807	9986 to 10792	Origin of replication (similar to Accession No. V00268.1 [NCBI 2012]) that permits replication of plasmids in *E. coli* (Itoh and Tomizawa 1979).
Intervening sequence	112	10793 to 10904	Noncoding intervening sequence with restriction sites used for cloning.
Border region			
Right border	25	1 to 25	Right border region of T-DNA from the *A. tumefaciens* nopaline Ti-plasmid (Accession No. J01826.1 [NCBI 2012]). Short direct repeat that flanks the T-DNA and is required for transfer of the T-DNA into the plant cell (Wang *et al.* 1984).

(ii) 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.

The plasmid vector pSYN15954 that was used in soybean event SYHT0H2 is shown in Figure 3 below.

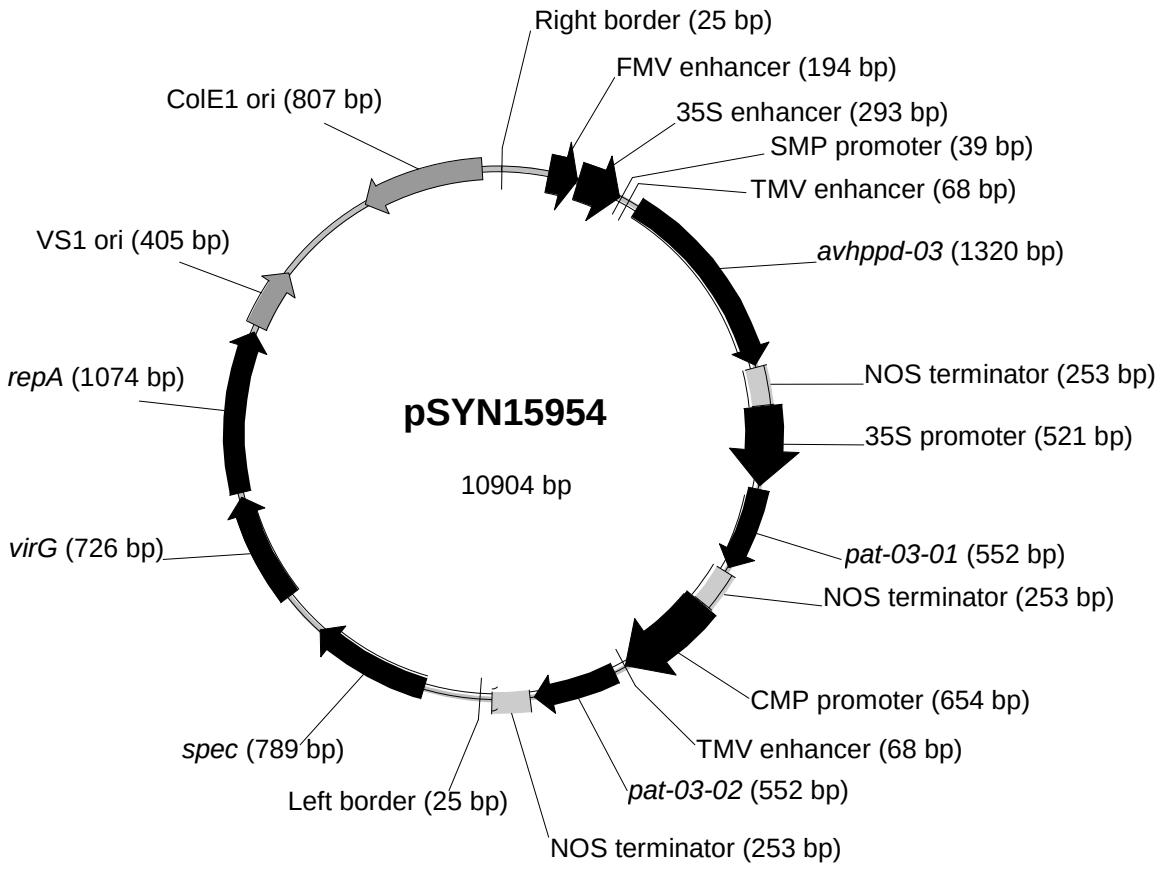


Figure 3 Map of plasmid vector pSYN15954 used in event SYHT0H2

(d) A full molecular characterisation of the genetic modification in the new organism, including:

(i) Identification of all transferred genetic material and whether it has undergone any rearrangement;

An extensive genetic characterization of the DNA insert in SYHT0H2 soybean was performed by means of Southern blot analyses and nucleotide sequencing. The genetic stability of the insert was assessed both by Southern blot analyses and by examining the inheritance patterns of the transgenes over three generations of SYHT0H2 soybean. In addition, the soybean genomic sequences flanking the SYHT0H2 insert were identified and characterized. It was determined that the SYHT0H2 insert did not disrupt the function of any known soybean gene. These data collectively demonstrate that no deleterious changes occurred in the SYHT0H2 soybean genome as a result of the DNA insertion.

Nucleotide Sequence of the DNA Insert

Nine overlapping fragments that covered the entire SYHT0H2 DNA insert were amplified via PCR from genomic DNA extracted from SYHT0H2 T_4 soybean ([redacted]). These fragments were cloned, and the sequences of the clones were assembled to generate a consensus sequence for the SYHT0H2 insert. This sequence was then compared with the sequence of the T-DNA in plasmid pSYN15954, the transformation plasmid used to create SYHT0H2 soybean.

Comparison of the SYHT0H2 insert sequence with the transformation plasmid pSYN15954 T-DNA sequence showed that the SYHT0H2 insert consists of two inverted and truncated copies of the pSYN15954 T-DNA centered on the right border proximal regions. The two copies are truncated at their right borders. The 5′ copy lacks the right border, the entire *avhppd-03* cassette, a portion of the 35S promoter, and the left border. The 3' copy lacks the right border, the FMV enhancer and a portion of the 35S enhancer from the *avhppd-03* cassette, and the left border. In addition, a 44-bp DNA sequence with similarity to *avhppd-03* is located between the two copies, and a 17-bp DNA insertion is located in the 35S promoter of the 3′ copy. The last 15 bp of the 17-bp insertion duplicate the sequence just upstream of this insertion.

Thus, insert sequence analysis indicated that the SYHT0H2 insert contains a single copy of *avhppd-03*, four copies of *pat*, a single copy of the *avhppd-03* enhancer complex sequence, two copies of the 35S promoter, two copies of the CMP promoter, two copies of the TMV enhancer, and five copies of the NOS terminator.

Copy Number of Functional Elements and Absence of Plasmid Backbone Sequence

Southern blot analyses were performed to determine the number of T-DNA integration sites, the number of copies of each functional element of the transformation plasmid pSYN15954, and the presence or absence of plasmid backbone sequence in the SYHT0H2 soybean genome ([redacted]).

Eight element-specific probes were used in the Southern blot analyses: (1) an *avhppd-03*–specific probe, (2) a *pat*-specific probe, (3) an *avhppd-03* enhancer complex–specific probe (consisting of the TMV enhancer, SMP promoter, and 35S enhancer), (4) a 35S promoter–specific probe, (5) a CMP promoter + TMV enhancer–specific probe, (6) an NOS terminator–specific probe, (7) an FMV enhancer–specific probe, and (8) a plasmid pSYN15954 backbone sequence probe. Each functional-element-specific probe except the *pat*-specific probe covered every base of the functional element present in the plasmid pSYN15954 T-DNA. Because the pSYN15954 T-DNA included two *pat* genes (*pat-03-01* and *pat-03-02*) differing by only two base pairs, only one probe was used; due to the high similarity of these genes, the probe could not distinguish between them. The plasmid-backbone-specific probe contained every base pair of the plasmid pSYN15954 backbone outside of the T-DNA.

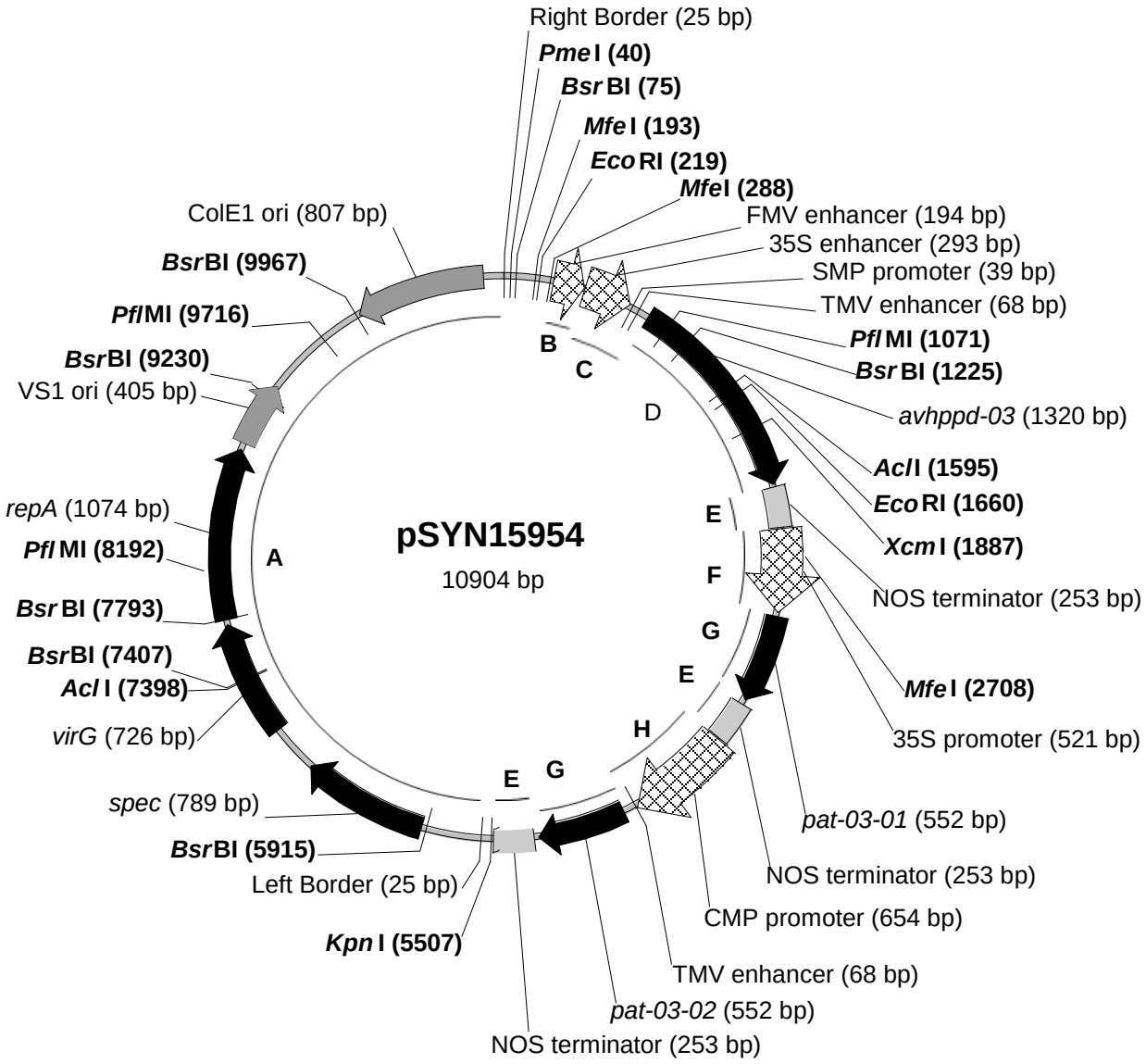
Each Southern blot analysis was performed with genomic DNA extracted from SYHT0H2 T_4 soybean and from nontransgenic ‘Jack’ soybean, which was used as a negative control to identify possible endogenous soybean DNA sequences that hybridized with the probes. Each analysis also included a positive control, to demonstrate the sensitivity of the analysis. The positive control consisted of the pSYN15954 plasmid digested with *Kpn*I and *Pme*I plus digested DNA from nontransgenic ‘Jack’ soybean (which was included so that the migration speed of the positive control DNA would more accurately reflect the migration speed of the restriction fragment containing the target sequence in the soybean genome).

Soybean genomic DNA was analyzed via two restriction enzyme digestion strategies. In the first strategy, the genomic DNA was digested with an enzyme that cut within the SYHT0H2 insert and in the soybean genome flanking the SYHT0H2 insert. This strategy was used twice, with two different enzymes, to determine the numbers of copies of the functional elements and the presence or absence of extraneous

DNA fragments of the functional elements in other regions of the SYHT0H2 soybean genome. The enzymes used were *Eco*RI, *Mfe*I, *Xcm*I, *Acl*I, and *Pfl*MI. In the second strategy, the genomic DNA was digested with a restriction enzyme that cut within the insert to release DNA fragments of predictable size. This strategy was used to determine the presence or absence of any closely linked extraneous DNA fragments of the functional elements. The enzymes used were *Kpn*I and *Kpn*I + *Bsr*BI. Figure 4 is a map of plasmid pSYN15954 showing the locations of the restriction endonuclease sites and probe annealing sites. Figure 5 is a map showing the locations of the restriction sites and probes in the SYHT0H2 soybean insert.

Table 5 shows the expected and observed numbers and sizes of the hybridization bands for SYHT0H2 soybean and the pSYN15954 positive control in the analyses with the eight element-specific probes. Additional, unexpected bands in any of these analyses would indicate the presence of additional copies of these elements in the SYHT0H2 soybean genome. Because the FMV enhancer is not present in SYHT0H2 soybean as discussed above, no hybridization bands were expected in analyses with the FMV enhancer–specific probe; unexpected bands would indicate the presence of this functional element in the SYHT0H2 soybean genome.

No hybridization bands were expected in any of the analyses of genomic DNA from nontransgenic ‘Jack’ soybean (the negative control). The positive control for each analysis contained 14.87 pg of digested plasmid pSYN15954 DNA, equivalent to one copy of a fragment of known size in the soybean genome, plus digested DNA from nontransgenic ‘Jack’ soybean. The positive control was expected to result in one hybridization band of approximately 5.5 kilobase pairs (kb) in all of the copy-number analyses and approximately 5.4 kb in the plasmid-backbone-sequence analyses.



A	pSYN15954-backbone-specific probe	E	NOS terminator–specific probe
B	FMV enhancer–specific probe	F	35S promoter–specific probe
C	*avhppd-03* enhancer complex–specific probe	G	*pat*-specific probe
D	*avhppd-03* –specific probe	H	CMP promoter + TMV enhancer–specific probe

Figure 4 Map of plasmid pSYN15954 showing the restriction sites and probes used in Southern blot analyses

Restriction enzymes and sites are indicated by bold type.

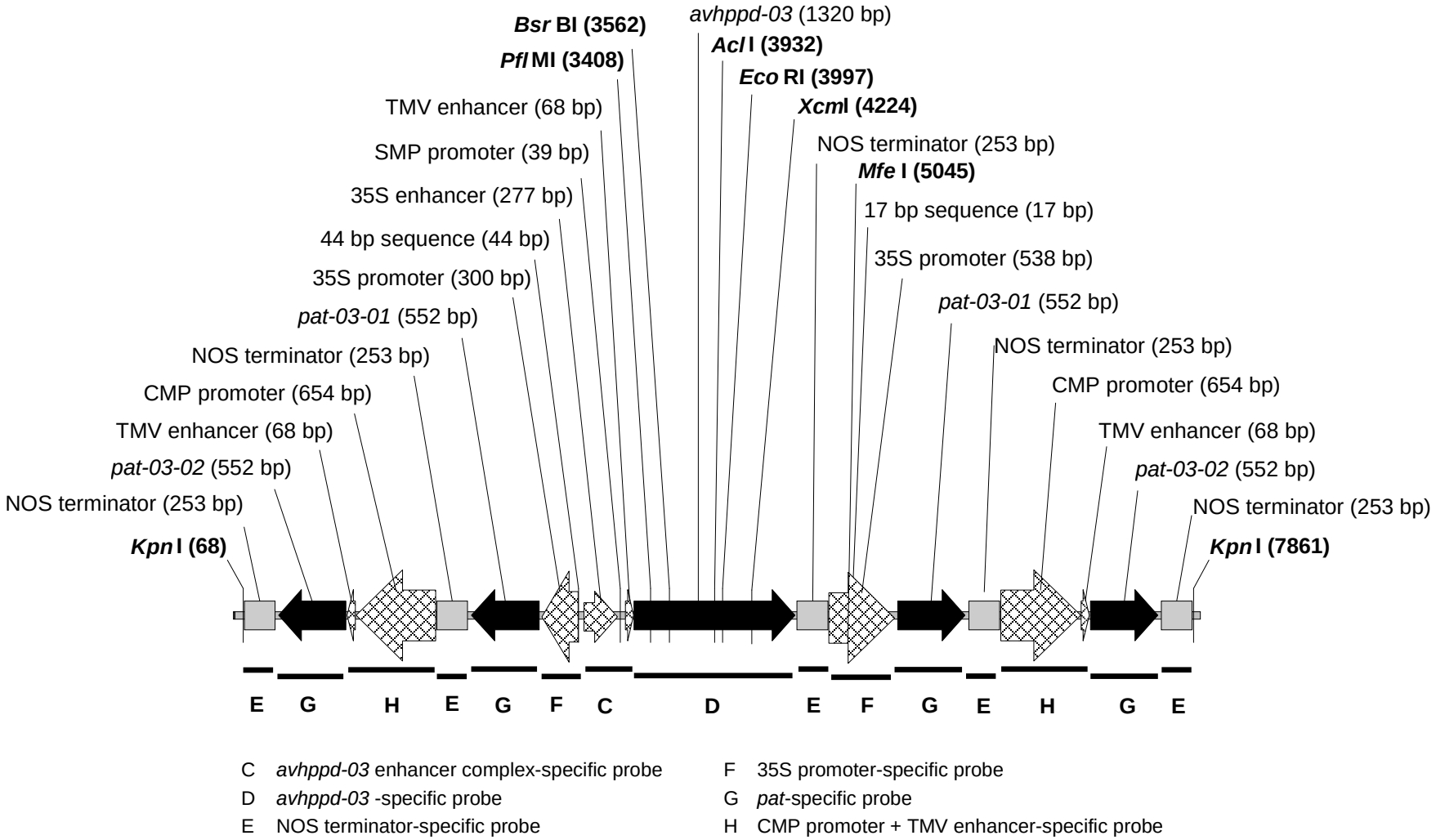


Figure 5 Map of the SYHT0H2 DNA insert showing the restriction sites and probes used in Southern blot analyses

Restriction enzymes and sites are indicated by bold type.

Table 5 Expected and observed hybridization bands in Southern blot analyses for copy number of functional elements and absence of plasmid backbone

Probe	Restriction enzyme(s)	Source of DNA[a]	Figure & Lane	Expected no. of bands	Expected band size (kb)	Observed band size (kb)
avhppd-03	*Eco*RI	SYHT0H2 T_4	V-3A, 3	2	>4.0, >3.9	~4.9, ~8.3
		positive control	V-3A, 5	1	~5.5	~5.5
	*Mfe*I	SYHT0H2 T_4	V-3B, 3	1	>5.0	~6.2
		positive control	V-3B, 5	1	~5.5	~5.3[b]
	*Kpn*I	SYHT0H2 T_4	V-3C, 3	1	~7.8	~7.8
		positive control	V-3C, 5	1	~5.5	~5.4[b]
pat	*Acl*I	SYHT0H2 T_4	V-5A, 3	2	>3.9, >4.0	~7.6, ~10
		positive control	V-5A, 5	1	~5.5	~5.3[b]
	*Eco*RI	SYHT0H2 T_4	V-5B, 3	2	>4.0, >3.9	~4.8, ~8.3
		positive control	V-5B, 5	1	~5.5	~5.3[b]
	*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-5C, 3	2	~3.5, ~4.3	~3.5, ~4.3
		positive control	V-5C, 5	1	~5.5	~5.4[b]
avhppd-03 enhancer complex	*Eco*RI	SYHT0H2 T_4	V-7A, 3	2	>4.0, >3.9	~4.8, ~8.2
		positive control	V-7A, 5	1	~5.5	~5.3[b]
	*Xcm*I	SYHT0H2 T_4	V-7B, 3	2	>4.2, >3.7	~4.3, ~5.7
		positive control	V-7B, 5	1	~5.5	~5.3[b]
	*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-7C, 3	2	~3.5, ~4.3	~3.5, ~4.3
		positive control	V-7C, 5	1	~5.5	~5.3[b]
35S promoter	*Eco*RI	SYHT0H2 T_4	V-9A, 3	2	>4.0, >3.9	~4.8, ~8.3
		positive control	V-9A, 5	1	~5.5	~5.3[b]
	*Xcm*I	SYHT0H2 T_4	V-9B, 3	2	>4.2, >3.7	~4.3, ~5.7
		positive control	V-9B, 5	1	~5.5	~5.3[b]
	*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-9C, 3	2	~3.5, ~4.3	~3.5, ~4.3
		positive control	V-9C, 5	1	~5.5	~5.4[b]
CMP promoter + TMV enhancer	*Eco*RI	SYHT0H2 T_4	V-11A, 3	2	>4.0, >3.9	~4.8, ~8.3
		positive control	V-11A, 5	1	~5.5	~5.3[b]
	*Mfe*I	SYHT0H2 T_4	V-11B, 3	2	>5.0, >2.9	~5.2, ~6.2
		positive control	V-11B, 5	1	~5.5	~5.3[b]
	*Kpn*I+*Bsr*BI	SYHT0H2 T_4	V-11C, 3	2	~3.5, ~4.3	~3.5, ~4.3
		positive control	V-11C, 5	1	~5.5	~5.4[b]
NOS terminator	*Acl*I	SYHT0H2 T_4	V-13A, 3	2	>3.9, >4.0	~7.6, ~10
		positive control	V-13A, 5	1	~5.5	~5.3[b]
	*Eco*RI	SYHT0H2 T_4	V-13B, 3	2	>4.0, >3.9	~4.8, ~8.3
		positive control	V-13B, 5	1	~5.5	~5.5
	*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-13C, 3	2	~3.5, ~4.3	~3.5, ~4.3
		positive control	V-13C, 5	1	~5.5	~5.5

Probe	Restriction enzyme(s)	Source of DNA[a]	Figure & Lane	Expected no. of bands	Expected band size (kb)	Observed band size (kb)
FMV	*Acl*I	SYHT0H2 T_4	V-16A, 3	0	N/A	N/A
		positive control	V-16A, 5	1	~5.5	~5.5
	*Pfl*MI	SYHT0H2 T_4	V-16B, 3	0	N/A	N/A
		positive control	V-16B, 5	1	~5.5	~5.5
	*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-16C, 3	0	N/A	N/A
		positive control	V-16C, 5	1	~5.5	~5.4[b]
Plasmid backbone	*Acl*I	SYHT0H2 T_4	V-18A, 3	0	N/A	N/A
		positive control	V-18A, 5	1	~5.4	~1.9, ~3.5, ~5.5 [b]
	N/A	positive control	V-18A, 7	1	~5.4	~5.5[b]
	*Pfl*MI	SYHT0H2 T_4	V-18B, 3	0	N/A	N/A
		positive control	V-18B, 5	1	~5.4	~5.7[b]
	*Kpn*I	SYHT0H2 T_4	V-18C, 3	0	N/A	N/A
		positive control	V-18C, 5	1	~5.4	~5.7[b]

N/A = not applicable.
[a]Positive control samples contained 14.87 pg of pSYN15954 (representing one copy of the T-DNA in the soybean genome) digested with *Kpn*I + *Pme*I plus 'Jack' digested with the indicated enzyme(s).
[b]The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

Copy Number of Functional Elements: avhppd-03

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the avhppd-03-specific probe are shown in Figure 6.

In the analysis of genomic DNA digested with EcoRI, two bands of approximately 4.9 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 6(A), Lane 3). These bands were absent from the lane containing DNA from nontransgenic 'Jack' soybean (Figure 6(A), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.5 kb was observed in the lane containing the positive control (Figure 6(A), Lane 5).

In the analysis of genomic DNA digested with MfeI, one band of approximately 6.2 kb was observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 6(B), Lane 3). This band was absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 6(B), Lane 4) and was therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 6(B), Lane 5).

In the analysis of genomic DNA digested with KpnI, one band of approximately 7.8 kb was observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 6(C), Lane 3). This band was absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 6(C), Lane 4) and was therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (Figure 6(C), Lane 5).

In the Southern blot analyses with the avhppd-03-specific probe, the expected numbers and sizes of hybridization bands were detected with both restriction enzyme digestion strategies. These results demonstrate that SYHT0H2 soybean contains a single copy of avhppd-03. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of avhppd-03.

Copy Number of Functional Elements: pat

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the *pat*-specific probe are shown in Figure 7.

In the analysis of genomic DNA digested with *Acl*I, two bands of approximately 7.6 and 10 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 7(A), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 7(A), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 7(A), Lane 5).

In the analysis with genomic DNA digested with *Eco*RI, two bands of approximately 4.8 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 7(B), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 7(B), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 7(B), Lane 5).

In the analysis with genomic DNA digested with *Kpn*I+ *Bsr*BI, two bands of approximately 3.5 and 4.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 7(C), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 7(C), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (Figure 7(C), Lane 5).

In the Southern blot analyses with the *pat*-specific probe, the expected numbers of hybridization bands were detected with both restriction enzyme digestion strategies. These results support the results of the insert sequence analysis, which determined that SYHT0H2 soybean contains four copies of *pat*. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of *pat*.

Copy Number of Functional Elements: avhppd-03 Enhancer Complex

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the *avhppd-03* enhancer complex–specific probe are shown in Figure 8.

Only one copy of the *avhppd-03* enhancer complex is present in SYHT0H2 soybean; however, because of sequence similarity between the 35S enhancer and SMP promoter (elements in the *avhppd-03* enhancer complex) and the 35S promoter, analyses with the *avhppd-03* enhancer complex–specific probe were expected to result in two hybridization bands in SYHT0H2 soybean, one corresponding to a copy of the *avhppd-03* enhancer complex and the other to a copy of the 35S promoter.

In the analysis of genomic DNA digested with *Eco*RI, two bands of approximately 4.8 and 8.2 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 8(A), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 8(A), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 8(A), Lane 5).

In the analysis of genomic DNA digested with *Xcm*I, two bands of approximately 4.3 and 5.7 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 5; Figure 8(B), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 8(B), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 8(B), Lane 5).

In the analysis of genomic DNA digested with *Kpn*I + *Bsr*BI, two bands of approximately 3.5 and 4.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 8(C), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 8(C), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 8(C), Lane 5).

In the Southern blot analyses with the *avhppd-03* enhancer complex–specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction digestion enzyme strategy, as expected. These results demonstrate that SYHT0H2 soybean contains a single copy of the *avhppd-03* enhancer complex. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of the *avhppd-03* enhancer complex.

Copy Number of Functional Elements: 35S Promoter

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the 35S promoter–specific probe are shown in Figure 9.

In the analysis of genomic DNA digested with *Eco*RI, two bands of approximately 4.8 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 9(A), Lane 3). These bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 9(A), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 9(A), Lane 5).

In the analysis of genomic DNA digested with *Xcm*I, two bands of approximately 4.3 and 5.7 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 9(B), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 9(B), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 9(B), Lane 5).

In the analysis of genomic DNA digested with *Kpn*I + *Bsr*BI, two bands of approximately 3.5 and 4.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 9(C), Lane 3). These bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 9(C), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (Figure 9(C), Lane 5).

In the Southern blot analyses with the 35S promoter–specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction enzyme digestion strategy, as expected. These results demonstrate that SYHT0H2 soybean contains two copies of the 35S promoter. No unexpected bands were detected, indicating that the SYHT0H2 soybean contains no extraneous DNA fragments of the 35S promoter.

Copy Number of Functional Elements: CMP Promoter + TMV Enhancer

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the CMP promoter + TMV enhancer–specific probe are shown in Figure 10.

In the analysis of genomic DNA digested with *Eco*RI, two bands of approximately 4.8 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 10(A), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 10(A), Lane 4)

and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 10(A), Lane 5).

In the analysis of genomic DNA digested with *Mfe*I, two bands of approximately 5.2 and 6.2 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 10(B), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 10(B), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 10(B), Lane 5).

In the analysis of genomic DNA digested with *Kpn*I + *Bsr*BI, two bands of approximately 3.5 and 4.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 10(C), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 10(C), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (Figure 10(C), Lane 5).

In the Southern blot analyses with the CMP promoter + TMV enhancer–specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction enzyme digestion strategy, as expected. These results demonstrate that SYHT0H2 soybean contains two copies of the CMP promoter and TMV enhancer. No unexpected bands were detected, indicating that the SYHT0H2 soybean contains no extraneous DNA fragments of the CMP promoter or TMV enhancer.

Copy Number of Functional Elements: NOS Terminator

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the NOS terminator–specific probe are shown in Figure 11.

In the analysis of genomic DNA digested with *Acl*I, two bands of approximately 7.6 and 10 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 11(A), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 11(A), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (Figure 11(A), Lane 5).

In the analysis of genomic DNA digested with *Eco*RI, two bands of approximately 4.8 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 11(B), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 11(B), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.5 kb was observed in the lane containing the positive control (Figure 11(B), Lane 5).

In the analysis of genomic DNA digested with *Kpn*I+ *Bsr*BI, two bands of approximately 3.5 and 4.3 kb were observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 5; Figure 11(C), Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 11(C), Lane 4) and were therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.5 kb was observed in the lane containing the positive control (Figure 11(C), Lane 5).

In the Southern blot analyses with the NOS terminator–specific probe, two hybridization bands were detected with both restriction enzyme digestion strategies, as expected. These results support the conclusions of the insert sequence analysis, which determined that SYHT0H2 soybean contains five copies of the NOS terminator. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of the NOS terminator.

Copy Number of Functional Elements: FMV Enhancer

The results of the Southern blot analyses of SYHT0H2 genomic DNA with the FMV enhancer–specific probe are shown in Figure 12.

The FMV enhancer is not present in SYHT0H2 soybean. In the analyses of genomic DNA digested with *Acl*I, *Pfl*MI, or *Kpn*I + *Bsr*BI, no bands were observed in the lanes containing DNA from SYHT0H2 T_4 soybean (Table 5; Figures 12(A) through 12(C), Lane 3) or in the lanes containing DNA from nontransgenic 'Jack' soybean (Figures 12(A) through 12(C), Lane 4), as expected. In all three analyses, one band of approximately 5.4 or 5.5 kb was observed in the lanes containing the positive control (Figures 12(A) through 12(C), Lane 5), as expected.

These results demonstrate that SYHT0H2 soybean does not contain DNA sequences from the FMV enhancer from the transformation plasmid pSYN15954.

Absence of Plasmid Backbone Sequence

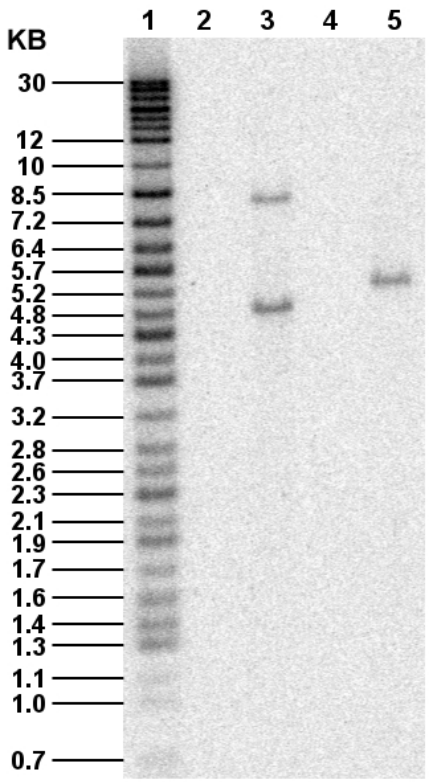
The results of the Southern blot analyses of SYHT0H2 genomic DNA with the pSYN15954 plasmid-backbone-specific probe are shown in Figure 13.

In the analyses of genomic DNA digested with *Acl*I, *Pfl*MI, or *Kpn*I, no hybridization bands were observed in the lanes containing DNA from SYHT0H2 T_4 soybean (Table 5; Figures 13(A) through IV-14(C), Lane 3) or in the lanes containing DNA from nontransgenic 'Jack' soybean (Figures 13(A) through 13(C), Lane 4). In the analyses of genomic DNA digested with *Pfl*MI or *Kpn*I, one band of approximately 5.7 kb was observed in the lanes containing the positive control (Figures 13(B) and 13(C), Lane 5), as expected.

However, in the analysis of genomic DNA digested with *Acl*I, three bands of approximately 5.5, 3.5, and 1.9 kb were observed in the lane containing the positive control (Figure 13(A), Lane 5). In this analysis, the positive-control plasmid pSYN15954 DNA digest was loaded with DNA from nontransgenic 'Jack' soybean that was digested with *Acl*I, and the *Acl*I also cut the plasmid DNA, resulting in the 3.5- and 1.9-kb bands. When an additional positive control without digested genomic DNA from nontransgenic 'Jack' soybean was included, a hybridization band of approximately 5.5 kb was observed, as expected for digestion of plasmid pSYN15954 with *Kpn*I + *Pme*I (Figure 13(A), Lane 7).

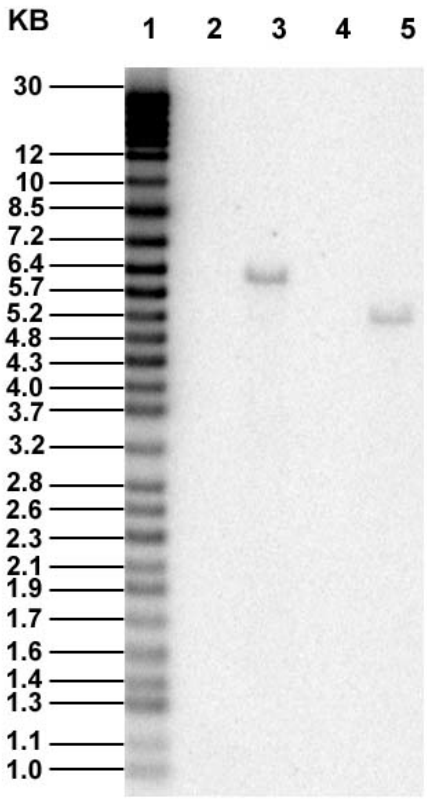
These results demonstrate that SYHT0H2 soybean does not contain any backbone sequences from the transformation plasmid pSYN15954.

(**A) *Eco*RI**



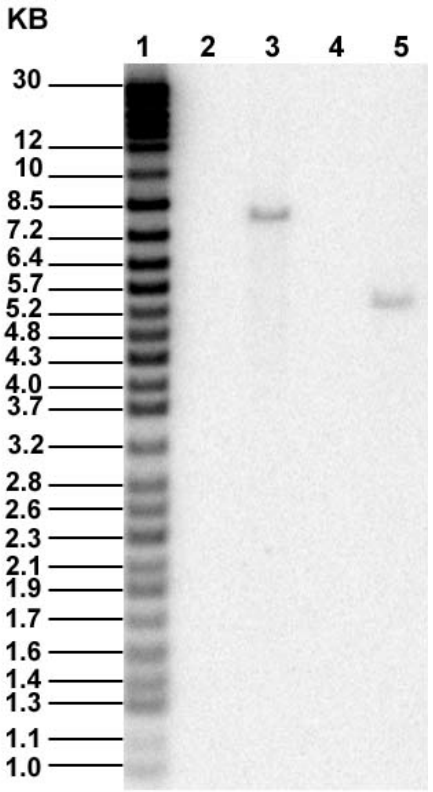
Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(**B) *Mfe*I**



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Mfe*I
Lane 4 = 'Jack' digested with *Mfe*I
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Mfe*I)

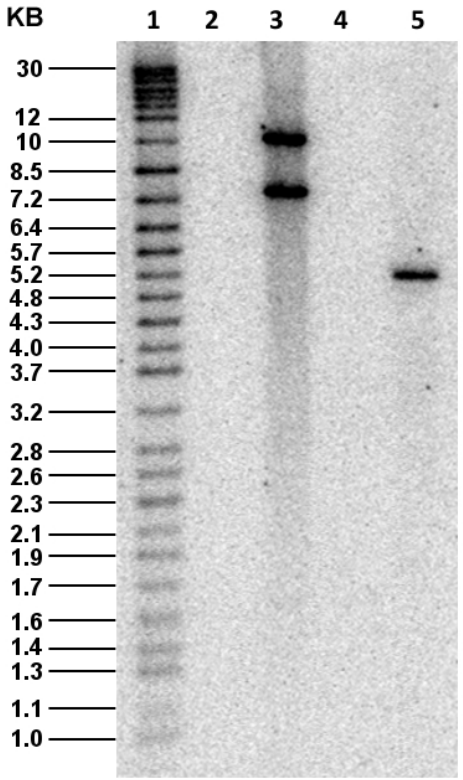
(C) *Kpn*I



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Kpn*I
Lane 4 = 'Jack' digested with *Kpn*I
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I)

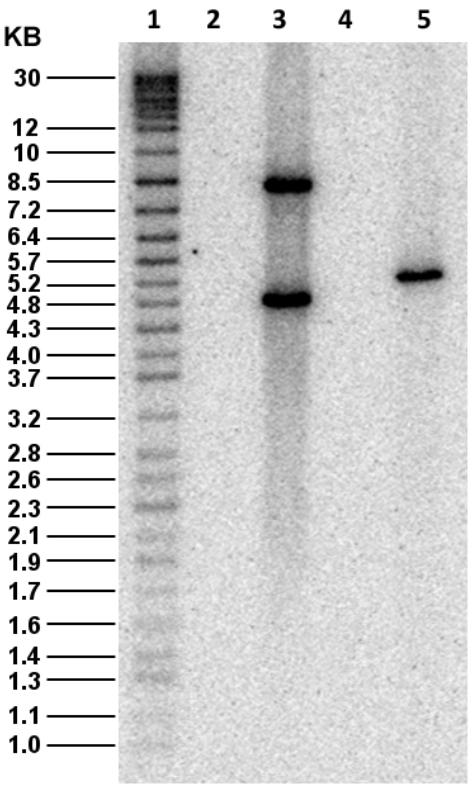
Figure 6 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *avhppd-03*–specific probe and the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I

(A) *Acl*I



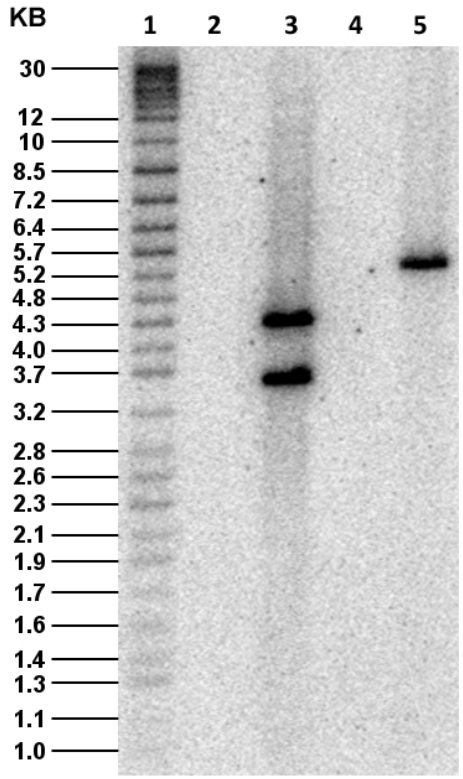
Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Acl*I
Lane 4 = 'Jack' digested with *Acl*I
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Acl*I)

(B) *Eco*RI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

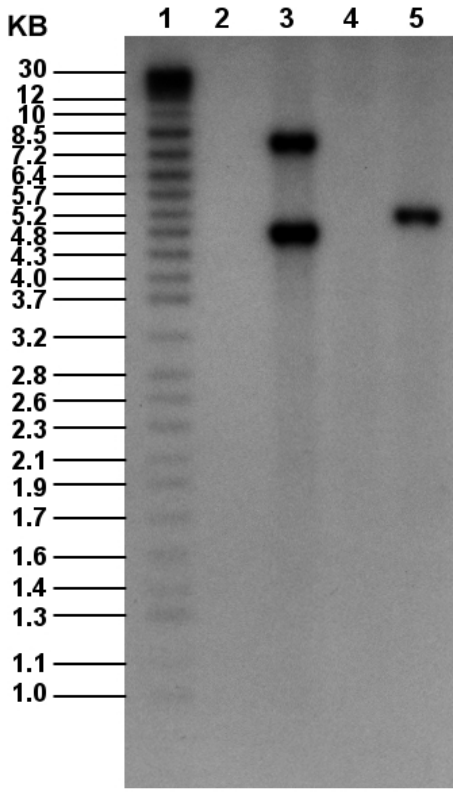
(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

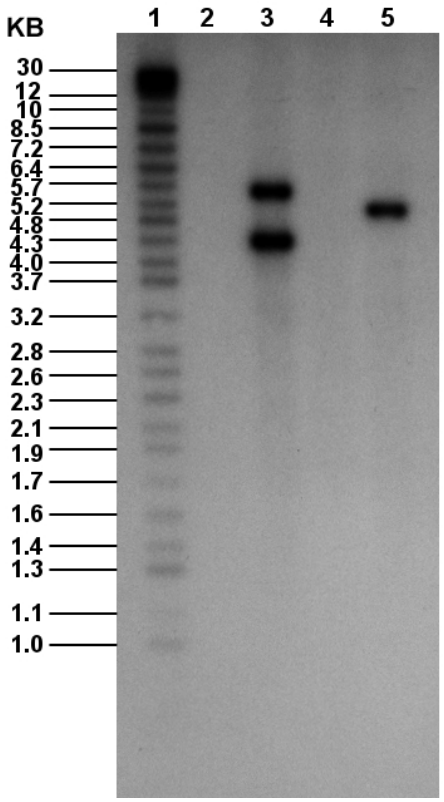
Figure 7 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *pat*-specific probe and the restriction enzymes AclI, *Eco*RI, and *Kpn*I + *Bsr*BI

(A) *Eco*RI



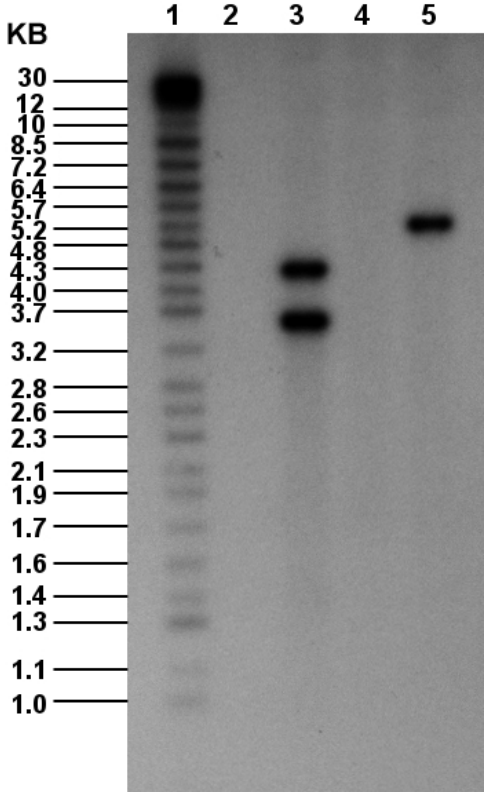
Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(B) *Xcm*I



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Xcm*I
Lane 4 = 'Jack' digested with *Xcm*I
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Xcm*I)

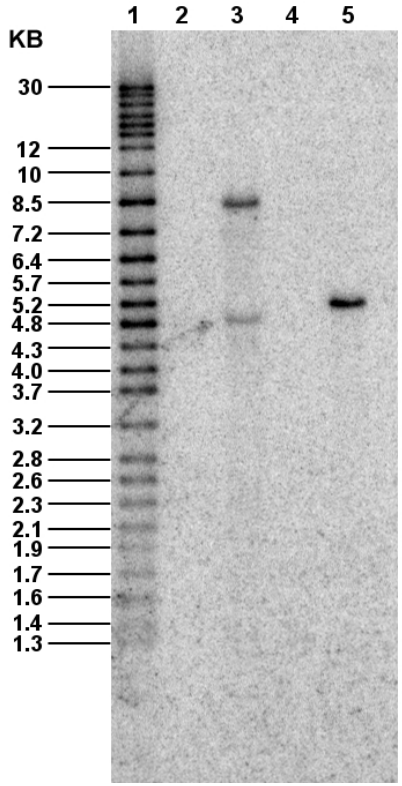
(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

Figure 8 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *avhppd-03* enhancer complex–specific probe and the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI

(A) *Eco*RI



Lane 1 = molecular-weight markers

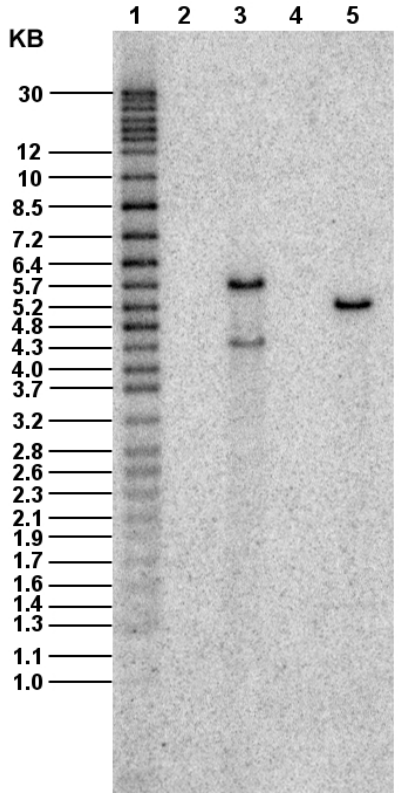
Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Eco*RI

Lane 4 = 'Jack' digested with *Eco*RI

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(B) *Xcm*I



Lane 1 = molecular-weight markers

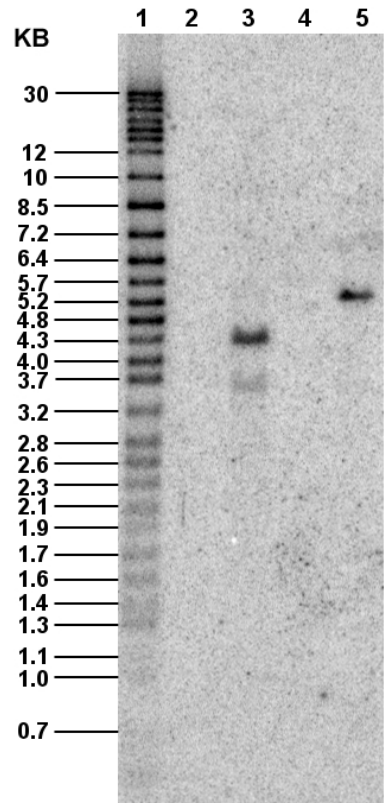
Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Xcm*I

Lane 4 = 'Jack' DNA digested with *Xcm*I

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Xcm*I)

(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers

Lane 2 = blank

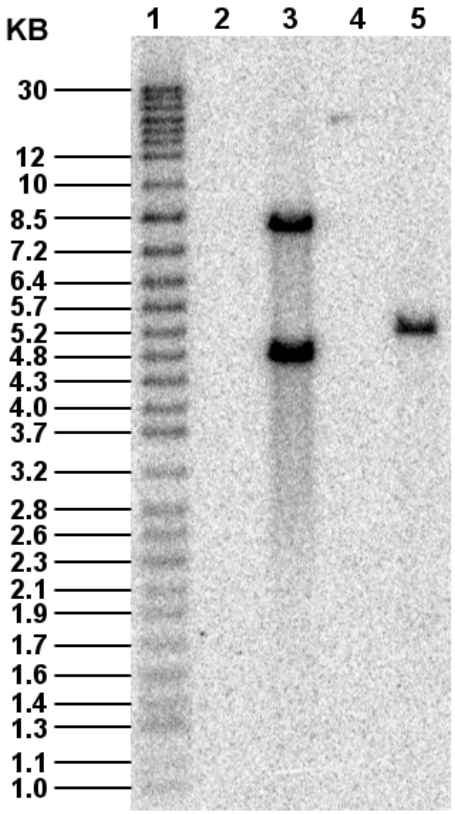
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI

Lane 4 = 'Jack' DNA digested with *Kpn*I + *Bsr*BI

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

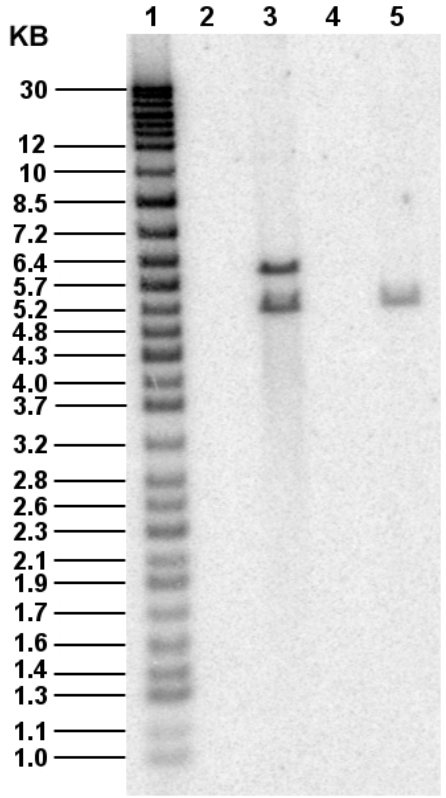
Figure 9 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 35S promoter–specific probe and the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI

(A) *Eco*RI



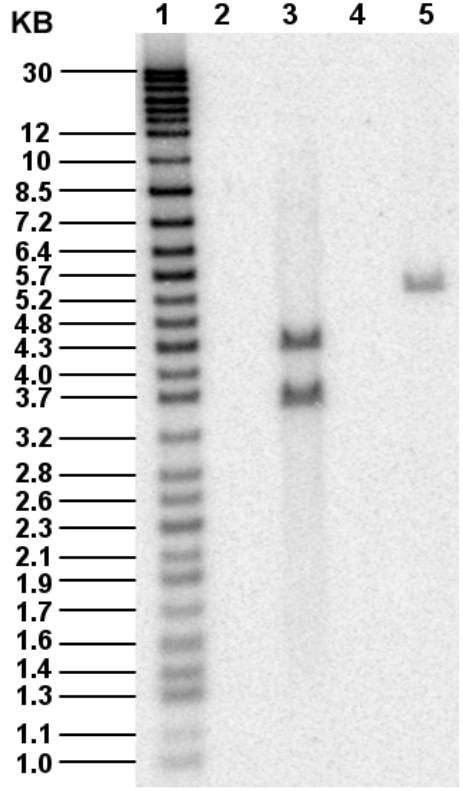
Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(B) *Mfe*I



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Mfe*I
Lane 4 = 'Jack' digested with *Mfe*I
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Mfe*I)

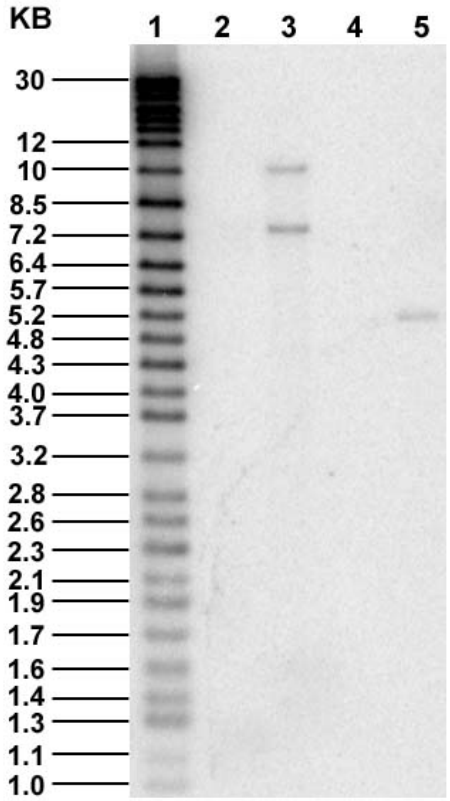
(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

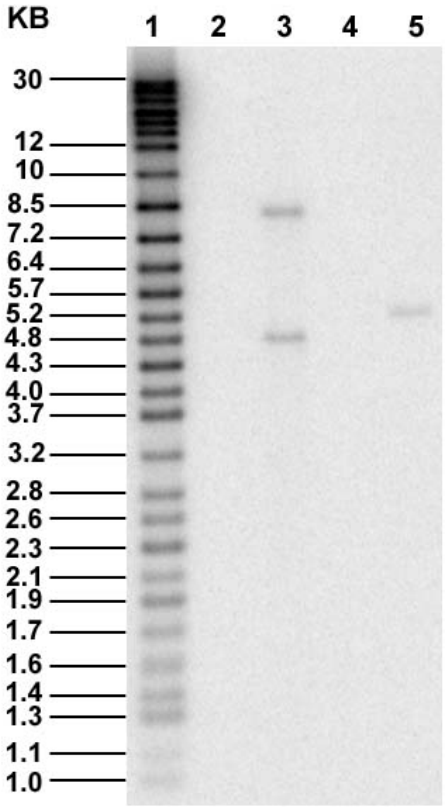
Figure 10 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the CMP promoter + TMV enhancer–specific probe and the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I + *Bsr*BI

(A) *Acl*I



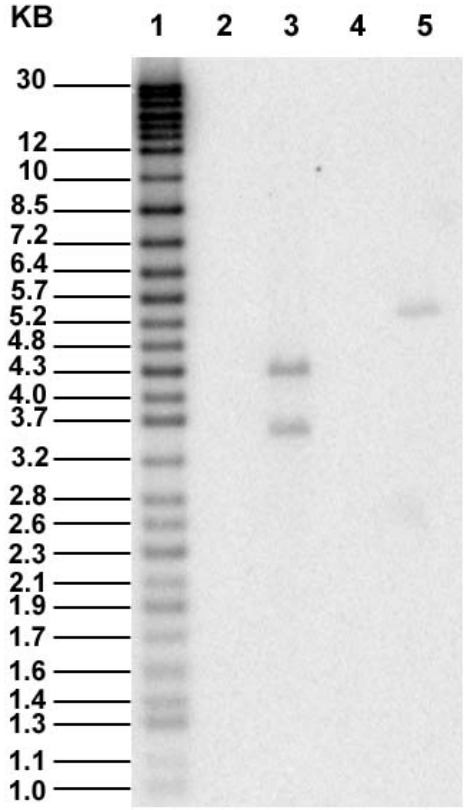
Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with AclI
Lane 4 = 'Jack' digested with AclI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with AclI)

(B) *Eco*RI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

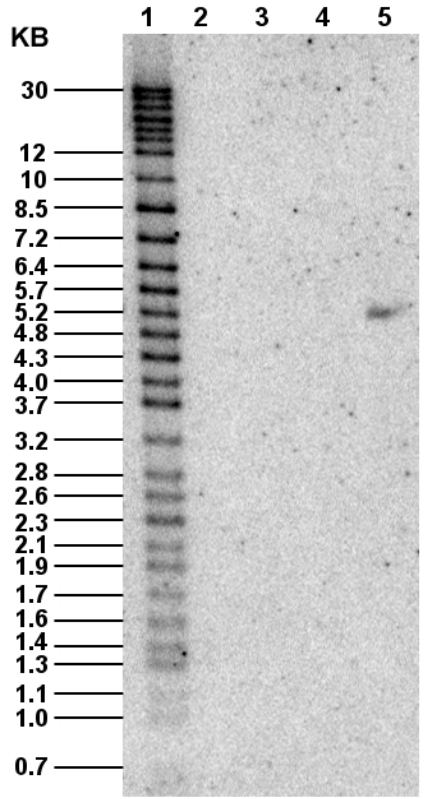
(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

Figure 11 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the NOS terminator–specific probe and the restriction enzymes AclI, *Eco*RI, and *Kpn*I + *Bsr*BI

(A) *Acl*I



Lane 1 = molecular-weight markers

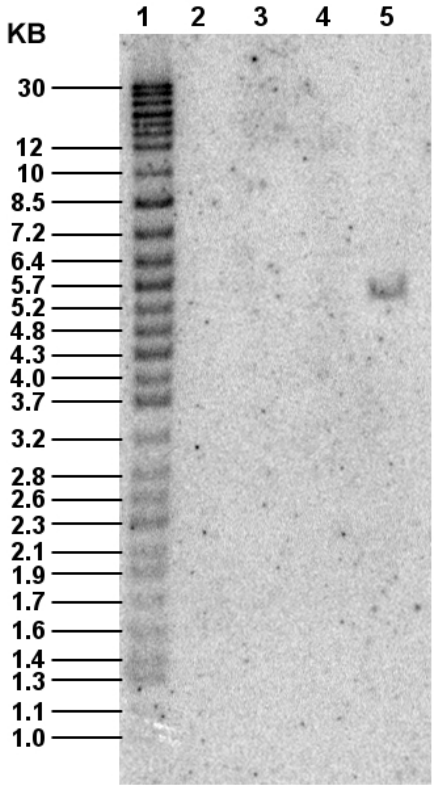
Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Acl*I

Lane 4 = 'Jack' digested with *Acl*I

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Acl*I)

(B) *Pfl*MI



Lane 1 = molecular-weight markers

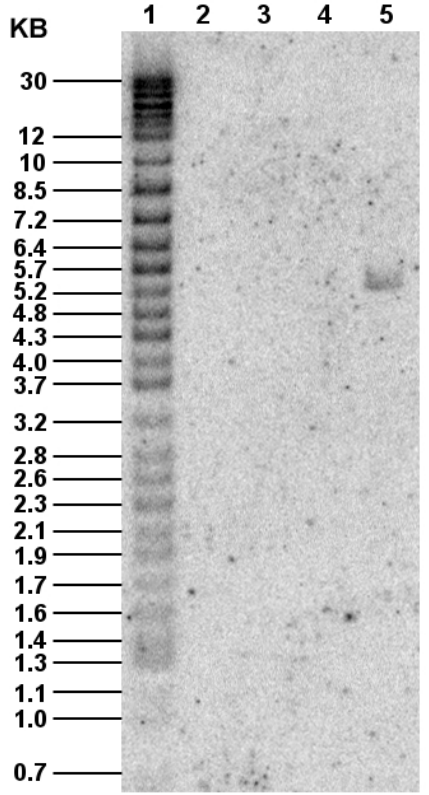
Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Pfl*MI

Lane 4 = 'Jack' digested with *Pfl*MI

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Pfl*MI)

(C) *Kpn*I + *Bsr*BI



Lane 1 = molecular-weight markers

Lane 2 = blank

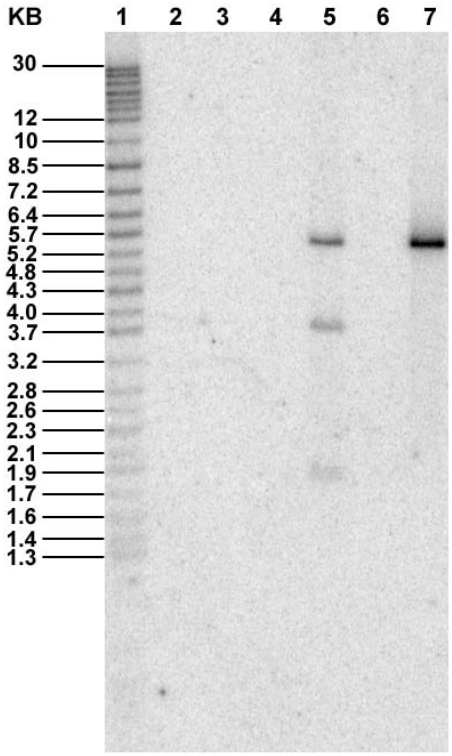
Lane 3 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI

Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I + *Bsr*BI)

Figure 12 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the FMV enhancer–specific probe and the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI

(A) *Acl*I



Lane 1 = molecular-weight markers

Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Acl*I

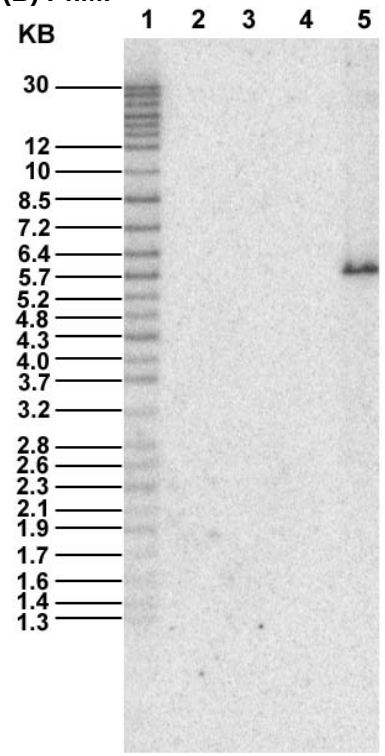
Lane 4 = 'Jack' digested with *Acl*I

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Acl*I)

Lane 6 = blank

Lane 7 = positive control without 'Jack' (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I)

(B) PflMI



Lane 1 = molecular-weight markers

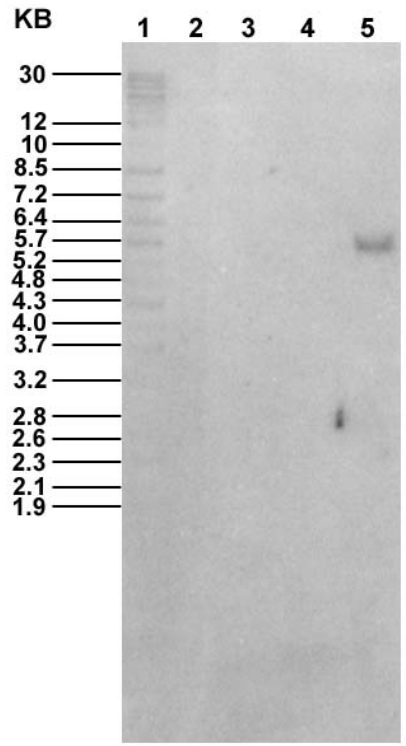
Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Pfl*MI

Lane 4 = 'Jack' digested with *Pfl*MI

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Pfl*MI)

(C) *Kpn*I



Lane 1 = molecular-weight markers

Lane 2 = blank

Lane 3 = SYHT0H2 T4 digested with *Kpn*I

Lane 4 = 'Jack' digested with *Kpn*I

Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Kpn*I)

Figure 13 Southern blot analysis of SYHT0H2 soybean with the pSYN15954 plasmid-backbone-specific probe and the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I

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

As detailed above in Section 2.3 (d)(i), Southern blot analysis and DNA sequencing of the single SYHT0H2 transgenic locus revealed that the inserted genetic material consists of a single copy of *avhppd-03*, four copies of *pat*, a single copy of the *avhppd-03* enhancer complex sequence, two copies of the 35S promoter, two copies of the CMP promoter, two copies of the TMV enhancer, and five copies of the NOS terminator.

(iii) 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 final food;

[redacted]

(iv) A map depicting the organisation of the inserted genetic material at each insertion site; and

The organisation of the SYHT0H2 transgenic locus is demonstrated in Figure 5 above.

(v) The identification and characterisation 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.

Bioinformatic analysis of the DNA sequence in the SYHT0H2 soybean T-DNA and at the T-DNA-to-genomic-DNA junctions was used to identify putative open reading frames (ORFs) that occurred between known or putative start (ATG) and stop (TAG, TAA, or TGA) codons and would code for a putative sequence of at least 30 amino acids. This analysis identified 47 putative ORFs in the T-DNA sequence (excluding the 5 ORFs for AvHPPD-03 and PAT) and 1 putative ORF at the 5′ junction [redacted]).

Each putative ORF sequence was translated into its putative amino acid sequence and then systematically compared with the protein sequences of known or putative allergens or toxins in (1) the Food Allergy Research and Resource Program (FARRP) Protein Allergen Database, v. 12 (FARRP 2012) and (2) a toxin database created from NCBI Entrez Protein database (NCBI 2012). The allergen comparison consisted of two alignment searches: (1) a full-length sequence search using the FASTA algorithm (Pearson and Lipman 1988) to identify any alignments of at least 80 amino acids with greater than 35% shared amino acid identity, and (2) a search for exact matches to 8 or more contiguous amino acids. Neither search found a significant level of shared amino acid sequence between any putative ORF amino acid sequences and any entry in the FARRP Allergen Protein Database. The Basic Local Alignment Search Tool for Proteins (BLASTP) program (Altschul *et al.* 1997) was used to search the toxin database; a statistically significant *E*-value of 1×10^{-5} was used as the initial threshold to identify potentially relevant alignments. No significant sequence similarity was observed to any entry in the toxin database. The likelihood that a novel protein would be expressed from any of the putative ORFs in the SYHT0H2 insert was determined by analysing each ORF's proximity to known promoters and the genetic context of the start codon. Of the 47 putative unintended ORFs identified, bioinformatic analysis ruled out the potential for expression of 46. The remaining ORF sequence showed no relevant biological similarity to any known or putative allergen or toxin.

(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).

Progeny of multiple transformants (T_0 plants) were field tested for tolerance to applications of mesotrione and glufosinate-ammonium herbicides and for agronomic performance. SYHT0H2 soybean was selected as a lead commercial candidate and underwent further development. Key steps in the development of SYHT0H2 soybean are shown in Figure 14. All shipments and field releases of SYHT0H2 soybean in the U.S. were carried out under USDA notifications.

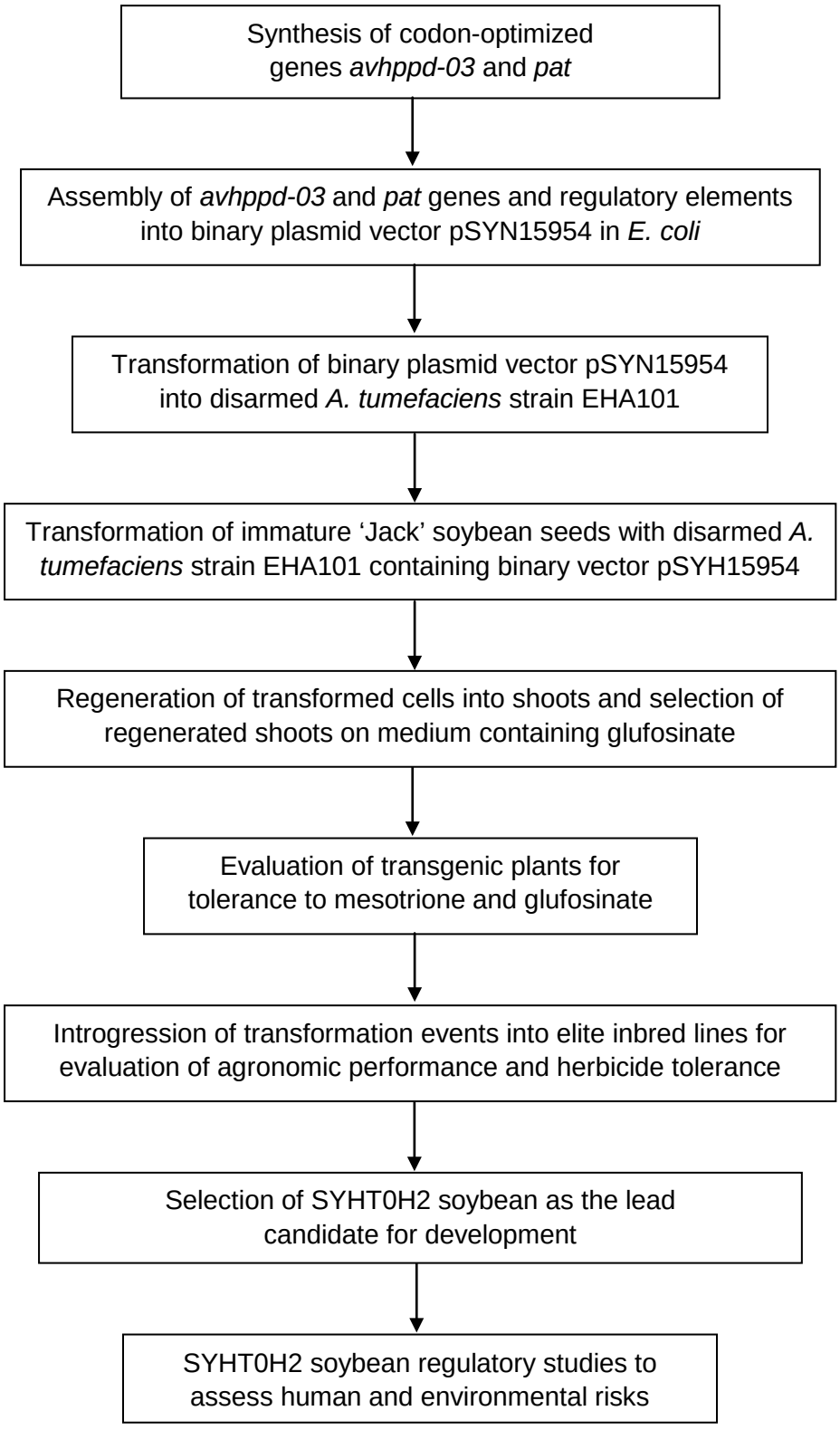


Figure 14 Steps in the development of SYHT0H2 soybean

Production of all SYHT0H2 soybean and nontransgenic control soybean seed lots used in the studies described in this petition was carried out under controlled and isolated conditions under the direction of Syngenta breeders and field researchers. Figure 15 shows the pedigree of SYHT0H2 seed materials. For all regulatory studies except the test for Mendelian inheritance, SYHT0H2 soybean was in the genetic background 'Jack.' Nontransgenic 'Jack' soybean was used as a near-isogenic control material in all regulatory studies. Nontransgenic control soybean seed lots were produced at the same time and location as the SYHT0H2 soybean seed lots to which they were compared.

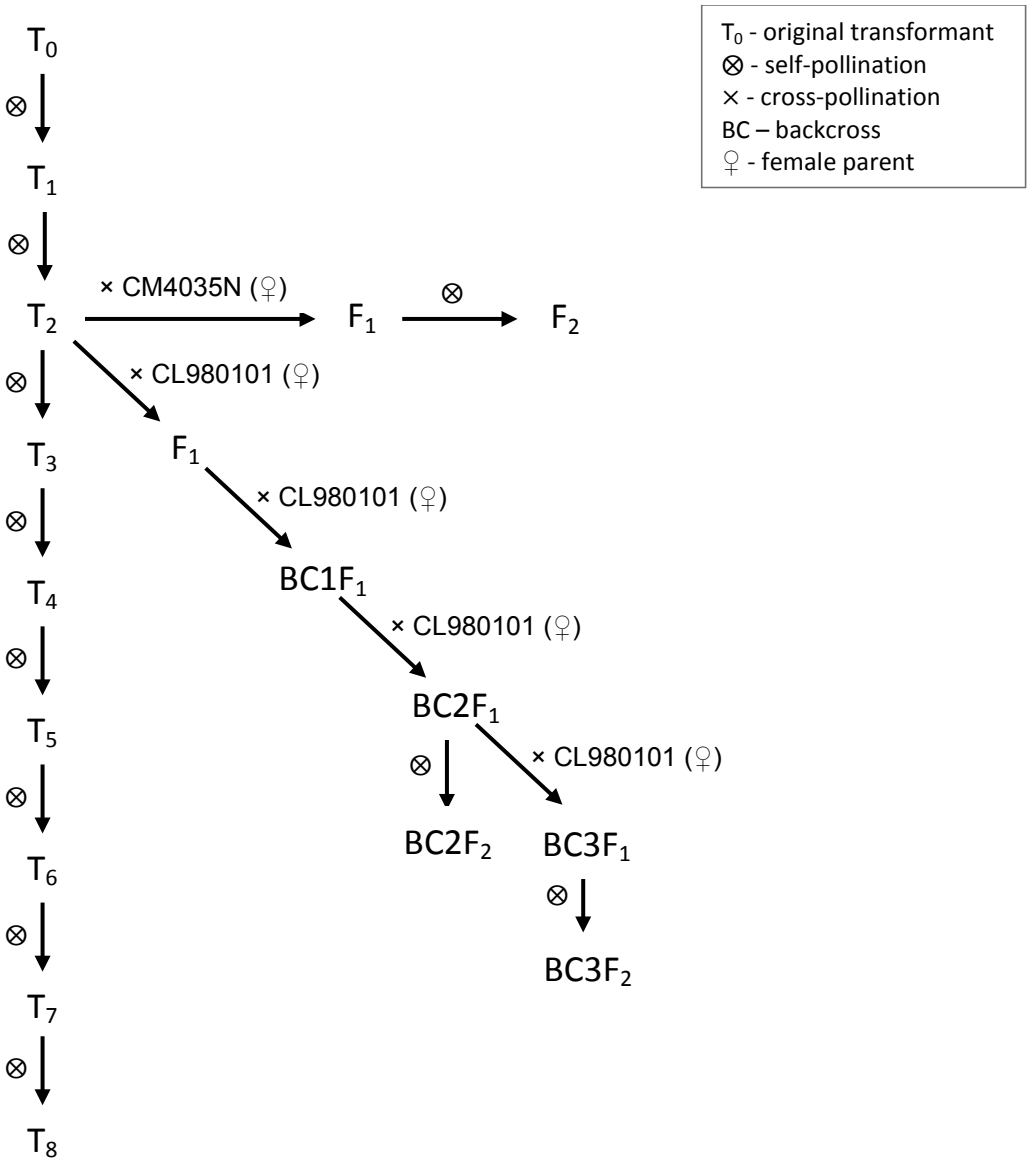


Figure 15 Pedigree of the SYHT0H2 plant materials used in regulatory studies

The transformation recipient line was 'Jack' soybean. CL4035N and CL980101 are commercial nontransgenic soybean lines.

(f) Evidence of the stability of the genetic changes, including:

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

Three generations of SYHT0H2 soybean were individually analysed for the presence of *avhppd-03* and *pat* by real-time PCR analysis ([redacted] The results from real-time PCR analysis were used to determine the segregation ratios of *avhppd-03* and *pat*. SYHT0H2 soybean populations that were hemizygous for the transgenes were self-pollinated to create the generations analysed in this study (F_2, $BC2F_2$, and $BC3F_2$, as shown in the pedigree diagram, Figure 15); therefore, the expected segregation ratio for each gene was 3:1 in each generation (i.e., 75% of the plants in each generation were expected to carry the gene). Chi-square analysis of the segregation data was performed to test the hypothesis that the SYHT0H2 insert is inherited in a predictable manner according to Mendelian principles and consistent with insertion into a chromosome within the soybean nuclear genome. The goodness-of-fit of the observed to the expected segregation ratios was tested by chi-square analysis (Strickberger 1976) with Yates' correction factor as in Armitage and Berry (1987):

$$\chi^2 = \text{sum } [|(\text{observed} - \text{expected})| - 0.5]^2 \div \text{expected}$$

The expected and observed segregation ratios are shown for *avhppd-03* in Table 6 and for *pat* in Table 7. The critical value for rejection of the hypothesis of segregation according to Mendelian inheritance at α = 0.05 was 3.84 (Strickberger 1976). All of the chi-square values were less than 3.84, indicating that *avhppd-03* and *pat* were inherited in a predictable manner according to Mendelian principles. These results support the conclusion that the SYHT0H2 soybean insert integrated into a chromosome within the soybean nuclear genome.

Table 6 Observed and expected frequencies of *avhppd-03* in three generations of SYHT0H2 soybean

	F_2		$BC2F_2$		$BC3F_2$	
Trait	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	115	123	99	104.25	134	131.25
Negative	49	41	40	34.75	41	43.75
Total	164	164	139	139	175	175
χ^2	1.83		0.87		0.15	

Table 7 Observed and expected frequencies of *pat* in three generations of SYHT0H2 soybean

	F_2		$BC2F_2$		$BC3F_2$	
Trait	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	115	123	99	104.25	134	131.25
Negative	49	41	40	34.75	41	43.75
Total	164	164	139	139	175	175
χ^2	1.83		0.87		0.15	

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

Southern blot analyses were performed to demonstrate the genetic stability of the SYHT0H2 insert over three generations ([redacted]). Two T-DNA–specific probes were used that collectively covered every base of the pSYN15954 T-DNA. The analysis was performed with genomic DNA extracted from SYHT0H2 T_4, T_5, and T_6 soybean (as shown in the pedigree diagram, Figure 15) and from nontransgenic 'Jack' soybean, as a negative control to identify any endogenous soybean DNA sequences that hybridized with the probes. One or more positive controls, equivalent to one copy of a fragment of known size in the soybean genome, were included to demonstrate the sensitivity of each analysis. The positive control contained 0.89 pg of CMP promoter–specific and 1.80 pg of *avhppd-03*–specific DNA fragments plus digested DNA from nontransgenic 'Jack' soybean.

Two restriction enzyme digestion strategies were used in these Southern blot analyses. In the first strategy, soybean genomic DNA was digested with an enzyme that cut at least once within the SYHT0H2 insert; the other recognition sites for this enzyme were located in the soybean genome flanking the SYHT0H2 insert. This strategy was used twice, with two different enzymes. Analyses with the T-DNA–specific probe were used to determine the copy number of the SYHT0H2 insert and the presence or absence of extraneous plasmid pSYN15954 T-DNA fragments in other regions of the SYHT0H2 soybean genome. The restriction enzymes used were *Eco*RI and *Xho*I.

In the second strategy, soybean genomic DNA was digested with enzymes that cut within the insert to release DNA fragments of predictable size. This strategy was used to determine the intactness of the SYHT0H2 insert and the presence or absence of any closely linked extraneous DNA fragments of plasmid pSYN15954. The enzyme combination used was *Kpn*I + *Bsr*BI.

Figure 16 shows the locations of the T-DNA–specific probes and restriction enzymes in the T-DNA region of the SYHT0H2 transformation plasmid pSYN15954. Figure 17 shows the locations of the T-DNA–specific probes and restriction sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in SYHT0H2 soybean insert. Table 8 shows the expected and observed numbers and sizes of the hybridization bands, and Figure 18 shows the results of the corresponding Southern blot analyses. No hybridization bands were expected in the analyses of genomic DNA from nontransgenic 'Jack' soybean.

In the analysis of genomic DNA digested with EcoRI, the lanes containing DNA from SYHT0H2 T4, T5, and T6 soybean (Table 8; Figure 18, Lanes 2 through 4) showed two hybridization bands of approximately 4.8 and 8.5 kb, as expected. In the analysis of genomic DNA digested with *Xho*I, the lanes containing DNA from these three generations of SYHT0H2 soybean (Table 8; Figure 18, Lanes 2 through 4) showed four hybridization bands of approximately 2.2, 3.7, 6.6, and 20 kb, as expected. In the analysis of genomic DNA digested with *Kpn*I + *Bsr*BI, two hybridization bands of approximately 3.5 and 4.3 kb were observed in the lanes containing DNA from SYHT0H2 T_4, T_5, and T_6 soybean (Table 8; Figure 18, Lanes 2 through 4), as expected.

As expected, no hybridization bands were observed in any of the analyses with nontransgenic 'Jack' soybean, indicating that all of the bands observed for SYHT0H2 T_4, T_5, and T_6 soybean DNA were specific to the SYHT0H2 insert. Also as expected, the positive control resulted in two bands of approximately 1.3 and 0.7 kb (one band for each T-DNA probe) in all of the analyses (Figure 18(A) through (C), Lane 6); the hybridization intensities corresponded to one copy each of the CMP promoter–specific and *avhppd-03*–specific DNA fragments.

In the Southern blot analyses with the T-DNA–specific probes, the expected numbers and sizes of hybridization bands were detected with both restriction enzyme digestion strategies. These results confirm that two partial copies of the SYHT0H2 insert integrated into a single locus in the soybean genome. No unexpected bands were observed, indicating that the SYHT0H2 soybean genome contains no extraneous fragments of the SYHT0H2 insert. Furthermore, the hybridization bands specific to the insert were identical in lanes containing DNA extracted from plants grown from all three generations of SYHT0H2 soybean tested, indicating that the SYHT0H2 insert is stably inherited from one generation to the next.

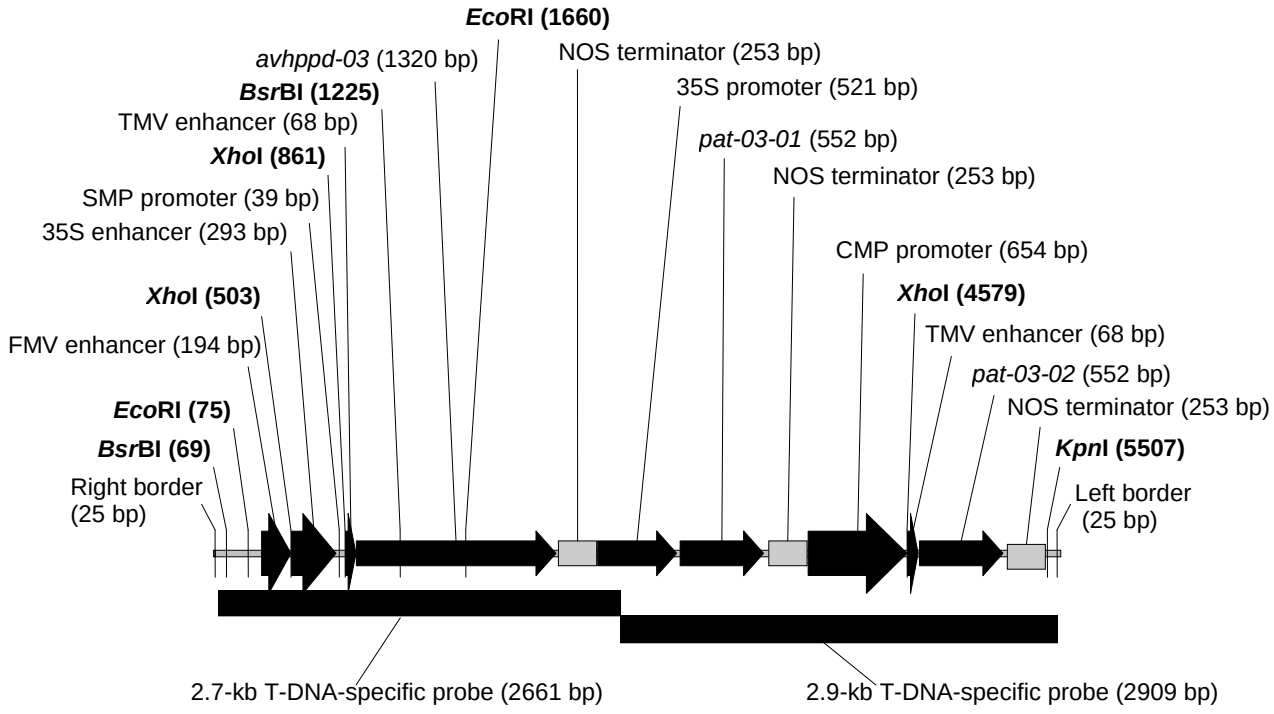


Figure 16 Locations of the 2.7- and 2.9-kb T-DNA–specific probes and the restrictions sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in the pSYN15954 transformation plasmid

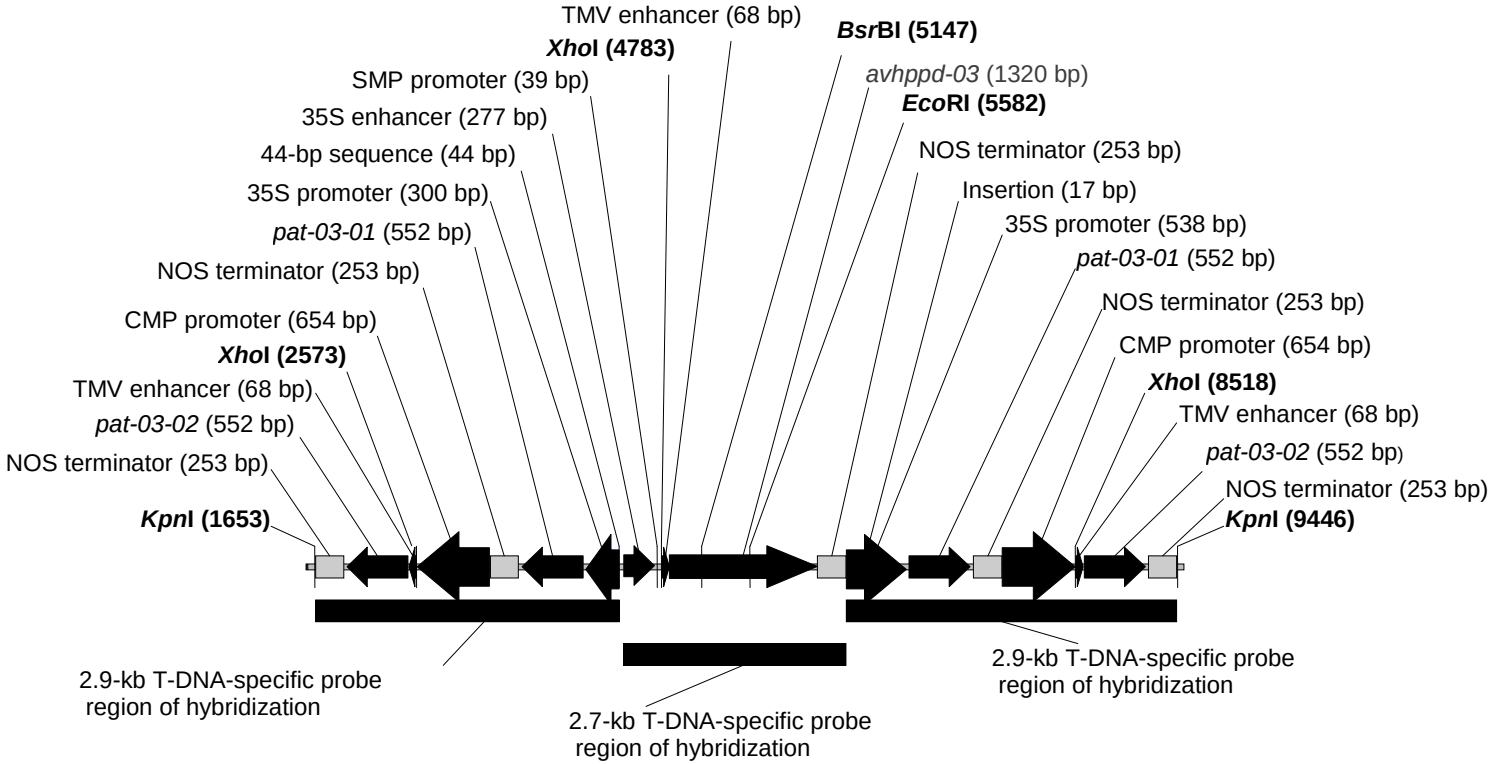


Figure 17 Locations of the 2.7- and 2.9-kb T-DNA–specific-probes and the restriction sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in the SYHT0H2 soybean insert

Table 8 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean with T-DNA–specific probes and restriction enzymes *Eco*RI, XhoI, and *Kpn*I + *Bsr*BI

Restriction enzyme	Source of DNA[a]	Figure & Lane	Expected no. of bands	Expected band size (kb)	Observed band size (kb)
*Eco*RI	SYHT0H2 T_4	V-13A, 2	2	>3.9	~4.8
				>4.0	~8.5
	SYHT0H2 T_5	V-13A, 3	2	>3.9	~4.8
				>4.0	~8.5
	SYHT0H2 T_6	V-13A, 4	2	>3.9	~4.8
				>4.0	~8.5
	positive control	V-13A, 6	0	~0.7, ~1.3	~0.7, ~1.3
*Xho*I	SYHT0H2 T_4	V-13B, 2	4	~2.2	~2.2
				~3.7	~3.7
				>1.0	~6.6
				>1.0	~20
	SYHT0H2 T_5	V-13B, 3	4	~2.2	~2.2
				~3.7	~3.7
				>1.0	~6.6
				>1.0	~20
	SYHT0H2 T_6	V-13B, 4	4	~2.2	~2.2
				~3.7	~3.7
				>1.0	~6.6
				>1.0	~20
	positive control	V-13B, 6	1	~0.7, ~1.3	~0.7, ~1.3
*Kpn*I + *Bsr*BI	SYHT0H2 T_4	V-13C, 2	2	~3.5	~3.5
				~4.3	~4.3
	SYHT0H2 T_5	V-13C, 3	2	~3.5	~3.5
				~4.3	~4.3
	SYHT0H2 T_6	V-13C, 4	2	~3.5	~3.5
				~4.3	~4.3
	positive control	V-13C, 6	2	~0.7, ~1.3	~0.7, ~1.3

[a]The positive control consists of 0.89 pg of CMP promoter–specific and 1.80 pg of *avhppd-03*–specific DNA fragments (representing one copy ofeach of the elements in the soybean genome) plus 'Jack' digested with the indicated enzyme.

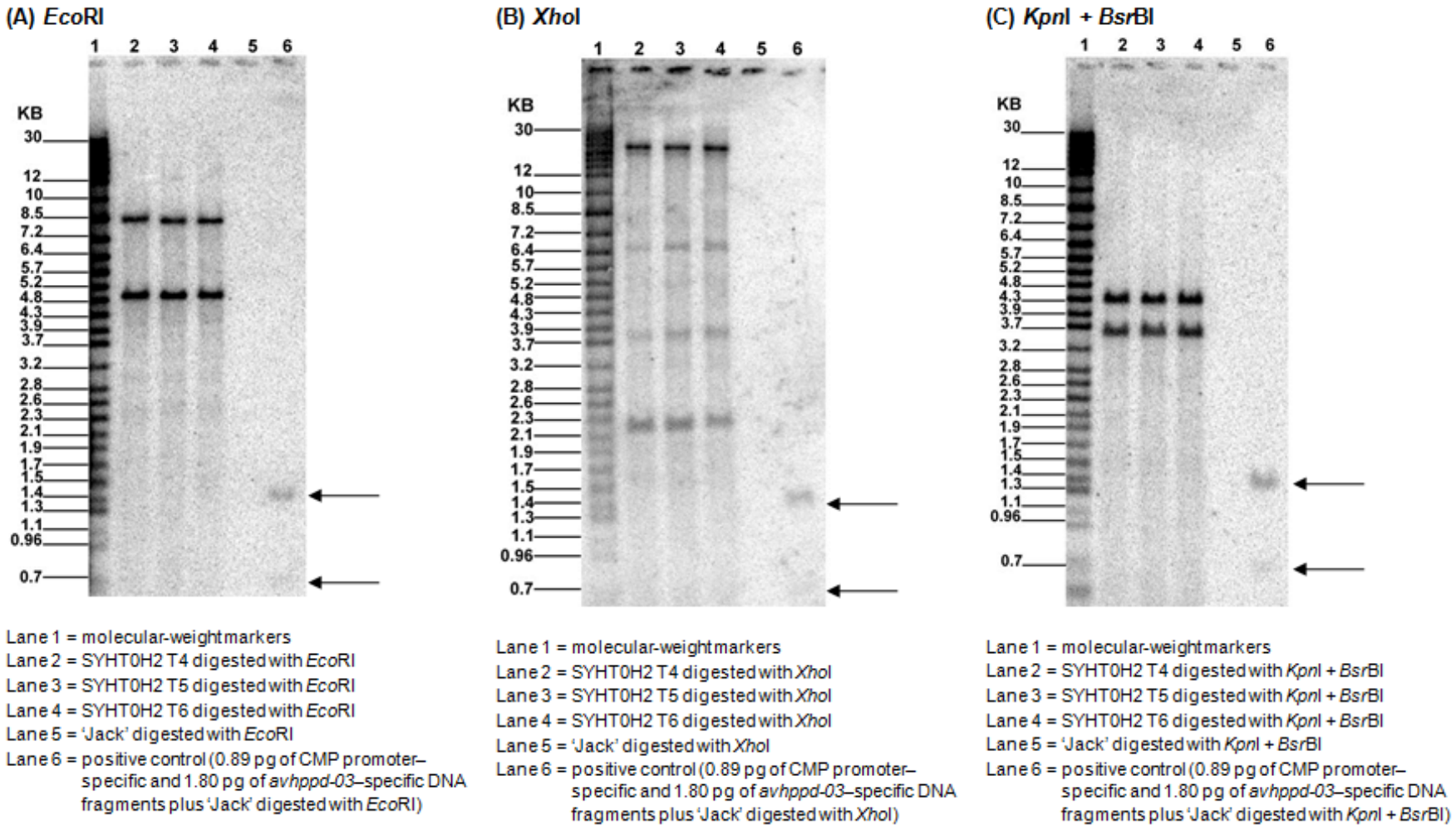


Figure 18 Genetic stability Southern blot analysis of SYHT0H2 soybean with the 2.7- and 2.9-kb T-DNA–specific probes and the restriction enzymes *Eco*RI, *Xho*I, and *Kpn*I + *Bsr*BI

The horizontal arrows indicate the locations of the *avhppd-03*–specific and CMP promoter–specific positive controls.

2.4 Information on the Labeling of the GM Food

(a) Information on whether novel DNA or protein is likely to be present in final food.

To enable an assessment of the potential exposure of humans and animals to the recombinant proteins expressed in soybean event SYHT0H2, seed samples and products derived from them were analysed for the content of the AvHPPD-03 and PAT protein [redacted]

The primary food product derived from soybean for human consumption is soybean oil. In the course of processing soybeans to produce refined vegetable oil of food grade quality, all protein components are destroyed by the high temperature and pressure of the screw pressing, or separated by extraction with a non-polar solvent and destroyed by the temperature of the solvent recovery. Remaining traces of protein in the crude oil are removed in the alkali treatment and deodorization steps of oil refining. Consequently, an intake of these recombinant proteins is not possible via soybean food grade oil or products containing this oil grade.

Laboratory-scale milling methodology equivalent to industry-standard processing was used to process the seed of SYHT0H2 soybean and of a nontransgenic, near-isogenic control soybean into the following commercially representative food and feed fractions: milk, tofu, hulls, full-fat flour, flakes, white flakes, defatted toasted meal, protein concentrate, and protein isolate. Enzyme-linked immunosorbent assay was used to quantify AvHPPD-03 and PAT in the processed soybean fractions and in the seed from which they were produced. AvHPPD-03 and PAT were detected in SYHT0H2 soybean seed and in the hulls, full-fat flour, flakes, white flakes, and defatted toasted meal processed from SYHT0H2 soybean seed. AvHPPD-03 and PAT were not detected in the milk, tofu, protein concentrate, or protein isolate processed from SYHT0H2 soybean seed (Table 9). AvHPPD-03 and PAT were not detected in any samples processed from nontransgenic soybean seed.

Table 9 Concentrations of AvHPPD-03 and PAT in SYHT0H2 soybean seed and in food and feed fractions processed from SYHT0H2 soybean seed

Sample	AvHPPD-03		PAT	
	Mean ± SD µg/g DW	Mean ± SD µg/g FW	Mean ± SD µg/g DW	Mean ± SD µg/g FW
Seed	20.36±1.76	18.91±1.63	9.25±0.45	8.59±0.42
Milk	<LOD[a]	-	<LOD[b]	-
Tofu	<LOD[a]	-	<LOD[b]	-
Hulls	2.53±1.14	2.27±1.02	1.10±0.45	0.99±0.40
Full-fat flour	21.03±5.61	20.34±5.43	6.74±3.32	6.51±3.21
Flakes	14.68±4.48	13.84±4.23	3.18±0.28	3.00±0.26
White flakes	17.36±5.46	16.55±5.20	5.82±1.79	5.55±1.71
Defatted toasted meal	1.42±0.09	1.41±0.09	0.09±0.02	0.09±0.02
Protein concentrate	<LOD[a]	-	<LOD[b]	-
Protein isolate	<LOD[a]	-	<LOD[b]	-

N=3 replicate analyses for each sample

The concentrations were adjusted for extraction efficiency.

- = Because lyophilized samples were analysed, LOD values were not determined on a FW basis.

[a]LOD = 0.0313 µg/g of sample.

[b]LOD = 0.025 µg/g of sample.

Using the "Pulses" information provided in the GEMS/Food Regional Diets publication of WHO, the potential intake of recombinant protein via the consumption of whole soybeans as pulses and, additionally, for all kind of oilseeds except groundnuts, was calculated. The highest recombinant protein concentrations measured were used for the calculation. These calculations were based on worst-case scenarios taking the highest recombinant protein amounts determined in the soybean commodity, assuming that all commercial soybean seeds taken to produce food or animal feed would be the soybean event SYHT0H2, and that all kinds of oilseeds with the exception of groundnuts consumed by humans would be soybean seeds.

Table 10 shows the predicted dietary intake of the AvHPPD-03 and PAT proteins in different regional diets. The predicted AvHPPD-03 protein intake via whole soybeans as pulses was between 1.89 and 85.1 µg per person per day for the various regional diets (WHO, 2003). Based on the consumption of all kinds of oilseeds (except groundnuts), the AvHPPD-03 protein intake was between 9.45 and 96.44 µg per person per day. The PAT protein intake via whole soybeans as pulses for the various regional diets was between 0.86 and 38.65 µg per person per day. Based on the consumption of all kinds of oilseeds (except groundnuts), the PAT protein intake was between 4.29 and 43.81 µg per person per day.

Table 10 Predicted dietary intake of AvHPPD-03 and PAT proteins

Parameter	Regional Diets				
	Europe [a]	Latin America	Middle East	Far East	Africa
Consumption of whole soybeans as pulses in gram per person per day	0.1[b]	0.1 [b]	4.5	2	0.5
Consumption of all kind of oilseeds (except groundnuts) in gram per person per day	3.1	0.5	5.1	1.2	3.1
Highest AvHPPD-03 protein content in SYHT0H2 seeds is 18.91 µg/g fw [c]					
Predicted daily intake of AvHPPD-03 protein via whole soybeans (µg per person per day)	1.89	1.89	85.1	37.82	9.45
Predicted daily intake of AvHPPD-03 protein via all kind of oilseeds (µg per person per day)	58.62	9.45	96.44	22.69	58.62
Highest PAT protein content in SYHT0H2 seeds is 8.59 µg/g fw [c]					
Predicted daily intake of PAT protein via whole soybeans (µg per person per day)	0.86	0.86	38.65	17.18	4.29
Predicted daily intake of PAT protein via all kind of oilseeds (µg per person per day)	26.63	4.29	43.81	10.31	26.63

[a] The European diet includes countries with European-type diets, such as Australia, Canada and the USA
[b] Since whole soybean is not consumed in Europe or Latin America a default value of 0.1 g per person per day was assigned.
[c] Highest AvHPPD-03 and PAT contents in soybeans taken from Table 9.

(b) Detection methodology for the GM food suitable for analytical purposes.

A gel-based, event-specific polymerase chain reaction (PCR) method was developed to detect SYHT0H2 soybean deoxyribonucleic acid (DNA). This method uses two oligonucleotide primers to amplify a 140 base pair (bp) DNA fragment that spans one of the junctions between the soybean genome and the SYHT0H2 insert ([redacted]).

Part 3 Information Related to the Safety of the Genetically Modified Food

3.1 Information on Antibiotic Resistance Marker Genes (if used)

(a) Information on the clinical and veterinary importance, if any, in Australia and New Zealand of the antibiotic to which any transferred antibiotic resistance genes confer resistance.

Not applicable. No antibiotic resistance genes were transferred in the production of SYHT0H2 soybean.

(b) Information on whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of the orally administered antibiotic.

Not applicable.

(c) Information on the safety of the gene product.

Not applicable.

(d) If the new GM organism is a micro-organism, information on whether it will remain viable in the final food.

Not applicable.

3.2 The Characterisation of Novel Proteins or Other Novel Substances

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

AvHPPD-03 Protein

Herbicides that competitively inhibit endogenous plant *p*-hydroxyphenylpyruvate dioxygenase enzymes provide pre- and post-emergence control of broadleaf weeds in many crop systems and are widely used for weed control in maize. The endogenous HPPDs of maize, oat, and other grass species are relatively insensitive to inhibition by such herbicides, in comparison with the endogenous HPPDs of soybean and other broadleaf species. SYHT0H2 soybean produces an HPPD enzyme, AvHPPD-03, derived from oat (*Avena sativa*), which confers tolerance to commercial application rates of HPPD-inhibiting herbicides, such as mesotrione.

HPPD is an enzyme in the tyrosine catabolic pathway (Mitchell *et al.* 2001). HPPD enzymes are found in nearly all aerobic forms of life (Lindstedt and Odelhog 1987, Ruetschi *et al.* 1993, Garcia *et al.* 1999) and catalyze the conversion of 4-hydroxyphenylpyruvate (HPP) to homogentisic acid (homogentisate, HGA), the aromatic precursor to plastoquinone and tocochromanol biosynthesis (Moran 2005). Tocopherols and tocotrienols are collectively known as tocochromanols; they are lipid-soluble molecules that comprise the group of vitamin E compounds.

Eukaryotic organisms catabolize tyrosine to HGA as a central intermediate in the tyrosine catabolic pathway; a simplified outline of the pathway is shown in Figure 19 (Cahoon *et al.* 2003, Arias-Barrau *et al.* 2004, Moran 2005, DellaPenna and Pogson 2006, Zbierzak *et al.* 2010). Plants can synthesize HGA from the enzymatic activity of HPPDs (including AvHPPD-03) via tyrosine and *p*-hydroxyphenylpyruvate (Valentin and Qi 2005). Overall, the primary biosynthetic products of the catabolic pathway are the eight tocochromanols, plastoquinone, or acetoacetate and fumarate. This biosynthetic pathway including HPPD is found in nearly all aerobic organisms, including plants, animals, and bacteria, and is important in both photosynthesis and cellular metabolism via the citric acid cycle. The HPPD pathway converges on the citric acid cycle with the production of fumarate and acetoacetate, which is degraded to acetyl coenzyme A (acetyl-CoA). Fumarate is also generated in the urea cycle, and acetyl-CoA is a product of polysaccharide, lipid, and protein metabolism.

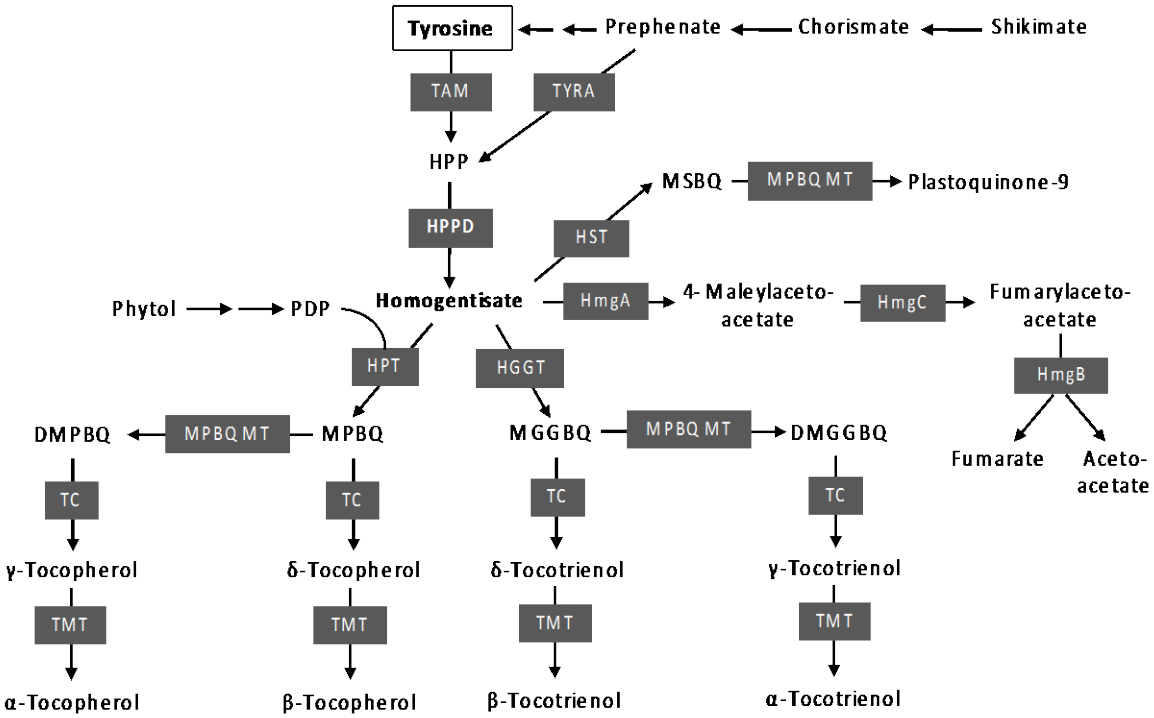


Figure 19 Tyrosine catabolic pathway including HPPD metabolism

Reaction products:

HPP = 4-hydroxyphenylpyruvate

PDP = phytyldiphosphate

MSBQ = 2-methyl-6-solanyl-1,4-benzoquinone

MPBQ = 2-methyl-6-phytyl-1,4-benzoquinone

DMPBQ = 2,3-dimethyl-5-phytyl-1,4-benzoquinone

MGGBQ = 2-methyl-6-geranylgeranyl-1,4-benzoquinone

DMGGBQ = 2,3-dimethyl-5-gernaylgeranyl-1,4-benzoquinone

Enzymes:

TAM = L-tyrosine aminotransferase

TYRA = chorismate mutase-prephenate dehydrogenase

HPPD = *p*-hydroxyphenylpyruvate dioxygenase

HST = homogentisate solanyltransferase

MPBQ MT = MPBQ methyltransferase

HmgA = homogentisate dioxygenase

HmgB = fumarylacetoacetate hydrolase

HmgC = maleylacetoacetate isomerase

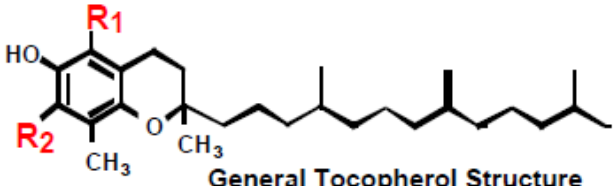
HPT = homogentisate phytyltransferase

HGGT = homogentisate geranylgeranyl transferase

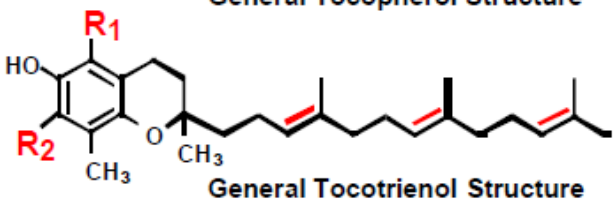
TC = tocopherol cyclase

TMT = tocopherol methyltransferase

Plastoquinone is involved in the electron transport chain in the light-dependent reactions of photosynthesis. Tocochromanols, more commonly known as vitamin E, consist of four tocopherol isoforms and four tocotrienol isoforms (shown in Figure 20). They are lipophilic antioxidants that are synthesized exclusively in photosynthetic organisms and are an essential part of the mammalian diet. Vitamin E, in the form of α-tocopherol, is essential in the human diet. Although tocochromanol content and composition vary considerably among plant species, oilseeds such as soybean are particularly rich in tocochromanols (Karunanandaa *et al.* 2005), and tocochromanol content has been reported to be as high as 1200 µg/g of oil in one soybean variety (DellaPenna 2005). In higher-order plants, the predominant vitamin E isoform is γ-tocopherol in seeds and α-tocopherol in green leaf tissue. The predominant form of tocopherol in soybean oil is γ-tocopherol (70% of total tocopherol).



General Tocopherol Structure



General Tocotrienol Structure

Tocochromonol isoform	R_1	R_2	Relative activity (tocopherol vs. tocotrienol)
α-tocopherol / tocotrienol	$-CH_3$	$-CH_3$	100% vs. 30%
β-tocopherol / tocotrienol	$-CH_3$	–H	50% vs. 5%
γ-tocopherol / tocotrienol	–H	$-CH_3$	10% vs. 0%
δ-tocopherol / tocotrienol	–H	–H	3% vs. 0%

Figure 20 Tocochromanol structure and isoforms in soybean

The saturated aliphatic side chain distinguishes tocopherols from tocotrienols. Structural differences in the isoforms are indicated by R_1 and R_2 and are defined in the table. Relative activity refers to the vitamin E activity of each tocopherol and tocotrienol isoform compared with the activity of α-tocopherol (DellaPenna 2005).

The fact that HPPD is present in nearly all aerobic life forms, that it is commonly consumed by both herbivorous and carnivorous animals, and that its expression in plants and animals exerts no known toxic effects all support the prediction that no adverse health effects will result from exposure to the AvHPPD-03 protein present in SYHT0H2 soybean.

PAT Protein

The gene *pat* contained in SYHT0H2 soybean encodes the enzyme phosphinothricin acetyltransferase, which inactivates the herbicide glufosinate-ammonium (L-phosphinothricin), an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. SYHT0H2 soybean contains two PAT genes, *pat-03-01* and *pat-03-02*, both of which were derived from *Streptomyces viridochromogenes* strain Tü494 and encode the selectable marker PAT (Wohlleben *et al.* 1988). The native coding sequences

were codon-optimized for enhanced expression, and *pat-03-02* was altered to remove restriction sites. Both *pat-03-01* and *pat-03-02* encode the identical PAT amino acid sequence.

PAT is a highly specific enzyme for acetylation of glufosinate-ammonium herbicide, and it does not acetylate glutamate (the closest structural analog to glufosinate-ammonium) or other L-amino acids (Wehrmann *et al.* 1996, Hérouet *et al.* 2005). PAT belongs to the class of acetyltransferase enzymes common in plants and animals, and it shares very similar three-dimensional structure, molecular weight, and functional properties with other acetyltransferase enzymes, which are present as natural components of human and animal diets. There are no reports of toxicity or allergenicity associated with the acetyltransferase class of enzymes.

The enzyme activity of PAT follows Michaelis-Menten kinetics in the pH range from 7 to 8.5 and shows a tolerance to pH values ranging from 6 to 11. Glutamate and analogues such as methionine sulfoximine and hydroxylysine are much poorer substrates than glufosinate-ammonium. These enzymatic properties establish that the activity of PAT and PAT homologues is limited to acetylation of the glufosinate-ammonium substrate (Hérouet *et al.* 2005).

(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.

As detailed in previous sections on the molecular characterisation of the soybean event SYHT0H2 (see Sections 2.3(d)(v), no novel genes apart from the *avhppd-03* and *pat* genes are expressed as a result of the genetic modification event.

(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.

The concentrations of AvHPPD-03 in various SYHT0H2 plant tissues were quantified via enzyme-linked immunosorbent assay (ELISA) ([redacted]). The tissues analysed were leaves (at four growth stages), roots (at two growth stages), forage, and seed. Tissues were collected from SYHT0H2 soybean and a nontransgenic, near-isogenic (control) soybean that were field grown in the 2011–2012 growing season concurrently at four locations in Argentina according to local agronomic practices. Concurrent analysis of tissues from nontransgenic soybean confirmed the absence of plant-matrix effects on the ELISA methods and the specificity of the ELISA methods for AvHPPD-03.

The mean tissue and whole-plant concentrations of AvHPPD-03 in SYHT0H2 soybean across all four locations were determined on a fresh-weight (FW) and dry-weight (DW) basis (as shown in Table 11). All values were corrected for extraction efficiency. On a fresh-weight basis, the concentration of AvHPPD-03 in individual samples across all locations and plant stages ranged from 4.93 to 135.84 µg/g in leaves, 0.42 to 45.65 µg/g in roots, 4.31 to 44.32 µg/g in forage, and 0.55 to 24.94 µg/g in seed. Variability of AvHPPD-03 concentrations was observed among replicate samples, as indicated by the wide ranges and large standard deviations. This variability could not be attributed to the study conduct, as several levels of bias control were implemented throughout the study. Although considerable variability was observed in tissue concentrations of AvHPPD-03, consistent performance of the herbicide-tolerance trait has been demonstrated in replicated efficacy field trials conducted by Syngenta.

Table 11 Concentrations of AvHPPD-03 in SYHT0H2 soybean tissue samples at several growth stages, across four locations, on dry-weight and fresh-weight bases

Stage	µg/g DW		µg/g FW	
(*N* = 20)	Mean ± SD	Range	Mean ± SD	Range
Leaves, V4	242.00 ± 140.99	20.23–585.46	59.01 ± 32.86	6.39–135.84
Leaves, V8	212.98 ± 102.03	53.77–386.15	56.65 ± 29.95	11.72–116.85
Leaves, V10	165.14 ± 66.11	55.96–302.90	41.38 ± 15.71	12.11–74.46
Leaves, R6	105.32 ± 67.18	16.94–255.30	29.50 ± 20.15	4.93–75.67
Roots (V8)	79.49 ± 47.33	15.43–201.47	17.72 ± 10.96	3.24–45.65
Roots (R6)	22.50 ± 20.82	1.50–69.95	5.87 ± 5.55	0.42–18.26
Forage (R6)	79.66 ± 44.43	16.76–164.01	20.86 ± 11.72	4.31–44.32
Seed (R8)	8.18 ± 8.36	0.62–28.30	7.16 ± 7.31	0.55–24.94

SD = standard deviation.

The concentrations of PAT protein in various SYHT0H2 plant tissues were quantified via ELISA ([redacted]). The tissues analysed were leaves (at four growth stages), roots (at two growth stages), forage, and seed. Tissues were collected from SYHT0H2 soybean and a nontransgenic, near-isogenic (control) soybean that were field grown in the 2011–2012 growing season concurrently at four locations in Argentina according to local agronomic practices. Concurrent analysis of tissues from nontransgenic soybean confirmed the absence of plant-matrix effects on the ELISA methods and the specificity of the ELISA methods for PAT.

The mean tissue and whole-plant concentrations of PAT in SYHT0H2 soybean across all four locations were determined on fresh-weight and dry-weight bases (as shown in Table 12). All values were corrected for extraction efficiency. On a fresh-weight basis, the concentration of PAT in individual samples across all locations and plant stages ranged from 0.22 to 41.43 µg/g in leaves, 0.07 to 11.98 µg/g in roots, 0.29 to 16.46 µg/g in forage, and 0.06 to 13.13 µg/g in seed. Variability of PAT concentrations was observed among replicate samples, as indicated by the wide ranges and large standard deviations. This variability could not be attributed to the study conduct, as several levels of bias control were implemented throughout the study. Although considerable variability was observed in tissue concentrations of PAT, consistent performance of the herbicide-tolerance trait has been demonstrated in replicated efficacy field trials conducted by Syngenta.

Table 12 Concentrations of PAT in SYHT0H2 soybean tissue samples at several growth stages, across four locations, on dry-weight and fresh-weight bases

Stage	µg/g DW		µg/g FW	
N = 20	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Leaves, V4	52.21 ± 53.28	0.89–167.97	12.56 ± 12.55	0.22–41.43
Leaves, V8	23.00 ± 22.84	2.04–83.43	6.05 ± 5.97	0.47–21.17
Leaves, V10	38.23 ± 31.10	4.77–115.86	9.76 ± 8.21	1.41–31.35
Leaves, R6	29.41 ± 27.51	0.77–101.58	8.23 ± 8.09	0.22–30.84
Roots(V8)	21.16 ± 18.17	0.33–46.07	4.77 ± 4.21	0.07–11.98
Roots (R6)	9.12 ± 8.50	0.32–29.35	2.40 ± 2.29	0.07–8.45
Forage (R6)	19.17 ± 18.61	1.12–60.91	5.03 ± 4.88	0.29–16.46
Seed (R8)	2.70 ± 4.04	0.07–14.85	2.36 ± 3.55	0.06–13.13

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

AvHPPD-03

A series of analytical methods were used to characterize the AvHPPD-03 protein produced in SYHT0H2 soybean seed and to demonstrate that an AvHPPD-03 test substance produced from recombinant *E. coli* is a suitable surrogate for use in food and feed safety studies ([redacted]). The use of a microbially produced test substance was necessary because SYHT0H2 soybean produces low levels of AvHPPD-03, making it infeasible to extract the plant-produced protein in quantities sufficient for safety studies.

The identities of the plant-produced and microbially produced AvHPPD-03 proteins were confirmed by apparent molecular weight, immunoreactivity, peptide mass mapping, and *N*-terminal amino acid sequence analyses. The AvHPPD-03 present in the microbially produced test substance (identified as AvHPPD-03-0209) was identical to that produced in SYHT0H2 soybean except for a minor (four-amino-acid) truncation at the *N*-terminus of the AvHPPD-03 protein as expressed *in planta*. Western blot analysis demonstrated that the apparent molecular weights of both the plant-produced and microbially produced AvHPPD-03 proteins were consistent with the predicted molecular weight of 47.0 kDa, and both proteins cross-reacted with the same antibody (as shown in Figure 21). The peptide mass mapping analysis verified 55% and 65% of the predicted amino acid sequence of AvHPPD-03 for the plant-produced and microbially produced proteins, respectively (as shown in Figures 22 and 23). Except for the apparent post-translational cleavage of the first four amino acids from the *N*-terminus of the plant-produced protein, the *N*-terminal sequence of AvHPPD-03 from both sources was consistent with the expected sequence (as shown in Figure 24).

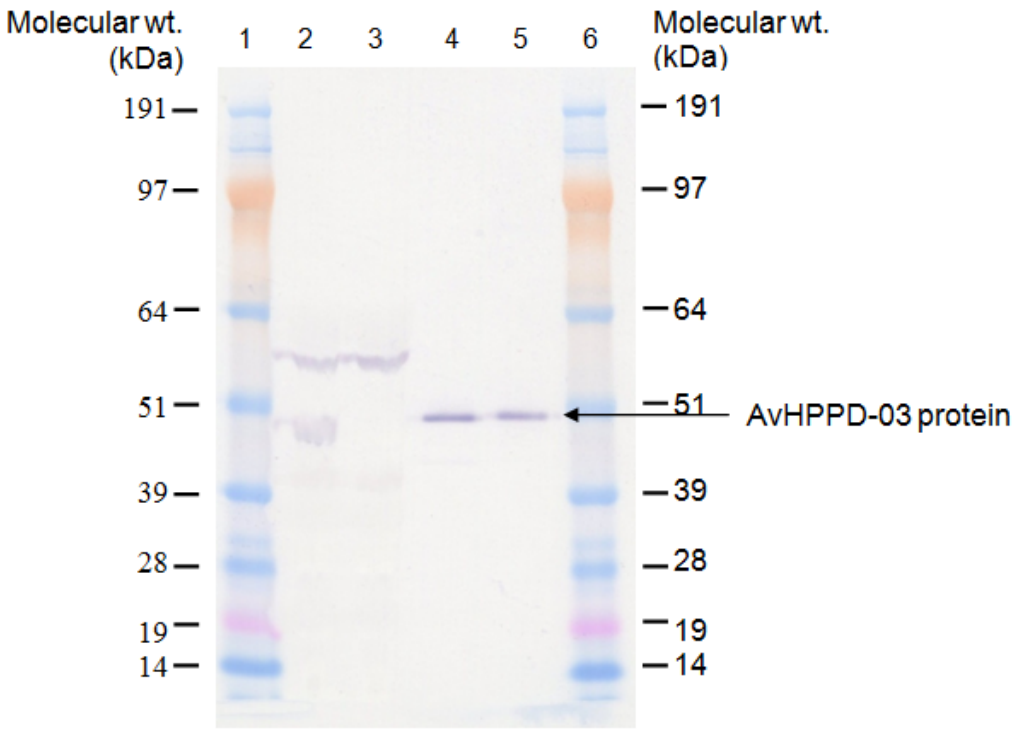


Figure 21 Western blot analysis of plant-produced and microbially produced AvHPPD-03

Lanes 1 & 6: Molecular-weight standard.

Lane 2: Crude SYHT0H2 soybean seed extract (10 ng AvHPPD-03, 83 µg total protein).

Lane 3: Nontransgenic soybean seed extract (83 µg total protein).

Lane 4: AvHPPD-03 purified preparation from SYHT0H2 soybean seed extract (10 ng AvHPPD-03).

Lane 5: Microbially produced AvHPPD-03 (10 ng AvHPPD-03).

```
  1    MPPTPATATG AAAAAVTPEH AARSFPRVVR VNPRSDRFPV LSFHHVELWC
 51    ADAASAAGRF SFALGAPLAA RSDLSTGNSA HASLLLRSGA LAFLFTAPYA
101    PPPQEAATAA TASIPSFSAD AARTFAAAHG LAVRSVGVRV ADAAEAFRVS
151    VAGGARPAFA PADLGHGFGL AEVELYGDVV LRFVSYPDET DLPFLPGFER
201    VSSPGAVDYG LTRFDHVVGN VPEMAPVIDY MKGFLGFHEF AEFTAEDVGT
251    TESGLNSVVL ANNSEAVLLP LNEPVHGTKR RSQIQTYLEY HGGPGVQHIA
301    LASNDVLRTL REMRARTPMG GFEFMAPPQA KYYEGVRRIA GDVLSEEQIK
351    ECQELGVLVD RDDQGVLLQI FTKPVGDRPT FFLEMIQRIG CMEKDEVGQE
401    YQKGGCGGFG KGNFSELFKS IEDYEKSLEV KQSVVAQKS
```

Figure 22 Amino acid sequence of plant-produced AvHPPD-03 identified by peptide mass mapping analysis

Identified AvHPPD-03 fragments are bold and underlined.

```
  1    MPPTPATATG AAAAAVTPEH AARSFPRVVR VNPRSDRFPV LSFHHVELWC
 51    ADAASAAGRF SFALGAPLAA RSDLSTGNSA HASLLLRSGA LAFLFTAPYA
101    PPPQEAATAA TASIPSFSAD AARTFAAAHG LAVRSVGVRV ADAAEAFRVS
151    VAGGARPAFA PADLGHGFGL AEVELYGDVV LRFVSYPDET DLPFLPGFER
201    VSSPGAVDYG LTRFDHVVGN VPEMAPVIDY MKGFLGFHEF AEFTAEDVGT
251    TESGLNSVVL ANNSEAVLLP LNEPVHGTKR RSQIQTYLEY HGGPGVQHIA
301    LASNDVLRTL REMRARTPMG GFEFMAPPQA KYYEGVRRIA GDVLSEEQIK
351    ECQELGVLVD RDDQGVLLQI FTKPVGDRPT FFLEMIQRIG CMEKDEVGQE
401    YQKGGCGGFG KGNFSELFKS IEDYEKSLEV KQSVVAQKS
```

Figure 23 Amino acid sequence of microbial AvHPPD-03 identified by peptide mass mapping analysis

Identified AvHPPD-03 fragments are bold and underlined.

Predicted sequence:	MPPTPATATGAAAAAV
Plant-produced AvHPPD-03:	PATATGAAAAAV
Microbially produced AvHPPD-03:	MPPTPATATGAA

Figure 24 *N*-terminal amino acid sequence of plant-produced and microbially produced AvHPPD-03

The plant-produced and microbially produced AvHPPD-03 proteins were also compared with respect to glycosylation status. The plant-produced AvHPPD-03 was analysed to ensure that no post-translational glycosylation of the protein had occurred *in planta*; *E. coli* cannot produce glycosylated proteins. As shown in Figure 25, this analysis demonstrated the absence of post-translational glycosylation of the plant-produced AvHPPD-03 protein and therefore, equivalence with the microbially produced AvHPPD-03 in this regard.

In addition, the AvHPPD-03 proteins from both sources were demonstrated to have comparable enzymatic activity when characterized in a standard substrate turnover assay ($^{14}CO_2$ capture assay). The activity of the microbially produced AvHPPD-03 was evaluated in the presence of extract of nontransgenic, near-isogenic soybean seed to control for possible seed matrix effects from the AvHPPD-03 protein preparation from soybean seeds. The specific activity was 1.22 units/mg for the plant-produced AvHPPD-03 and 1.38 units/mg for the microbially produced AvHPPD-03 (see Table 13). These results confirmed that the truncation of four amino acids from the *N*-terminus of the plant-produced AvHPPD-03 did not affect the function of this enzyme.

These results verified the identities of the plant-produced and microbially produced AvHPPD-03 proteins, and it was concluded that the AvHPPD-03 proteins produced in SYHT0H2 soybean and in recombinant *E. coli* were biochemically and functionally equivalent. Therefore, the microbially produced test substance containing AvHPPD-03 was a suitable surrogate for AvHPPD-03 in SYHT0H2 soybean and was appropriate for use in studies supporting the safety of AvHPPD-03.

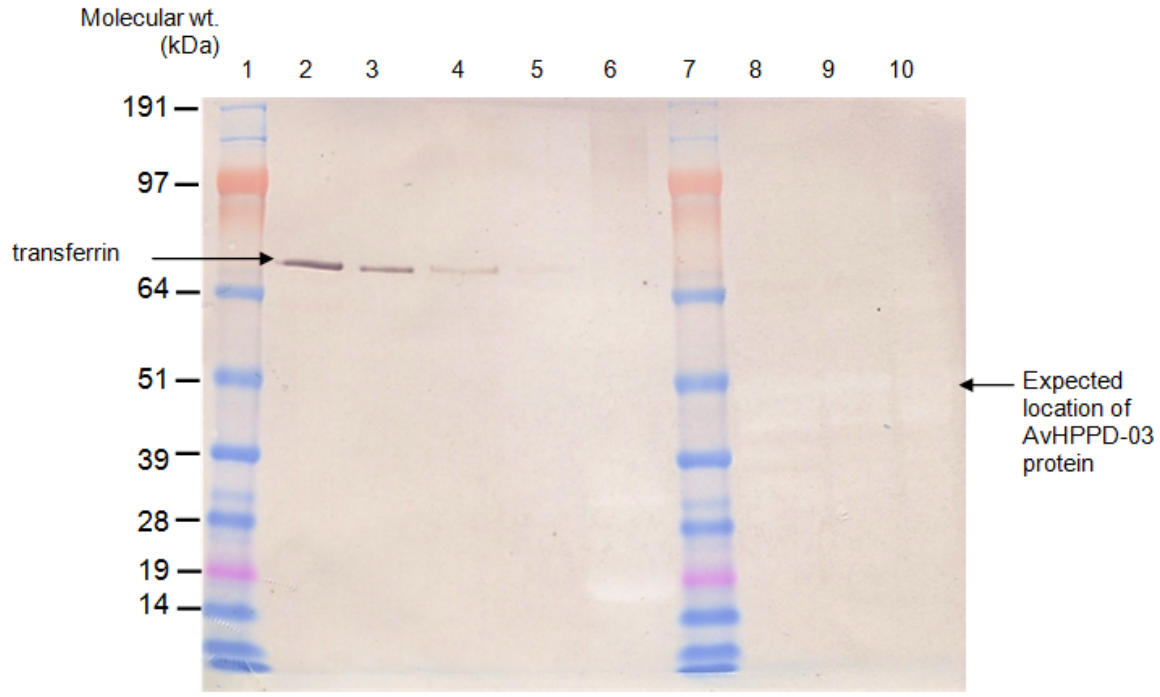


Figure 25 Glycosylation analysis of plant-produced and microbially produced AvHPPD-03

Lane 1: Molecular-weight standard

Transferrin (positive control):

Lane 2: 100 ng

Lane 3: 50 ng

Lane 4: 25 ng

Lane 5: 10 ng[a]

Lane 6: Soybean trypsin inhibitor (negative control), 1000 ng

Lane 7: Molecular-weight standard

AvHPPD-03 purified preparation from SYHT0H2 soybean seed extract:

Lane 8: 1000 ng

Lane 9: 500 ng

Lane 10: 1000 ng

[a]Because of limitations in printer resolution, the faint band visible at approximately 80 kDa may not be visible on the printed copy.

Table 13 Specific enzyme activity of the plant-produced and microbially produced AvHPPD-03

Test substance	Assay replicate	HPPD specific activity (U/mg HPPD)[a]	Mean HPPD specific activity (U/mg HPPD)	RSD (%)[b]
Plant-produced AvHPPD-03	1	1.26	1.22	4.36
	2	1.18		
Microbially produced AvHPPD-03	1	2.45	2.58	7.17
	2	2.71		
Nontransgenic soybean seed extract + microbially produced AvHPPD-03	1	1.44	1.38	6.80
	2	1.31		
Nontransgenic soybean seed extract	1	0.39	0.41	6.74
	2	0.43		

[a]One unit of HPPD activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of HPP to produce 1 µmol of HGA and 1 µmol of CO_2 per minute.
[b]RSD = relative standard deviation.

PAT

The PAT characterization and safety studies reported by Hérouet *et al.* (2005) were conducted with a purified microbially produced PAT test substance. Hérouet *et al.* demonstrated that this PAT test substance, produced in an *E. coli* expression system, was biochemically and functionally equivalent to PAT as encoded by *pat* in Event T25 maize. A similar comparison of plant-produced and microbially produced PAT was conducted to justify the use of the existing PAT safety and characterization data in support of the safety of SYHT0H2 soybean. The microbially produced PAT used in this comparison was the same test substance that was characterized and evaluated by Hérouet *et al.* (2005). PAT was extracted from SYHT0H2 soybean seed and compared with the microbially produced PAT in analyses of apparent molecular weight, immunoreactivity, peptide mass mapping, glycosylation, enzyme activity, and *N*-terminal amino acid sequence ([redacted]).

Western blot analysis demonstrated that the apparent molecular weights of both plant-produced and microbially produced PAT were consistent with the predicted molecular weight of 20.6 kDa, as shown in Figure 26. The peptide mass mapping analysis identified 63% and 77% of the predicted amino acid sequence of PAT for the plant-produced and microbially produced proteins, respectively (as shown in Figures 27 and 28). The *N*-terminal sequencing analysis revealed that the plant-produced PAT lacked the *N*-terminal methionine (Figure 29).

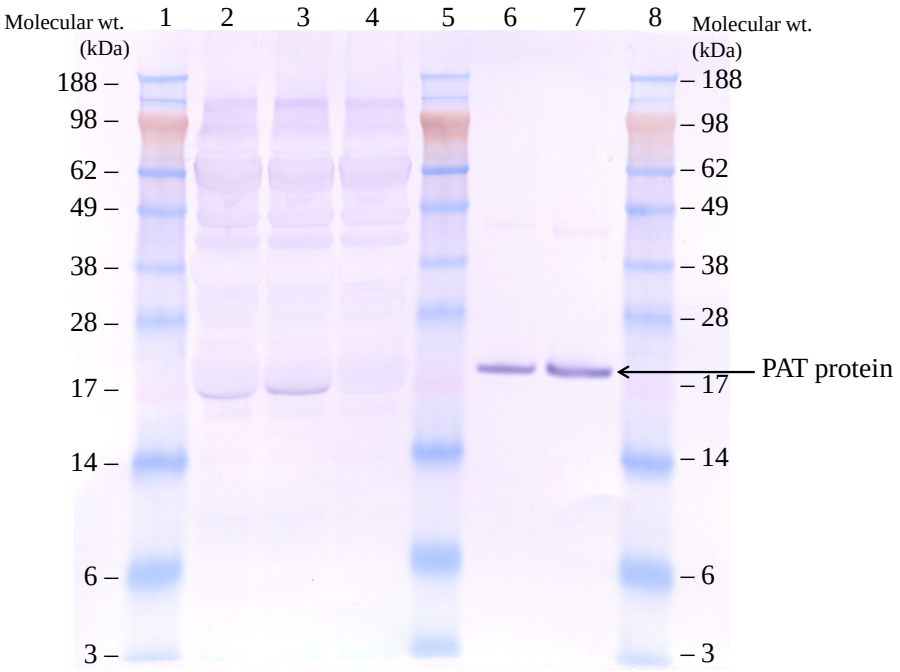


Figure 26 Western blot analysis of plant-produced and microbially produced PAT

Lanes 1, 5 & 8: Molecular-weight standard

Lane 2: Crude SYHT0H2 soybean seed extract (10 ng PAT, 64.52 µg total protein)

Lane 3: Nontransgenic soybean seed extract fortified with microbially produced PAT (10 ng PAT, 64.52 µg total protein)

Lane 4: Nontransgenic soybean seed extract (64.52 µg total protein)

Lane 6: PAT purified preparation from SYHT0H2 extract (10 ng PAT)[a]

Lane 7: Microbially produced PAT (10 ng PAT)[a]

[a]Because of limitations in printer resolution, the faint band at approximately 43 kDa may not be visible on the printed copy. Because this protein cross-reacted with a PAT-specific antibody and its apparent molecular weight is consistent with that of two PAT molecules, it most likely represents a dimer of PAT.

```
  1     MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
 51     ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101     GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151     AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI
```

Figure 27 Amino acid sequence of a plant-produced PAT identified by peptide mass mapping analysis

Identified PAT fragments are bold and underlined.

```
  1     MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
 51     ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101     GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151     AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI
```

Figure 28 Amino acid sequence of the microbially produced PAT identified by peptide mass mapping analysis

Identified PAT fragments are bold and underlined.

Predicted sequence:	MSPERRPVEIR
Microbially produced PAT:	MSPER
Plant-produced PAT:	SPERRPVEIR

Figure 29 *N*-terminal amino acid sequence of plant-produced and microbially produced PAT

The PAT proteins from both sources were also compared with respect to glycosylation status. The plant-produced PAT was analysed to ensure that no post-translational glycosylation of the protein had occurred *in planta*, as *E. coli* cannot produce glycosylated proteins. As shown in Figure 30, this analysis demonstrated the absence of post-translational glycosylation of the plant-produced PAT, and therefore equivalence with the microbially produced PAT in this regard.

The plant-produced and microbially produced PAT proteins were shown to have comparable enzyme activity when evaluated in a standardized substrate turnover assay. The activity of microbially produced PAT was evaluated in the presence of extract of nontransgenic, near-isogenic soybean seed to control for seed matrix effects in the PAT protein extract from soybean seeds. The specific activity was 30.58 units/mg for the plant-produced PAT and 22.13 units/mg for the microbially produced PAT (Table 14).

These results verified the identities of the plant-produced and microbially produced PAT, and it was concluded that the PAT proteins produced in SYHT0H2 soybean and in recombinant *E. coli* were biochemically and functionally equivalent. Therefore, the microbially produced test substance containing PAT that was used in the safety studies reported by Hérouet *et al.* (2005) was a suitable surrogate for PAT in SYHT0H2 soybean.

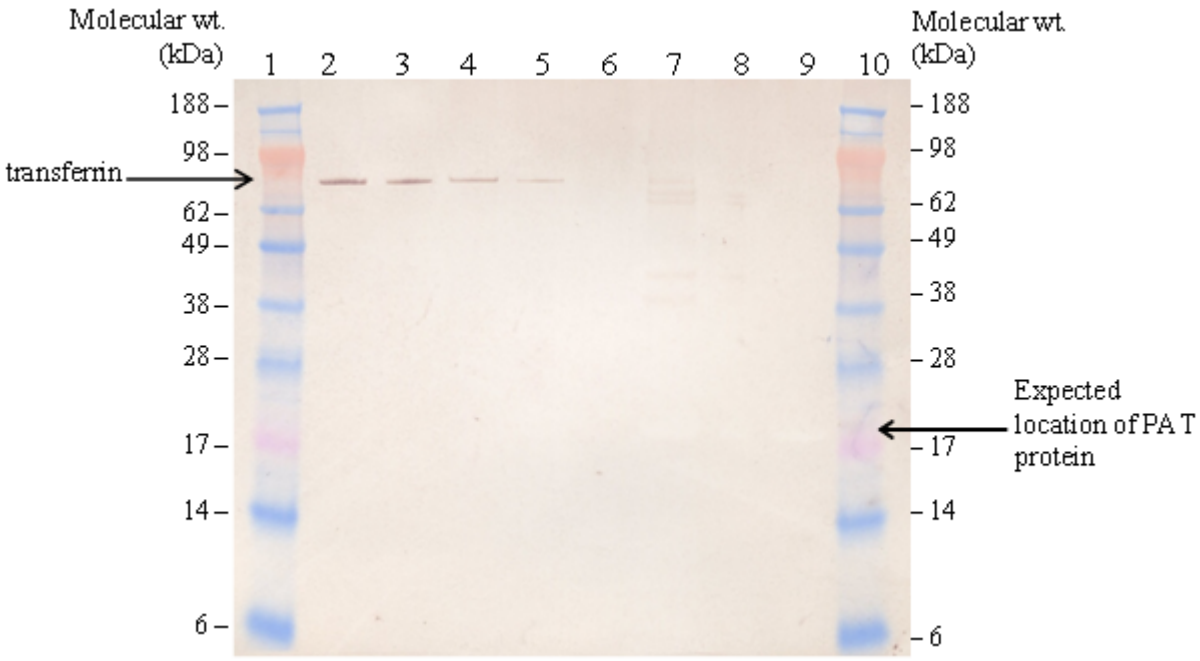


Figure 30 Glycosylation analysis of plant-produced and microbially produced PAT

Lanes 1 & 10: Molecular-weight standard

Transferrin (positive control):

Lane 2: 100 ng

Lane 3: 50 ng

Lane 4: 25 ng

Lane 5: 10 ng

Lane 6: Soybean trypsin inhibitor (negative control), 1000 ng

PAT purified preparation from SYHT0H2 soybean seed extract:

Lane 7: 1000 ng

Lane 8: 500 ng

Lane 9: PAT in the microbially produced test substance, 1000 ng

Table 14 Specific enzyme activity of the plant-produced and microbially produced PAT

Test substance	Assay replicate	PAT-specific activity (units/mg PAT)[a]	Mean PAT specific activity (units/mg PAT)	RSD (%)
Plant-produced PAT	1	31.33	30.58	2.1
	2	30.22		
	3	30.20		
Microbially produced PAT	1	20.77	20.84	1.4
	2	20.60		
	3	21.16		
Nontransgenic soybean seed extract + microbially produced PAT	1	20.69	22.13	5.8
	2	23.19		
	3	22.52		
Nontransgenic soybean seed extract[b]	1	<LOD	<LOD	–
	2	<LOD		
	3	<LOD		

[a]One unit of PAT activity is defined as the amount of enzyme required to acetylate 1 µmol of phosphinothricin per minute (equivalent to 1 µmol of 5,5′-dithiobis(2-nitrobenzoic acid) reduced or 1 µmol of 2-nitro-5-thiobenzoate anion produced per minute).
[b]LOD = 15.3 µM 2-nitro-5-thiobenzoate anion.

(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' roles or is in a non-functional form).

Not applicable. The two transferred genes, *avhppd-03* and *pat*, are functional as intended.

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

AvHPPD-03

The gene *avhppd-03* was codon-optimized for expression in soybean and was synthetically constructed. This synthetic gene encodes the AvHPPD-03 protein, which is 99.7% identical to the native oat (*A. sativa*) HPPD in amino acid sequence; the two proteins differ by a single amino acid residue that is not part of the enzyme's active site. Oat contains no endogenous proteins that are listed in the FARRP Allergen Protein Database (FARRP 2012) and therefore is not considered to be a known allergenic food. Oat has been implicated as a potential source of proteins that cause celiac disease in humans; however, a recent review of the literature clarified that this risk has likely been confounded by the use of test materials that were not pure oats (Health Canada 2007), and Health Canada stated that pure oats can be consumed by celiac disease patients who are otherwise sensitive to foods such as wheat and barley (which contain the proteins associated with celiac disease and are listed in the FARRP Allergen Protein Database). The endogenous oat HPPD protein is not a known allergen or celiac related protein. AvHPPD-03 is not related to putative oat allergens, either by structural protein family or by bioinformatics, and therefore is not considered to have any specific safety risk for allergy due to being highly similar to the endogenous oat HPPD protein. There is also no putative risk for AvHPPD-03 due to being derived from an endogenous oat HPPD protein.

PAT

A comprehensive characterization and safety assessment of the PAT protein is available in a 2005 article published in *Regulatory Toxicology and Pharmacology* (Hérouet *et al.* 2005). It is likely that small amounts of acetyltransferase enzymes from various sources have always been present in the food and feed supply, because of the ubiquitous occurrence of PAT proteins in nature. PAT has a long history of safe exposure as part of the endogenous proteome of microorganisms that are widely distributed taxonomically and as part of many existing commercially available transgenic crop plants, including maize, canola and soybean. A list of transgenic crops containing PAT that have been approved for food and feed use globally is shown in Table 15. The safety of PAT in existing commercial transgenic crop products is supported by a permanent exemption from food tolerances for PAT in all crops in the United States (US EPA 2007) and by regulatory approvals of numerous transgenic crops containing PAT (encoded by either *pat* or by a similar gene, *bar*) for U.S. cultivation beginning in 1995, as shown in Table 16 (ILSI 2011). There are no reports of concern about PAT as it exists in commercially available transgenic food crops.

To supplement the extensive data supporting the safety of PAT in food crops, additional studies specific to assessment of the safety of the PAT protein encoded by *pat* in SYHT0H2 soybean are described below.

Table 15 Transgenic crops approved for U.S. cultivation that contain PAT

Species	Events or crosses	Alternate designations	Source of PAT gene
Beta vulgaris	ACS-BVØØ1-3	T120-7	*S. viridochromogenes*
Brassica napus (oilseed rape/canola)	HCN1Ø		*S. viridochromogenes*
	ACS-BNØØ7-1	HCN92	*S. viridochromogenes*
	ACS-BNØØ4-7 × ACS-BNØØ1-4	MS1, RF1; PGS1	*S. hygroscopicus*
	ACS-BNØØ4-7 × ACS-BNØØ2-5	MS1, RF2; PGS2	*S. hygroscopicus*
	ACS-BNØØ5-8 × ACS-BNØØ3-6	MS8 × RF3	*S. hygroscopicus*
	PHY14, PHY35		*S. hygroscopicus*
	PHY36		*S. hygroscopicus*
	ACS-BNØØ8-2	T45, HCN28	*S. viridochromogenes*
Brassica rapa (bird rape, canola)	HCR-1		*S. viridochromogenes*
Cichorium intybus (chicory)	RM3-3, RM3-4, RM3-6		*S. hygroscopicus*
Glycine max (soybean)	ACS-GMØØ5-3	A2704-12, A2704-21, A5547-35	*S. viridochromogenes*
	ACS-GMØØ6-4	A5547-127	*S. viridochromogenes*
	ACS-GMØØ3-1	GU262	*S. viridochromogenes*
	ACS-GMØØ1-8, ACS-GMØØ2-9	W62, W98	*S. hygroscopicus*
Gossypium hirsutum (cotton)	DAS-24236-5	281-24-236	*S. viridochromogenes*
	DAS 21Ø23-5	3006-210-23	*S. viridochromogenes*
	DAS 21Ø23-5 × DAS-24236-5		*S. viridochromogenes*
	DAS 21Ø23-5 × DAS-24236-5 × MON-Ø1445-2		*S. viridochromogenes*
	DAS 21Ø23-5 × DAS-24236-5 × MON-88913-8		*S. viridochromogenes*
	ACS-GHØØ1-3	LLCotton25	*S. hygroscopicus*
	ACS-GHØØ1-3 × MON-15985-7	LLCotton25 × MON15985	*S. hygroscopicus*
Oryza sativa (rice)	ACS-OSØØ1-4, ACS-OSØØ2-5	LLRice06, LLRice62	*S. hygroscopicus*
	BCS-OSØØ3-7	LLRice601	*S. hygroscopicus*
Zea mays (maize, corn)	SYN-EV176-9	176	*S. hygroscopicus*
	PH-ØØØ676-7, PH-ØØØ678-9, PH-ØØØ68Ø-2	676, 678, 680	*S. viridochromogenes*
	DKB-8979Ø-5	B16, DLL25	*S. hygroscopicus*
	SYN-BTØ11-1	BT11 (X4334CBR, X4734CBR)	*S. viridochromogenes*
	SYN-BTØ11-1 × MON-ØØØ21-9	BT11 × GA21	*S. viridochromogenes*
	SYN-BTØ11-1 × SYN-IR162-4	BT11 × MIR162	*S. viridochromogenes*
	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5	BT11 × MIR162 × MIR604	*S. viridochromogenes*
	SYN-BTØ11-1 × SYN-IR6Ø4-5	BT11 × MIR604	*S. viridochromogenes*
	SYN-BTØ11-1 × SYN-IR6Ø4-5 × MONØØØ21-9	BT11 × MIR604 × GA21	*S. viridochromogenes*
	ACS-ZMØØ4-3	CBH-351	*S. hygroscopicus*

(continued)

Species	Events or crosses	Alternate designations	Source of PAT gene
	DAS-Ø6275-8		*S. hygroscopicus*
	DAS-59122-7		*S. viridochromogenes*
	DAS-59122-7, MON-ØØ6Ø3-6	DAS-59122-7 × NK603	*S. viridochromogenes*
	DAS-59122-7 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	DAS-59122-7 × TC1507 × NK603	*S. viridochromogenes*
	DKB-89614-9	DBT418	*S. hygroscopicus*
Zea mays (maize, corn)	MON-89Ø34-3 × DAS- Ø15Ø7-1 × MON-88017 88Ø17-3 × DAS-59122-7	MON89034 × TC1507 × MON88017 × DAS-59122-7	*S. viridochromogenes*
	ACS-ZMØØ1-9	MS3	*S. hygroscopicus*
	ACS-ZMØØ5-4	MS6	*S. hygroscopicus*
	MON-ØØ6Ø3-6 × ACS-ZMØØ3-2	NK603 × T25	*S. viridochromogenes*
	ACS-ZMØØ2-1, ACS-ZMØØ3-2	T14, T25	*S. viridochromogenes*
	ACS-ZMØØ3-2, MON-ØØ81Ø-6	T25 × MON810	*S. viridochromogenes*
	DAS-Ø15Ø7-1	TC1507	*S. viridochromogenes*
	DAS-Ø15Ø7-1, DAS-59122-7	TC1507 × DAS-59122-7	*S. viridochromogenes*
	DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	TC1507 × NK603	*S. viridochromogenes*

Taken in abbreviated form from ILSI (2011).

Table 16 Transgenic crops approved for food and feed use globally that contain PAT

Company[a]	Product name/ crop	First approval granted	Event/ stacked events	OECD unique identifier(s)	*pat* or *bar* gene	Other gene(s)	Countries with approvals for food and/or feed use[a]
BCS	LibertyLink® maize (corn)	1995	T25	ACS-ZMØØ3-2	*pat*	–	Argentina, Australia, Brazil, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, South Africa, Taiwan, U.S.
Syngenta	KnockOut® insect resistant corn	1995	176	SYN-EV176-9	*bar*	*cry1Ab*	Argentina, Australia, Canada, China, European Union, Japan, Korea, New Zealand, South Africa, Taiwan, U.S.
BCS	SeedLink®/ InVigor® canola	1997	Ms8/Rf3	ACS-BNØØ5-8 × ACS-BNØØ3-6	*bar*	*barnase* and *barstar*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, South Africa, U.S.
Syngenta	NK brand Bt corn with YieldGard or Agrisure CB/LL®	1996	Bt11	SYN-BTØ11-1	*pat*	*cry1Ab*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Indonesia, Japan, Korea, Mexico, New Zealand, Philippines, Russia, South Africa, Switzerland, Taiwan, Turkey, U.S., Uruguay

(continued)

Company[a]	Product name/ crop	First approval granted	Event/ stacked events	OECD unique identifier(s)	*pat* or *bar* gene	Other gene(s)	Countries with approvals for food and/or feed use[a]
BCS	LibertyLink® soybean	1996	A2704-12	ACS-GMØØ5-3	*pat*	–	Argentina, Australia, Brazil, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Russia, South Africa, Taiwan, U.S.
BCS	LibertyLink® soybean	1998	A5547-127	ACS-GMØØ6-4	*pat*	–	Argentina, Australia, Brazil, Canada, European Union, Japan, Mexico, New Zealand, Russia, U.S.
BCS	LibertyLink® rice	1999	LLRICE 62	ACS-OSØØ2-5	*bar*	–	Argentina, Australia, Canada, Colombia, Honduras, Mexico, New Zealand, Philippines, Russia, South Africa, U.S.
Dow	Herculex I corn	2001	TC1507	DAS-Ø15Ø7-1	*pat*	*cry1F*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Singapore, South Africa, Taiwan, U.S., Uruguay
BCS	LibertyLink® cotton	2003	LLCotton25	ACS-GHØØ1-3	*bar*	–	Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, U.S.
Dow	WideStrike cotton	2004	281-24-236 × 3006-210-23	DAS-21Ø23-5 × DAS-24236-5	*pat*	*cry1F/ cry1Ac*	Australia, Brazil, Canada, European Union, Japan, Korea, Mexico, New Zealand, U.S.
BCS	LibertyLink® × Bollgard II® cotton	2006	LLCotton25 × MON15985	ACS-GHØØ1-3 × MON 15985-7	*bar*	*cry1Ac/ cry2Ab*	Australia, Japan, Korea, Mexico, New Zealand, U.S.

[a]Bayer CropScience, Syngenta Seeds, or Dow AgroSciences, LLC.
[b]List of products and approving countries may be incomplete.

3.3 The Potential Toxicity of Novel Proteins or Other Novel Substances

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

AvHPPD-03

The potential toxicity of the AvHPPD-03 protein in SYHT0H2 soybean was evaluated through an extensive bioinformatic search to determine whether the amino acid sequence of AvHPPD-03 had significant sequence similarity to proteins identified as known or putative toxins ([redacted]).

The AvHPPD-03 amino acid sequence was systematically compared with the latest posting of the NCBI Entrez Protein Database (NCBI 2012). The BLASTP program (Altschul *et al.* 1997) was used to compare the NCBI Entrez Protein Database sequences with the AvHPPD-03 amino acid sequence as the query sequence. This analysis addressed two questions: (1) whether any protein(s) in the database had a high degree of sequence similarity to the AvHPPD-03 amino acid sequence, and (2) whether any proteins

demonstrating a high degree of sequence similarity to the AvHPPD-03 amino acid sequence were known or putative toxins.

The BLASTP searches were performed with the default parameters, and a statistically significant *E*-value (a measure of the probability that matches between sequences occurred by chance) of less than 0.4 was established by analysis of searches using randomly shuffled versions of the AvHPPD-03 amino acid sequence. Database sequences with a high degree of similarity to the AvHPPD-03 amino acid sequence ($E < 0.4$) were categorized by their biological function, ranked by *E*-value, and evaluated for source organism, percent sequence identity, and any other details regarding the potential for shared structure and function.

The NCBI Entrez Protein Database search identified 1,394 sequences with significant similarity to the AvHPPD-03 amino acid sequence ($E < 0.4$). None of these sequences corresponded to known or putative toxins.

Of the 1,394 significant alignments, 1,292 alignments from 674 species were to HPPD or similar proteins, including glyoxylases and members of the dioxygenase superfamily. The *E*-values for alignments between these sequences and the AvHPPD-03 amino acid sequence ranged from 1.20×10^{-175} to 0.3. Alignments were found to HPPDs from a wide variety of plants, animals, and microbial species, but the most similarly aligned HPPDs were from plants, including close relatives of oat (*A. sativa*), the source organism for AvHPPD-03. The sources of the 30 HPPD proteins most similarly aligned to AvHPPD-03, all plants, are listed in Table 17.

Table 17 The 30 HPPD proteins most similarly aligned to AvHPPD-03

Plant species	Common name or general description	GI number[a]	Amino acid length
Oryza sativa Japonica Group	rice (cultivated)	49387760	446
Hordeum vulgare, subsp. *vulgare*	barley (wild)	3334222	434
Triticum aestivum	common wheat	72256523	436
Sorghum bicolor	sorghum (milo)	242064140	440
Zea mays	maize (corn)	55669753	418
Zea mays	maize (corn)	162459274	444
Oryza sativa Japonica Group	rice (cultivated)	125580949	447
Zea mays	maize (corn)	224034593	426
Triticum aestivum	common wheat	157040846	381
Oryza sativa Indica Group	rice (cultivated)	218190140	601
Daucus carota	Queen Anne's lace (wild carrot)	3334219	442
Medicago truncatula	barrel medic (a Mediterranean legume)	357494205	437
Ricinus communis	Castorbean	255558690	441
Sorghum bicolor	sorghum (milo)	242048166	496
Coptis japonica var. *dissecta*	cutleaf Japanese goldthread	154240639	430
Mangifera indica	mango	309260073	432
Glycine max	soybean (cultivated)	351721017	443
Populus trichocarpa	black cottonwood (western balsam poplar)	224062651	444
Arabidopsis thaliana	thale-cress (mouse-ear cress)	52695552	424
Arabidopsis thaliana	thale-cress (mouse-ear cress)	3334223	445
Arabidopsis thaliana	thale-cress (mouse-ear cress)	30679736	473
Arabidopsis thaliana	thale-cress (mouse-ear cress)	22530912	473
Eutrema halophilum (Thellungiella halophila)	salt cress	312282469	445
Arabidopsis lyrata subsp. *lyrata*	lyre-leaved rock cress	297848936	445
Hevea brasiliensis	rubber tree	219842162	445
Salvia miltiorrhiza	redroot sage (Chinese salvia)	134284741	481
Lactuca sativa	lettuce (cultivated)	225001452	446
Solenostemon scutellarioides	coleus (painted nettle)	17366672	436
Brassica rapa subsp. *pekinensis*	Chinese cabbage	114324487	443
Vitis vinifera	wine grape	225446801	448

[a]GenBank protein sequence identification number (NCBI 2012).

An additional 14 alignments from 11 bacterial species were to proteins identified as putative hemolysins, related to hemolysins, hemolysin-like, or Vlly or Lly proteins (known as legiolysins). HPPD catalyzes the conversion of HPP to HGA in aerobic metabolism. In some Gram-negative bacteria, such as *Shewanella*, *Legionella*, and *Vibrio*, HGA then undergoes nonenzymatic oxidation and polymerization and is converted into melanin or melanin-like pigments, fluorescent substances, or hemolysins (Steinert *et al.* 2001, Kakidani and Hirai 2003). A similar process can occur in human blood *in vitro* or when a metabolic disorder prevents normal metabolism of HGA, whereby nonenzymatic oxidation and polymerization of HGA can induce spontaneous hemolysis and melanin production (Hegedus and Nayak 1994). Because HPPDs are required for production of HGA, which is subsequently converted to hemolysins in certain bacteria, bacterial HPPDs have sometimes been identified as putative hemolysins (Lee *et al.* 2008). It has also been suggested that the bacterial legiolysins function as HPPDs in the production of HGA (Steinert *et al.* 2001). However, although HPPD activity is required for production of HGA, neither HPPD

nor its metabolic product HGA itself causes hemolysis. AvHPPD-03 is no more similar to bacterial HPPDs than to HPPDs from a wide variety of plants and animals, and it is most similar to HPPDs from related crop plants. Therefore, the similarity of AvHPPD-03 to putative bacterial hemolysins or legiolysins is not indicative of any shared toxicity.

PAT

The BLASTP algorithm (Altschul *et al.* 1997) was used to compare the PAT amino acid sequence with all protein sequences present in the following large reference databases: UniProt Swiss-Prot and TrEMBL (UniProt 2012), DNA Data Bank of Japan (DDBJ 2012), and NCBI GenPept (NCBI 2012). A custom toxin database was also used which consisted of a select set of sequences identified by keyword searches of the UniProt Swiss-Prot and NCBI GenPept databases, and including sequences from the Animal Toxin Database (He *et al.* 2007). The scoring matrix used was BLOSUM62. The conservative criterion for selecting similar proteins was a threshold *E*-value of 0.1, and all aligned proteins with an *E*-value less than 0.1 were examined for potential biological relevance. Significant alignments were found only with other acetyltransferases from bacteria, and no records were found that identified potential hazards associated with this protein family. The PAT protein had no significant amino acid sequence similarity to any known toxin or any other protein known to cause adverse effects ([redacted]). The results of this updated bioinformatic analysis support previous analyses, including those reported by Hérouet *et al.* (2005).

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

Digestive Fate of AvHPPD-03 Protein

The susceptibility of AvHPPD-03 to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin and in simulated mammalian intestinal fluid (SIF) containing pancreatin (a mixture of intestinal proteases including trypsin, chymotrypsin, carboxypeptidase, and elastase) ([redacted]). Approximately 50% of ingested protein is digested and absorbed in the duodenum. *In vivo,* the peptides produced by pancreatic proteases are further digested to tripeptides, dipeptides, and amino acids by peptidases located in the brush border membrane of the intestinal epithelium (Kutchai 1998).

In the digestibility assays, the test substance was microbially produced AvHPPD-03. Degradation of the protein was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. The SDS-PAGE analysis, using a nonspecific Coomassie protein stain, allows for visualization of all proteins present in a sample. The Western blot method allows for specific analysis of the AvHPPD-03 protein; antibody specific for the AvHPPD-03 protein is used to detect the full-length protein and any immunoreactive fragments.

In Vitro *Digestibility of AvHPPD-03 in Simulated Gastric Fluid with Pepsin*

The SGF digestibility assay was performed at 37°C ± 2°C over a 60-minute time course, with samples taken at 0, 1, 2, 5, 10, 30, and 60 minutes. The SGF was prepared at pH 1.2 with pepsin at approximately 2,600 units/ml. The digestion was performed at a ratio of 1 µg of AvHPPD-03 per 10 pepsin activity units (Thomas *et al.* 2004). No intact AvHPPD-03 or immunoreactive fragments of AvHPPD-03 were present after incubation in SGF for 1 minute (as shown in Figure 31), indicating that AvHPPD-03 was rapidly and completely digested by pepsin.

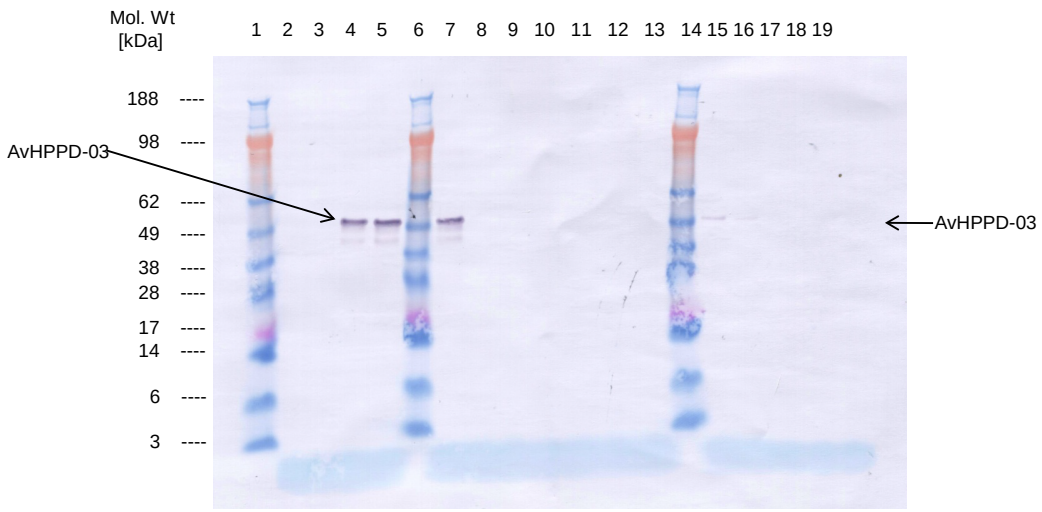


Figure 31 Immunoreactivity analysis by Western blot of AvHPPD-03 following digestion in SGF

Lanes 1, 6 & 14: molecular-weight standard

SGF control:

Lane 2: 0 min

Lane 3: 60 min

AvHPPD-03 control (in SGF without pepsin):

Lane 4: 0 min

Lane 5: 60 min

In vitro digestibility assay:

Lane 7: 0 min

Lane 8: 1 min

Lane 9: 2 min

Lane 10: 5 min

Lane 11: 10 min

Lane 12: 30 min

Lane 13: 60 min

LOD determination:

Lane 15: 0.16 ng AvHPPD-03[a]

Lane 16: 0.078 ng AvHPPD-03[a]

Lane 17: 0.039 ng AvHPPD-03

Lane 18: 0.020 ng AvHPPD-03

Lane 19: 0.0098 ng AvHPPD-03

[a]Because of limitations in printer resolution, the faint AvHPPD-03 bands in the original image may not be visible on the printed page.

The AvHPPD-03 protein band showed slightly lower mobility and therefore an apparently higher molecular weight than the expected 47.0 kDa when compared with the molecular weight standards. The difference between the expected and observed molecular weights can be explained by the limitations of SDS-PAGE for accurate determination of molecular weight. Dube and Flynn (1998) reviewed the reliability of SDS-PAGE for molecular weight determinations and concluded that the apparent molecular weight of a protein by this method is typically within 10% of its true molecular weight. This depends greatly on the similarity between the properties of the protein of interest and the proteins in the standard set (Sadeghi *et al.* 2003).

In Vitro *Digestibility of AvHPPD-03 in Simulated Intestinal Fluid with Pancreatin*

The SIF digestibility assay was performed over a 48-hour time course, with samples taken at 0, 1, 2, 5, 10, 30, and 60 minutes and 2, 3, 6, 24, and 48 hours ([redacted]). The SIF was prepared at pH 7.5 with pancreatin at 10 mg/ml, and the digestion was performed at a ratio of 38 µg of pancreatin to 1 µg of AvHPPD-03. No intact AvHPPD-03 was present after incubation in SIF for 1 minute. Three apparent AvHPPD-03 degradation products were detected after 1 minute and after 2 minutes, but were no longer present after 5 minutes (as shown in Figure 32), indicating that AvHPPD-03 was completely digested by intestinal proteases within 5 minutes.

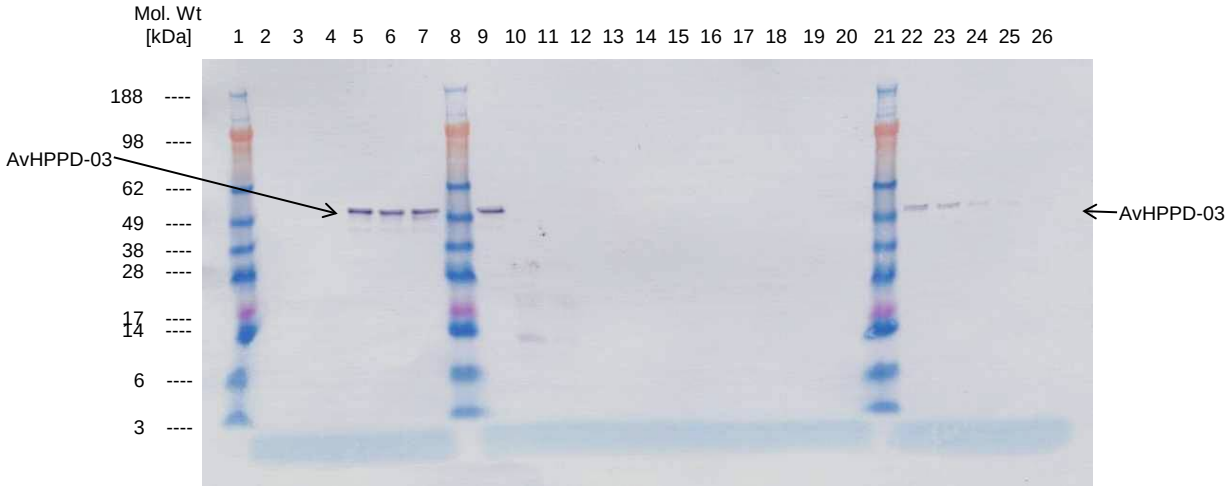


Figure 32 Immunoreactivity analysis by Western blot of AvHPPD-03 following digestion in SIF

Lanes 1, 8 & 21: molecular-weight standard

SGF control:

Lane 2: 0 min

Lane 3: 2 h

Lane 4: 48 h

AvHPPD-03 control (in SIF without pancreatin):

Lane 5: 0 min

Lane 6: 2 h

Lane 7: 48 h

In vitro digestibility assay:

Lane 9: 0 min

Lane 10: 1 min[a]

Lane 11: 2 min[a]

Lane 12: 5 min

Lane 13: 10 min

Lane 14: 30 min

Lane 15: 60 min

Lane 16: 2 h

Lane 17: 3 h

Lane 18: 6 h

Lane 19: 24 h

Lane 20: 48 h

LOD determination:

Lane 22: 0.63 ng AvHPPD-03

Lane 23: 0.31 ng AvHPPD-03

Lane 24: 0.16 ng AvHPPD-03

Lane 25: 0.078 ng AvHPPD-03

Lane 26: 0.039 ng AvHPPD-03

[a]Because of limitations in printer resolution, the faint bands representing AvHPPD-03 degradation products in the original image may not be visible on the printed page.

Effect of Temperature on the AvHPPD-03 Protein

The effects of temperature on the immunoreactivity and enzymatic activity of AvHPPD-03 were investigated ([redacted]). Although heat stability is not directly associated with allergenic potential (Privalle *et al.* 2011), an assessment of the heat stability of AvHPPD-03 provides a characterization of the potential exposure that is relevant to the consumption of SYHT0H2 soybean.

Aliquots of an aqueous solution of AvHPPD-03 were incubated for 30 minutes at 4°C (to establish a baseline), 25°C, 37°C, 65°C, and 95°C. Immunoreactivity was assessed via ELISA. Incubation at 37°C and 65°C resulted in 24.9% and 96.9% loss of immunoreactivity, respectively, and immunoreactivity fell to below the limit of quantitation at 95°C, indicating that the protein was substantially degraded.

In an enzyme activity assay, AvHPPD-03 retained 97.8% of its activity following incubation for 30 minutes at 25°C, but its activity was below the limit of detection following incubation at 65°C or 95°C.
These results support the conclusion that exposure of AvHPPD-03 to temperatures of 65°C or above, which are encountered during soy processing and cooking, would be expected to result in negligible amounts of intact and functional AvHPPD-03 protein in foods and feeds.

Digestive Fate of PAT Protein

The susceptibility of PAT to proteolytic degradation was evaluated in simulated mammalian gastric fluid containing pepsin ([redacted] and in simulated mammalian intestinal fluid containing pancreatin (a mixture of intestinal proteases including trypsin, chymotrypsin, carboxypeptidase, and elastase) ([redacted]). The time points used in both analyses were 0, 0.5, 2, 5, 10, 20, 30, and 60 minutes, and the samples were analysed for the presence of intact PAT and any immunoreactive PAT fragments by SDS-PAGE and Western blotting. PAT was completely digested in both SGF and SIF within 0.5 minute, the first time point sampled, indicating that PAT was rapidly and completely degraded by pepsin under mammalian gastric conditions and by pancreatin under simulated mammalian intestinal conditions.

Heat Stability of PAT Protein

PAT was evaluated for structural integrity and enzyme activity at temperatures up to 90°C for 60 minutes. Although intact PAT was observed by SDS-PAGE with Coomassie blue staining after exposure to a temperature of 90°C for 60 minutes, it was completely enzymatically inactivated after 10 minutes at 55°C, a relatively low temperature (Hérouet *et al.* 2005, Wehrmann *et al.* 1996). These results support the conclusion that exposure of PAT to temperatures of 55°C, which are encountered during soy processing and cooking, would be expected to inactivate PAT in foods and feeds.

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

Acute oral toxicity of AvHPPD-03 in mice

The human diet includes proteins from diverse plant, animal, and bacterial species. It is recognized that consumption of most food proteins, including many uncharacterized proteins, does not raise safety concerns (FAO/WHO 1996). When a protein is toxic, it usually acts via acute mechanisms and at very low dose levels (Sjoblad *et al.* 1992). To test for the potential toxicity of AvHPPD-03, an acute oral toxicity study was conducted in mice with attention to OECD Guideline 420 (OECD 2002) and U.S. EPA Test Guideline OPPTS 870.1100 (US EPA 2002) ([redacted]).

A microbially produced test substance, AVHPPD-03-0209, containing AvHPPD-03 (72.2% purity w/w) was administered to groups of 10 male and 10 female Crl:CD-1 mice (9 to 10 weeks old) by oral gavage

in deionized water. The doses of AvHPPD-03 were 500, 1500, or 2000 mg/kg of body weight (b.w.). The AvHPPD-03 present in this microbially produced test substance was previously characterized for use in safety studies and demonstrated to be equivalent to the plant-produced AvHPPD-03. A negative control group concurrently received the dosing vehicle alone. All dosing formulations were administered at a volume of 20 ml/kg b.w.

Half of the mice in each dose group were observed for a period of 2 days following dosing on day 0, and half were observed for a period of 14 days. Clinical observations, body weights, and food consumption were measured daily throughout the study. After the 2-day and 14-day observation periods, the mice were euthanized and examined *post mortem*. Complete necropsies were conducted on all mice, and selected tissues from all mice were examined microscopically. Histopathological evaluations were made of the esophagus, stomach, duodenum, jejunum, Peyer's patches, ileum, cecum, colon, rectum, mandibular and mesenteric lymph nodes, spleen, thymus, and gross lesions. A full suite of hematology parameters were evaluated, including hemoglobin distribution width, red cell distribution width, red cell morphology, erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

No mortality occurred during the 2- or 14-day observation periods, and no clinical signs of toxicity were observed in mice adminstered AvHPPD-03. No AvHPPD-03-related effects were observed on body weight, hematology parameters, or any gross or microscopic pathology findings at any time point. The only statistically significant difference observed was lower mean food consumption between day 0 and day 1 in high-dose females. However, this mean food consumption value was within the laboratory's historical control reference range and was mostly due to one very low individual value, which was below the reference range. This individual observation was an isolated occurrence, and no other significant differences in food consumption were noted during the study. Therefore, the difference was not considered to be related to AvHPPD-03.

It was concluded that AvHPPD-03 was not acutely toxic in mice. The no-observed-adverse-effect level (NOAEL) for a single oral gavage dose of AvHPPD-03 was 2000 mg/kg b.w., which was the highest dose level tested and the limit dose according to the OECD and U.S. EPA guidelines.

Conclusions of the Toxicological Assessment of AvHPPD-03

The source organism for AvHPPD-03, oat, is a safely consumed food crop, and the enzymatic mode of action of AvHPPD-03 is a native feature of *A. sativa* HPPD, with no toxicological significance to mammals. The bioinformatic analysis showed that AvHPPD-03 is most similar to other HPPD proteins in common food crops and does not have sequence similarity to any known or putative toxins. In mice, AvHPPD-03 was not acutely toxic when administered orally (NOAEL = 2000 mg/kg b.w.). Therefore, AvHPPD-03 is considered to be nontoxic.

Conclusions of the Toxicological Assessment of PAT

The PAT protein in SYHT0H2 soybean is from a source organism that is not known to be toxic. The PAT protein from *S. viridochromogenes* is a member of a well-characterized, safe class of enzymes with a high degree of substrate specificity, and shows significant homology with PAT proteins from other source organisms. Bioinformatic analysis revealed no amino acid sequence similarity to any known toxins or other proteins known to cause adverse effects, and PAT was not acutely toxic to mice. PAT is therefore considered to be nontoxic.

3.4 The Potential Allergenicity of Novel Proteins

(a) The source of the introduced protein.

Event SYHT0H2 soybean contains transgenes that encode two proteins:

(i) The AvHPPD-03 protein is 99.7% identical to the native oat (*A. sativa*) HPPD in amino acid sequence; the two proteins differ by a single amino acid.

(ii) The PAT protein is derived from *Streptomyces viridochromogenes* strain Tü494 gene. The native coding sequence (Wohlleben *et al.* 1988) was codon-optimized for enhanced expression. The synthetic gene *pat-03-01* was obtained from AgrEvo, Germany.

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

AvHPPD-03

To determine whether AvHPPD-03 had biologically relevant amino acid sequence similarity to known or putative allergens, two different bioinformatic comparison searches were performed against the FARRP Allergen Protein Database, v. 12.0, which contained 1,603 amino acid sequences of known and putative allergens (FARRP 2012) ([redacted]). First, a full-length sequence search using the FASTA algorithm (Pearson and Lipman 1988) was performed to identify any alignments of at least 80 amino acids with greater than 35% shared amino acid identity. Second, a search was performed for exact matches to eight or more contiguous amino acids. Alignments meeting these criteria indicate the potential for the protein of interest to possess immunologically relevant cross-reactivity (Codex Alimentarius Commission 2009). Neither search found a significant level of shared amino acid sequence between AvHPPD-03 and any entry in the FARRP Allergen Protein Database.

PAT Protein

To determine whether PAT had biologically relevant amino acid sequence similarity to known or putative allergens, two different bioinformatic comparison searches were performed against the FARRP Allergen Protein Database, version 12.0, which contained 1,603 amino acid sequences of known and putative allergens (FARRP 2012) ([redacted]). First, a full-length sequence search using the FASTA algorithm (Pearson and Lipman 1988) was performed to identify any alignments of at least 80 amino acids with greater than 35% shared amino acid identity. Second, a search was performed for exact matches to eight or more contiguous amino acids. Neither search found a significant level of shared amino acid sequence between PAT and any entry in the FARRP Allergen Protein Database.

(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.

The structural stability of the AvHPPD-03 and PAT proteins on exposure to heat, and degradation in simulated human gastric and intestinal fluids are detailed above in Section 3.3(b).

(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.

Not applicable. The newly expressed proteins encoded by the SYHT0H2 event, AvHPPD-03 and PAT, are not from sources known to be allergenic, nor do they show any homology to known allergens.

3.5 Compositional Analyses of the GM Food

(a) The levels of key nutrients, toxicants and anti-nutrients in the GM food compared with the levels in 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

Composition Study Design and Methods

Compositional analyses were conducted on soybean forage and seed samples harvested from replicated field trials planted at eight locations in the United States during 2010 ([redacted]). The test substance was SYHT0H2 soybean seed in the genetic background 'Jack', and the control substance was seed of the nontransgenic, near-isogenic soybean 'Jack.' Six nontransgenic commercial soybean varieties were included in the study design as references to establish a range of normal values for the components analysed. The test, control, and reference entries are listed in Table 18.

Table 18 Identification of test, control, and reference soybean varieties

Entry identification	Entry description	Variety	Maturity group
SYHT0H2	test substance	generation T_6 SYHT0H2/'Jack'	2.9
Control	nontransgenic, near-isogenic control	'Jack'	2.9
Reference variety 1	nontransgenic reference	03JR313108	3.5
Reference variety 2	nontransgenic reference	S23-T5	2.3
Reference variety 3	nontransgenic reference	03RM893031	3.1
Reference variety 4	nontransgenic reference	NE0800097	2.6
Reference variety 5	nontransgenic reference	WN0800099	2.9
Reference variety 6	nontransgenic reference	06RM934408	2.9

The locations selected were representative of where soybean is commercially grown and were suitable for planting of soybean varieties in maturity groups II to IV. The trials were planted on research or commercial farms where the soil type was typical for soybean production and where growth and maintenance of the plants could easily be monitored. At each location, the plots were planted in a randomized complete block design with four replicate plots per entry. The plots were six rows spaced 30 inches apart and 15 feet long, planted with approximately 105 seeds per row. The plots were managed according to local agricultural practices, and all plots at a given location were managed identically with regard to irrigation, fertilization, and pest control. Seed and forage samples were taken from rows 4 and 5 of each plot. A satellite view of the composition trial locations is shown in Figure 33. The soil type, previous year's crop, and planting date for each location are listed in Table 19.

Figure 33 Satellite view of composition trial locations in the United States

The location designated is the city nearest to the field plots.

Table 19 Composition field-trial locations

Location	Soil type	Previous crop	Planting date (2010)
Carlyle, Illinois	silt loam	milo	June 24
Fisk, Missouri	sandy loam	rice	June 21
Hamburg, Pennsylvania	loam	tomato, potato, sweet corn	June 18
Mebane, North Carolina	sand	corn	June 22
Richland, Iowa	silt loam	grain sorghum	June 25
Rockville, Indiana	silt loam	corn	June 27
Windsor, Illinois	loam	corn	July 2
York, Nebraska	silt loam	soybean	June 11

The forage samples collected from each plot consisted of the entire above-ground portions of 10 plants harvested at the R6 growth stage. The plants were chopped and pooled to create a composite sample for each plot. At full maturity (R8), the pods were collected from 30 plants per plot. The seeds were removed from the pods, shelled, and mixed to create a composite plot sample. The nutritional components chosen for analysis were those recommended by the Organisation for Economic Co-Operation and Development (OECD 2001) plus an additional few. The components analysed are listed in

Table 20. The component levels were converted to equivalent units of DW based on the moisture content of each FW sample. All compositional analyses were conducted according to methods published and approved by AOAC International or other industry-standard methods or according to methods based on literature references and developed and validated by the analytical laboratory.

Table 20 Nutritional components analyzed in soybean forage and seed

Forage and seed	Seed only			
Proximates[a]	**Minerals**	**Vitamins**	**Vitamin E isoforms**	**Antinutrients**
moisture	calcium	A (β-carotene)	α-tocopherol	daidzein
protein	Iron	B_1 (thiamine)	β-tocopherol	glycitein
fat	magnesium	B_2 (riboflavin)	γ-tocopherol	genistein
ash	phosphorus	B_9 (folic acid)	δ-tocopherol	lectin
carbohydrates	potassium	K_1 (phytonadione)	α-tocotrienol	phytic acid
ADF			β- tocotrienol	raffinose
NDF			γ- tocotrienol	stachyose
			δ-tocotrienol	trypsin inhibitor
	Amino acids		**Fatty acids**	
	alanine	lysine	8:0 caprylic	18:0 stearic
	arginine	methionine	10:0 capric	18:1 oleic
	aspartic acid	phenylalanine	12:0 lauric	18:2 linoleic
	cystine	proline	14:0 myristic	18:3 linolenic
	glutamic acid	serine	14:1 myristoleic	18:3 gamma linolenic
	glycine	threonine	15:0 pentadecanoic	20:0 arachidic
	histidine	tryptophan	15:1 pentadecenoic	20:1 eicosenoic
	isoleucine	tyrosine	16:0 palmitic	22:0 behenic
	leucine	valine	16:1 palmitoleic	20:2 eicosadienoic
			17:0 heptadecanoic	20:3 eicosatrienoic
			17:1 heptadecenoic	20:4 arachidonic

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber.

Data Analysis

Mean levels of each component across locations were computed. The mean levels in SYHT0H2 soybean and the nontransgenic control soybean were compared via analysis of variance (ANOVA) using the following mixed model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

where Y_{ijk} is the observed response for entry i at location j block k, U is the overall mean, T_i is the entry effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location-by-entry interaction effect, and e_{ijk} is the residual error. Entry was regarded as a fixed effect, while the effects of location, block within location, and location-by-entry interaction were regarded as random. In the across-

location analysis, only the control and SYHT0H2 entries were included, to avoid inflation of the residual error by any possible interaction between location and reference varieties.

For each component, *t*-tests were used to statistically compare the results for SYHT0H2 and nontransgenic control soybean. Significance was based on an alpha level of 0.05, and the denominator degrees of freedom were determined by the Kenward-Roger method (Kenward and Roger 1997). The standard error of the mean (SEM) also was determined for each component. In cases where the numbers of replicates per entry differed because of missing samples, the SEM for each component was determined separately for each entry.

SYHT0H2 soybean component across-location means were nonstatistically compared with the ranges of values observed in the six soybean reference varieties and with the values published in the International Life Sciences Institute (ILSI) Crop Composition Database (ILSI 2010). The ILSI database is a comprehensive source of crop composition data for most nutritional components. Statistically significant differences observed between the compositions of SYHT0H2 and control soybean were assessed in the context of the range of natural variation in the components to determine whether any differences could be biologically significant (Codex Alimentarius 2009).

Forage

Across-location means and statistics for the proximate components of forage are shown in Table 21. Forage component levels did not differ significantly between SYHT0H2 soybean and the nontransgenic, near-isogenic control soybean. Although some mean levels for SYHT0H2 soybean were outside of the ILSI database ranges, all were within the ranges for the six reference varieties.

Table 21 Proximate composition of forage from SYHT0H2 and nontransgenic control soybean (% DW)[a]

Entry & sample size	Statistic	Moisture	Protein	Fat	Ash	Carbohydrates	ADF	NDF
SYHT0H2	mean	70.4	18.9	6.04	6.39	68.7	26.8	33.0
N = 32	range	60.8–75.1	14.4–22.8	4.03–8.72	4.78–8.39	63.8–74.2	21.7–31.9	26.5–38.5
Control	mean	69.9	18.4	6.15	6.73	68.7	27.3	32.6
N = 32	range	58.5–74.5	13.5–22.1	3.22–8.84	5.34–8.18	63.9–74.8	22.6–35.8	26.9–37.5
	P	0.315	0.203	0.595	0.065	0.966	0.464	0.686
	SEM	0.94	0.53	0.477	0.251	0.82	0.61	0.71
Reference varieties	mean	70.7	19.6	6.82	6.77	66.8	26.3	31.6
N = 192	range	53.2–76.4	12.0–25.1	2.68–11.40	5.06–8.88	58.9–75.2	18.4–38.3	23.0–44.2
ILSI (2010)[b]	mean	77.0	19.38	3.138	9.036	68.5	ND	ND
N = 72	range	73.5–81.6	14.38–24.71	1.302–5.132	6.718–10.782	59.8–74.7		

[a]Except moisture, which is reported as % FW.
[b]ND = no data were available.

Seed

Numerous statistically significant differences were observed between SYHT0H2 soybean and the nontransgenic control soybean in seed component levels. However, the magnitudes of the differences were less than 10% for all components except the differences in mean tocopherol levels.

Proximates, Minerals, and Vitamins

As shown in Tables 22 and 23, ADF, iron, and potassium levels differed significantly between SYHT0H2 and control soybean seed. However, all mean levels of proximates and minerals in SYHT0H2 soybean seed were within the reference-variety and ILSI database ranges except for potassium levels. The mean potassium levels in SYHT0H2, control, and reference-variety soybean seed all were below the ILSI database range, and the difference between SYHT0H2 and control soybean was small (2.7%).

As shown in Table 25, the levels of vitamins other than E did not differ significantly between SYHT0H2 and control soybean seed. All mean levels in SYHT0H2 soybean seed were within the reference-variety and ILSI database ranges except for the levels of vitamins B_1 and B_2, which were above the ILSI database range in SYHT0H2, control, and reference-variety soybean seed.

Tocopherol levels are highly influenced by environment and genotype and vary widely in conventional soybean (Dolde *et al.* 1999, Ujie *et al.* 2005, Carrão-Panizzi and Erhan 2007, Seguin *et al.* 2010). Rani *et al.* (2007) reported a 6-fold range in γ-tocopherol levels and a 9-fold range in δ-tocopherol levels across 66 conventional soybean varieties. The mean level of α-tocopherol was 11.6% lower in SYHT0H2 than in control soybean, but was well within the reference-variety and ILSI database ranges, as shown in Table 25. Therefore, the difference is not considered to be an effect of transformation. The higher levels of γ-tocopherol (12.4%) and δ-tocopherol (29.1%) in SYHT0H2 soybean seed were consistent with reports that overexpression of genes encoding HPPD in tobacco (Falk *et al.* 2003) and *Arabidopsis* (Tsegaye *et al.* 2002, Collakova and DellaPenna 2003) result in increased seed tocopherol levels. Vitamin E antioxidant activity associated with the γ-tocopherol and δ-tocopherol isoforms is relatively small, amounting to only 10% and 3%, respectively, of that of α-tocopherol (DellaPenna 2005). The increases in these isoforms would have negligible impact on overall seed content of active vitamin E.

Table 22 Proximate composition of seed from SYHT0H2 soybean and nontransgenic control soybean (% DW)[a]

Entry & sample size	Statistic	Moisture	Protein	Fat	Ash	Carbohydrates	ADF	NDF
SYHT0H2	mean	8.66	38.6	20.5	5.29	35.5	13.9*	16.0
N = 31	range	6.84–12.2	32.6–41.4	18.0–22.9	4.29–6.92	32.5–39.7	10.0–18.2	13.0–19.6
Control	mean	8.70	38.2	20.7	5.25	35.7	14.8	16.7
N = 32[b]	range	5.90–12.6	32.2–44.7	18.9–22.8	4.08–6.62	29.3–40.1	10.3–18.0	12.6–21.3
	P	0.786	0.280	0.271	0.549	0.602	0.044	0.069
	SEM	0.533, 0.533	0.70, 0.70	0.31, 0.31	0.171	0.56, 0.56	0.40, 0.40	0.35, 0.35
Reference varieties	mean	9.18	38.1	20.4	5.26	36.2	14.6	16.3
N = 192	range	6.10–14.30	30.6–44.4	15.8–25.0	4.14–6.59	25.2–43.8	8.20–20.6	11.2–21.9
ILSI (2010)	mean	10.1	39.47	16.681	5.320	38.2	11.97	12.33
	range	4.7–34.4	33.19–45.48	8.104–23.562	3.885–6.994	29.6–50.2	7.81–18.61	8.53–21.25
	N	323	323	323	323	323	149	149

[a]Except moisture, which is reported as % FW.
[b]Except *N* = 31 for ash.
*Significantly different from control soybean at $P < 0.05$.

Table 23 Mineral composition of seed from SYHT0H2 soybean and nontransgenic control soybean (mg/kg DW)

Entry & sample size	Statistic	Ca	Fe	Mg	P	K
SYHT0H2	Mean	3062	80.5*	2433	6141	17261*
N = 31[a]	Range	2380–3840	68.5–109	2100–2920	4300–8760	14000–21100
Control	Mean	2990	83.4	2391	6117	17747
N = 32[b]	Range	2280–3910	72.5–117	1970–3070	4000–9130	14000–24000
	P	0.165	0.027	0.079	0.719	0.002
	SEM	117.6, 117.5	2.74, 2.74	76.6, 76.6	379.6, 379.5	572.3, 571.9
Reference varieties	Mean	2897	72.5	2394	5910	17793
	Range	2050–3860	48.0–110	1820–3090	4200–8570	13800–24700
N = 192						
ILSI (2010)	Mean	2170.5	78.10	2635.8	7148.0	20613.7
N = 80	Range	1165.5–3071.0	55.36–109.54	2194.0–3128.4	5067.4–9352.4	18680.1–23161.4

[a]Except *N* = 30 for iron.
[b]Except *N* = 31 for iron.
*Significantly different from control soybean at $P < 0.05$.

Table 24 Vitamin composition of seed from SYHT0H2 soybean and nontransgenic control soybean (mg/100 g DW)[a]

Entry & sample size	Statistic	Vitamin A[b] (β-carotene)	Vitamin B_1 (Thiamine)	Vitamin B_2 (Riboflavin)	Vitamin B_9 (Folic Acid)	Vitamin K_1 (Phytonadione)
SYHT0H2	Mean	–	0.515	0.384	0.440	0.411
N = 31	Range	<LOQ–0.135	0.277–0.749	0.280–0.521	0.251–0.631	0.181–0.724
Control	Mean	–	0.535	0.381	0.415	0.462
N = 32	Range	<LOQ–0.208	0.332–0.756	0.288–0.546	0.234–0.552	0.143–0.827
	P	–	0.205	0.845	0.112	0.094
	SEM	–	0.0341, 0.0340	0.0142, 0.0141	0.0300, 0.0300	0.0456, 0.0455
Reference varieties	Mean	–	0.472	0.384	0.410	0.388
	Range	<LOQ–0.104	0.253–1.02	0.270–0.532	0.224–0.680	0.106–0.886
N = 192						
ILSI (2010)[c]	Mean	ND	0.197	0.267	0.3589	ND
N = 80	Range		0.101–0.254	0.190–0.321	0.2386–0.4709	

[a]Except Vitamin K_1, which is reported as ppm.
[b]The LOQ for β-carotene was 0.0213−0.0233 mg/100 g DW; where some or all values were below the LOQ, the means could not be calculated or statistically compared, so only the range is shown.
[c]ND = no data were available.

Table 25 Vitamin E composition of seed from SYHT0H2 soybean and nontransgenic control soybean (mg/g DW)[a]

Entry & sample size	Statistic	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	α-tocotrienol	β-tocotrienol	γ-tocotrienol	δ-tocotrienol
SYHT0H2	Mean	0.0228*	–	0.226*	0.0789*	–	–	–	–
N = 31	Range	0.00996–0.0628	<LOQ	0.183–0.268	0.0518–0.107	<LOQ	<LOQ–0.549	<LOQ	<LOQ
Control	Mean	0.0258	–	0.201	0.0611	–	–	–	–
N = 32	Range	0.00934–0.0605	<LOQ	0.154–0.244	0.0312–0.0845	<LOQ	<LOQ	<LOQ	<LOQ
	P	0.019	–	<0.001	<0.001	–	–	–	–
	SEM	0.00470, 0.00470	–	0.0059, 0.0059	0.00547, 0.00547	–	–	–	–
Reference varieties	Mean	0.0299	–	0.176	0.0678	–	–	–	–
	Range	0.0115–0.0771	<LOQ–0.00779	0.127–0.236	0.0320–0.112	<LOQ	<LOQ	<LOQ	<LOQ
N = 192									
ILSI (2010)[b]	Mean	0.0191	ND	ND	ND	ND	ND	ND	ND
N = 234	Range	0.0019–0.0617							

[a]The LOQ for all tocopherols and tocotrienols was 0.0053–0.0058 mg/g DW; where some or all values were below the LOQ, the means could not be calculated or statistically compared, so only the range is shown.
[b]ND = no data were available.
*Significantly different from control soybean at $P < 0.05$.

Amino Acids, Fatty Acids, and Antinutrients

Nearly half of the significant differences in seed composition between SYHT0H2 soybean and the nontransgenic control soybean were due to slightly higher amino acid levels in SYHT0H2 soybean, as shown in Table 26. These differences (which ranged from 1.3% to 3.8%) corresponded to slightly (nonsignificantly) higher mean protein levels in SYHT0H2 soybean seed. However, the mean levels of all amino acids in SYHT0H2 soybean seed fell within the reference-variety and ILSI database ranges, and the overall amino acid profiles of SYHT0H2 and control soybean seeds did not differ, as shown in Figure 34.

Of the 22 fatty acids analysed, 13 were below the LOQ in all replicates of SYHT0H2 soybean; the results for the remaining nine fatty acids are shown in Table 27. The mean levels of seven of these fatty acids differed significantly between SYHT0H2 and control soybean seed; five were higher in SYHT0H2 soybean (by up to 6.1%), and two were lower (by up to 3.5%). However, the mean levels of these fatty acids in SYHT0H2 soybean seed were within the reference-variety and ILSI database ranges.

As shown in Table 28, the levels of antinutrients did not differ significantly between SYHT0H2 and control soybean seed. All mean levels in SYHT0H2 soybean seed were within the reference-variety and ILSI database ranges except for the levels of raffinose and stachyose, which were above the ILSI database range in SYHT0H2, control, and reference-variety soybean seed.

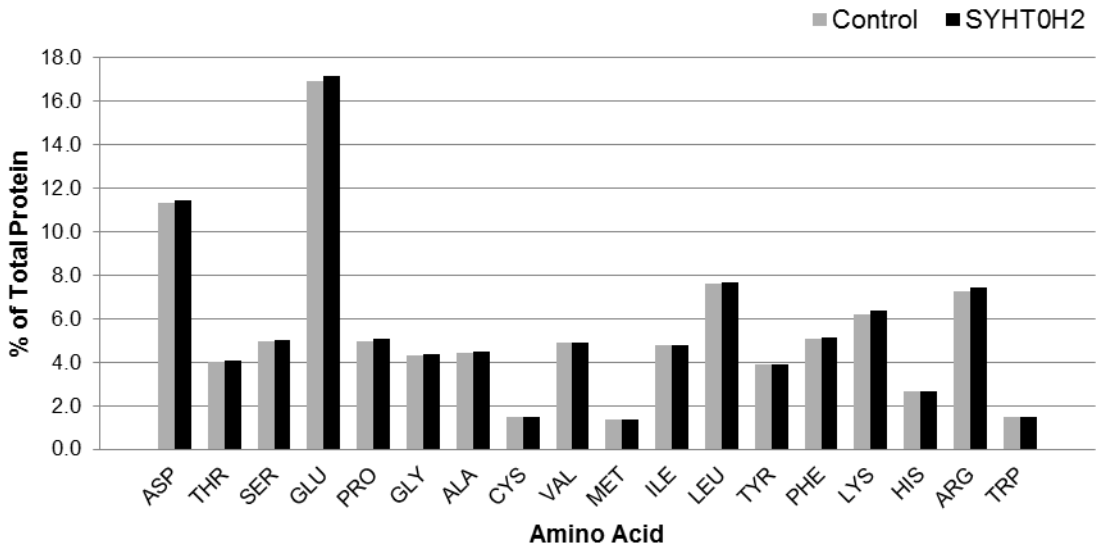


Figure 34 Amino acid profiles in SYHT0H2 and nontransgenic control soybean seed

Table 26 Amino acid composition of seed from SYHT0H2 soybean and nontransgenic control soybean (mg/g DW)

Entry & sample size	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
SYHT0H2	mean	44.1*	15.7*	19.4*	66.2*	19.6*	16.9	17.3*	5.73	19.0
N = 31	range	36.1–48.5	13.6–16.5	16.3–21.2	52.7–74.5	15.7–21.6	14.3–18.0	14.7–18.7	5.01–6.55	15.4–20.5
Control	mean	43.2	15.4	19.0	64.8	19.0	16.6	17.0	5.73	18.7
N = 32	range	36.7–47.3	13.5–16.6	16.4–20.8	52.9–72.6	16.1–21.2	14.5–18.0	14.7–18.4	4.99–6.45	16.1–20.2
	P	0.013	0.021	0.048	0.046	0.002	0.077	0.014	0.995	0.117
	SEM	0.82, 0.82	0.19, 0.19	0.32, 0.32	1.48, 1.48	0.38, 0.38	0.25, 0.25	0.23, 0.23	0.121, 0.121	0.31, 0.31
Reference varieties	mean	43.1	15.3	18.8	66.2	19.4	16.4	17.0	5.82	18.4
N = 192	range	34.8–49.6	12.9–16.7	15.3–21.6	50.5–78.3	15.4–22.6	13.8–18.5	14.4–18.5	4.79–7.36	15.0–20.6
ILSI (2010)	mean	44.93	14.73	20.19	70.88	20.01	16.88	17.16	5.87	19.10
N = 234	range	38.08–51.22	11.39–18.62	11.06–24.84	58.43–82.01	16.87–22.84	14.58–19.97	15.13–21.04	3.70– 8.08	15.97–22.04
		Met	**Ile**	**Leu**	**Tyr**	**Phe**	**Lys**	**His**	**Arg**	**Trp**
SYHT0H2	mean	5.18	18.6	29.6*	15.1*	19.9*	24.6*	10.4*	28.7*	5.72
N = 31	range	4.51–5.93	15.4–20.3	24.1–32.4	12.8–16.4	15.9–21.7	21.1–26.4	8.79–11.3	22.4–31.9	4.98–6.20
Control	mean	5.13	18.4	29.0	14.9	19.4	23.7	10.2	27.8	5.69
N = 32	range	4.53–5.68	15.8–20.1	24.9–31.5	13.0–16.0	16.0–21.3	21.2–25.7	8.56–11.1	22.8–31.5	5.04–6.33
	P	0.488	0.159	0.011	0.035	0.010	<0.001	0.007	0.005	0.657
	SEM	0.072, 0.071	0.31, 0.31	0.53, 0.53	0.22, 0.22	0.40, 0.40	0.35, 0.35	0.14, 0.14	0.64, 0.64	0.074, 0.073
Reference varieties	mean	5.37	18.2	28.7	14.7	19.3	24.5	10.0	28.3	5.67
N = 192	range	4.22–6.19	14.9–20.7	23.3–32.2	12.3–16.4	15.5–21.7	19.8–27.4	8.05–11.1	21.9–33.0	4.88–6.20
ILSI (2010)	mean	5.51	18.08	30.39	13.21	19.79	25.57	10.40	28.40	4.329
N = 234	range	4.31–6.81	15.39–20.77	25.90–36.22	10.16–16.13	16.32–23.46	22.85–28.39	8.78–11.75	22.85–34.00	3.563–5.016

*Significantly different from control soybean at $P < 0.05$.

Table 27 Fatty acid composition of seed from SYHT0H2 soybean and nontransgenic control soybean (% of total fatty acids)

Entry & sample size	Statistic	16:0 Palmitic	17:0 Heptadecanoic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic	20:0 Arachidic	20:1 Eicosenoic	22:0 Behenic
SYHT0H2	mean	10.5*	–	4.67*	24.3*	52.2*	7.35*	0.368*	0.183	0.372*
N = 31	range	10.2–11.0	<LOQ–0.122	4.08–5.62	21.5–29.5	47.5–54.4	5.88–9.03	0.320–0.454	0.150–0.234	0.345–0.431
Control	mean	10.0	–	4.50	23.0	54.1	7.51	0.347	0.181	0.357
N = 32	range	9.61–10.5	<LOQ–0.121	4.01–5.40	20.1–26.3	50.7–56.3	6.37–8.99	0.305–0.433	0.148–0.240	0.323–0.430
	P	<0.001	–	0.001	0.004	<0.001	<0.001	<0.001	0.444	0.001
	SEM	0.08, 0.08	–	0.144, 0.144	0.69, 0.68	0.56, 0.56	0.266, 0.266	0.0136, 0.0136	0.0077, 0.0076	0.0087, 0.0087
Reference varieties	mean	10.8	–	4.57	24.1	52.2	7.44	0.368	0.199	0.364
N = 192	range	8.93–12.2	<LOQ–0.127	3.75–6.32	18.1–35.2	45.0–56.7	5.30–10.1	0.288–0.534	0.153–0.286	0.304–0.498
ILSI (2010)	mean	11.12	0.114	4.01	20.7	53.3	8.34	0.323	0.204	0.402
	range	9.55–15.77	<LOQ–0.146	2.70–5.88	14.3–32.2	42.3–58.8	3.00–12.52	<LOQ–0.482	<LOQ–0.350	0.277– 0.595
	N[b]	234	97	234	234	234	234	233	221	233

[a]Where some or all values were below the LOQ, the means could not be calculated or statistically compared, so only the range is shown.
[b]Excludes values <LOQ.
*Significantly different from control soybean at $P < 0.05$.

Table 28 Antinutrient composition of seed from SYHT0H2 soybean and nontransgenic control soybean

Entry & sample size	Statistic	Daidzein (µg/g DW)	Glycitein (µg/g DW)	Genistein (µg/g DW)	Lectin (HU/mg DW)[a]	Phytic acid (% DW)	Raffinose (% DW)	Stachyose (% DW)	Trypsin inhibitor (TIU/mg DW)[b]
SYHT0H2	mean	391	181	569	26.1	1.38	0.816	3.76	35.9
N = 31	range	117–670	103–258	121–1020	12.3–46.5	0.819–2.14	0.576–1.13	3.13–4.25	21.8–55.1
Control	mean	375	196	556	25.8	1.41	0.801	3.72	34.4
N = 32	range	136–624	122–284	190–974	8.07–56.1	0.780–2.35	0.511–1.18	2.93–4.03	23.7–61.9
	P	0.273	0.138	0.548	0.924	0.259	0.303	0.562	0.397
	SEM	46.5, 46.5	10.3, 10.3	80.4, 80.4	2.18, 2.15	0.114, 0.114	0.0503, 0.0503	0.099, 0.099	1.64, 1.62
Reference varieties	mean	702	124	710	20.2	1.311	0.951	4.32	37.4
N = 192	range	229–1230	58.8–265	165–1240	4.19–61.3	0.766–2.21	0.607–1.58	3.15–5.13	18.9–68.3
ILSI (2010)	mean	834.8	156.6	976.8	1.718	1.121	0.355	2.19	48.33
	range	60.0–2453.5	<LOQ–310.0	144.3–2837.2	0.105-9.038	0.634–1.960	0.212–0.661	1.21–3.50	19.59–118.68
	N[c]	251	248	251	251	118	118	118	178

[a]HU = hemagglutinating unit.
[b]TIU = trypsin inhibitor unit.
[c]Excludes values <LOQ.

Conclusions from Compositional Analysis

All mean component levels in SYHT0H2 soybean forage and seed were within the range of mean levels for the six soybean reference varieties included in the study, and most were within the range of values in the ILSI database. These data indicate that forage from SYHT0H2 soybean and its nontransgenic, near-isogenic counterpart does not differ significantly in composition. The data indicate that seed from SYHT0H2 soybean differs slightly in composition from that of its nontransgenic, near-isogenic counterpart. However, comparisons with the ranges of component levels in other nontransgenic soybean varieties indicate that the nutrient and antinutrient composition of SYHT0H2 soybean is not materially different from that of conventional soybean.

(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.

Other than the intended presence of the Av-HPPD-03 and PAT proteins in soybean varieties containing event SYHT0H2, forage and seed have been shown to be compositionally and nutritionally equivalent to conventional soybean (see Section 3.5(a) directly above).

(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.

Soybean seed contains several known allergens, including abundant seed storage proteins, that either cause allergy directly or cross-react with IgE antibody that binds to other known protein allergens. The similarity in human serum IgE antibody binding to endogenous allergens contained in seeds of SYHT0H2 soybean and three nontransgenic soybean varieties was assessed ([redacted]). The objective was to provide a qualitative assessment of the human serum IgE binding patterns to soybean seed proteins using a 2-dimensional Western blot analysis. A 2-dimensional electrophoretic technique provides a broad spectrum separation of proteins by their molecular weight and by their isoelectric charge. The 2-dimensional separation provides a more discrete isolation of some proteins which have multiple isoforms differing by inherent molecular charge differences as compared with 1-dimensional separation. An IgE binding Western blot using human serum was employed to observe potential differences in the expression pattern of proteins that bind IgE antibody, most of which would represent known soybean allergens.

SYHT0H2 soybean was compared to nontransgenic control soybean and two conventional reference varieties using five sera known to contain IgE antibody specific for soybean proteins. Overall similarity was evident among all four soybean varieties for each of the allergic sera with most comparisons best observed with three sera showing the most varied and intense IgE binding. SYHT0H2 soybean was similar in IgE binding pattern and overall intensity of IgE binding to both the nontransgenic control soybean and the two reference soybean varieties. SYHT0H2 soybean did not show the presence of any shifted or unique protein banding patterns that would indicate a significant difference in endogenous allergen content.

In summary, SYHT0H2 soybean is similar to nontransgenic soybeans in its expression of endogenous allergens. There is no evidence that suggests SYHT0H2 soybean has significantly increased expression of endogenous allergens or reacts differently with soybean-reactive serum IgE antibody as compared with nontransgenic soybeans. The data supports the conclusion that SYHT0H2 soybean is similar to a nontransgenic, near-isogenic control soybean variety and unlikely to have greater allergenic potential than nontransgenic soybean varieties.

Part 4 Information Related to the Nutritional Impact of the Genetically-Modified Food

4.1 Data to Allow the Nutritional Impact of Compositional Changes in the Food to be Assessed

Section 3.5(a) provides data from a compositional analysis that was performed to compare the nutritional properties of raw agricultural commodity derived from SYHT0H2 soybean. Analysis of key nutritional components of forage and seed from SYHT0H2 soybean identified no differences from conventional, nontransgenic soybean that would affect human or animal health. No unintended, adverse consequences of the transformation process or expression of the transgenes in SYHT0H2 soybean were evident. Seed, forage, and soybean meal from SYHT0H2 soybean were found to be similar in composition to those same materials from conventional soybean. SYHT0H2 soybean exhibited a composition profile similar to that of reference soybean varieties grown concurrently in several locations and other soybean varieties represented in the historical ILSI Crop Composition Database. SYHT0H2 soybean nutritional equivalence to conventional soybean was further assessed in a 42-day poultry feeding study, described below.

4.2 Data from an Animal Feeding Study, if Available

Chickens (*Gallus domesticus*) consume large quantities of soybeans as processed soybean meal in commercial feeds. Broiler chickens, in particular, have relatively high soybean meal consumption, because conventional feeding regimens have been designed to provide maximal body-weight gain in the shortest amount of time, and soybean meal is a high-protein diet constituent that supports rapid growth in monogastric animals. Broiler chickens are highly sensitive to small nutrient changes in their diets because of their extremely rapid growth rates and for this reason are considered a sensitive species for assessing the nutritional impact of diet components. A broiler chicken study model has previously been used to assess whether consumption of transgenic maize grain (Brake and Vlachos 1998, Brake *et al.* 2005) or soybean meal processed from transgenic soybean varieties (Hammond *et al.* 1996, McNaughton *et al.* 2007, Taylor *et al.* 2007) in poultry diets could result in adverse effects.

A 42-day feeding study was performed to evaluate whether standard broiler poultry diets prepared with SYHT0H2 soybean meal had any adverse effects on male or female broiler chicken survival or growth in comparison with soybean meal processed from a nontransgenic, near-isogenic (control) soybean variety and a conventional (nontransgenic) commercial soybean reference variety ([redacted]). Seed of the three varieties of soybean was processed into meal, and meal from each variety was used to prepare three sets of poultry diets. The diets were formulated based on the individual nutrient analyses of each of the processed meals to meet standard nutritional recommendations for growing chickens. The diets were prepared with 29.0% to 33.5% soybean meal, depending on diet type and production batch, and were fed to groups of 60 male and 60 female birds for 42 consecutive days. The parameters evaluated were survival, body weight, feed conversion (an indicator of how efficiently a bird converts feed to live body weight), and carcass yield.

Broiler chickens fed diets prepared with SYHT0H2 soybean meal did not exhibit any adverse or unexpected effects in comparison with chickens fed diets prepared with soybean meal from either the control or the reference-variety soybean. Performance over the 42-day test period did not differ significantly (ANOVA, $P > 0.05$) between chickens fed diets containing SYHT0H2 soybean meal and chickens fed diets formulated with meal from the nontransgenic control or reference-variety soybean. A significant interaction between diet and gender was observed for thigh weight. However, in pairwise comparisons between males, thigh weight differed significantly only between male chickens fed SYHT0H2 soybean meal and those fed meal from the reference-variety soybean; the SYHT0H2

and nontransgenic control groups did not differ significantly, and no effect of diet on thigh weight was detected in females. No significant diet-related differences between the SYHT0H2 and control groups were observed in the other carcass measurements.

In addition, the concentrations of AvHPPD-03 and PAT were measured in samples of the soybean meal and the broiler chicken diets. The concentrations of AvHPPD-03 and PAT were below the limit of detection in all samples. However, real-time PCR analysis confirmed the presence of SYHT0H2-soybean-specific DNA in the SYHT0H2 soybean meal and in the broiler chicken diets prepared with that meal, and the absence of SYHT0H2-specific DNA in the control and reference-variety soybean-meal diets.

In summary, diets containing SYHT0H2 soybean meal supported rapid broiler chicken growth with low mortality rates and excellent feed conversion ratios, and no adverse effects on carcass yield were observed. No differences were observed between broiler chickens consuming diets prepared with SYHT0H2 soybean meal and those consuming diets prepared with control soybean meal. Analyses of soybean meal and diet samples indicated that the nutritional profile of SYHT0H2 soybean meal was similar to that of nontransgenic control soybean meal and that diets formulated from SYHT0H2 and control soybean meal were similar. The results of this study support the conclusion that SYHT0H2 soybean meal is nutritionally comparable to and as safe as conventional soybean meal.

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