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# REVISED PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR HERBICIDE-TOLERANT EVENT SYHT0H2 SOYBEAN

## OECD UNIQUE IDENTIFIER: SYN-ØØØH2-5

## PETITION 12-215-01P


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### SUBMISSION DATE

September 17, 2012

### SUBMITTER PETITION ID

SYHT0H2-USDA-3

## Certification

The undersigned submit this petition under 7 CFR Part 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that Event SYHT0H2 soybean should not be regulated under 7 CFR Part 340.[1]

The undersigned certify that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the Petitioners that are unfavorable to the petition.

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### Release of Information

Syngenta and BCS (the "Petitioners") are submitting the information in this petition for review by the USDA as part of a federal regulatory process. Except in accordance with the following, the Petitioners do not authorize the release, publication, or other distribution of this information (including internet posting) to any third party without prior notice and Petitioners' consent. In responding to a request under the Freedom of Information Act (FOIA), 5 U.S.C. §552 and 7 CFR Part 1, relating to all or some of the information in this petition, Petitioners expect that, in advance of the release of the information, USDA will provide Petitioners with a copy of the FOIA request and the material proposed to be released and opportunity to object to the release of information based on appropriate legal grounds (e.g., responsiveness, trade secret, and/or commercial concerns).

## Summary of the Petition

The Petitioners have co-developed Event SYHT0H2 soybean, a new cultivar that has been genetically modified to tolerate herbicides that inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD) and glufosinate-ammonium herbicide. Most soybeans currently grown in the United States are glyphosate-tolerant transgenic varieties. 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.

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. Although the vector agent and the sources of some genetic elements used to create SYHT0H2 soybean are listed as plant pests in 7 CFR §340.2, the introduced nucleotide sequences do not impart plant-pest properties.

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.

Investigations with SYHT0H2 soybean confirmed that there were no changes in seed, pollen, plant phenotypic, agronomic, or composition parameters suggestive of increased plant-pest risk or increased susceptibility of SYHT0H2 soybean to plant diseases or other pests. Soybean does not possess weedy properties or outcross to wild relatives in the United States; these properties have not been altered in SYHT0H2 soybean.

Analyses of seed and forage from several U.S. field testing sites demonstrate that SYHT0H2 soybean is not materially different in composition or nutritional value from 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. PAT is exempt from the requirement for food or feed tolerances in all crops (40 CFR 174.522) and has a history of safe use in numerous transgenic crop varieties that have been deregulated by the Animal and Plant Health Inspection Service (APHIS).

On the basis of the data and information described in this petition, Syngenta and BCS request a determination from APHIS that SYHT0H2 soybean, and any progeny derived from crosses between SYHT0H2 soybean and conventional soybean or deregulated soybean cultivars, should be granted nonregulated status under 7 CFR 340.

Syngenta and BCS intend to submit an Environmental Report to APHIS in the near future that is intended to assist the agency in fulfilling its obligations under the National Environmental Policy Act, 42 U.S.C. §4321, *et seq.*, as well as other applicable statutes and regulations. Syngenta and BCS are aware of no study results or observations associated with SYHT0H2 soybean that are anticipated to result in adverse consequences to the quality of the human environment, directly, indirectly, or cumulatively. No adverse effects are anticipated on endangered or threatened species listed by the U.S. Fish and Wildlife Service, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of soybean, farmer or consumer choice, herbicide resistance, or the economy, either within or outside of the United States.

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## Table of Abbreviations, Acronyms, and Symbols

| | |
|---|---|
| A | adenine |
| ac | acre |
| a.i. | active ingredient |
| acetyl-CoA | acetyl coenzyme A |
| ADF | acid detergent fiber |
| *adh1* | alcohol dehydrogenase gene 1 |
| a.i. | active ingredient |
| ANOVA | analysis of variance |
| APHIS | Animal and Plant Health Inspection Service |
| *avhppd-03* | gene derived from *Avena sativa* (oat) and that encodes *p*-hydroxyphenylpyruvate dioxygenase in SYHT0H2 soybean |
| AvHPPD-03 | *p*-hydroxyphenylpyruvate dioxygenase encoded by *avhppd-03* |
| BC | backcross |
| BCS | Bayer CropScience |
| BLASTN | Basic Local Alignment Search Tool for Nucleotides |
| BLASTP | Basic Local Alignment Search Tool for Proteins |
| bp | base pair |
| bu | bushel; 60 pounds for soybean |
| b.w. | body weight |
| C | cytosine |
| CaMV | Cauliflower Mosaic Virus |
| CFR | Code of Federal Regulations |
| CLA | CropLife America |
| CMP | Cestrum yellow leaf curling virus promoter |
| CTAB | cetyltrimethyl ammonium bromide |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| DMGGBQ | 2,3-dimethyl-5-gernaylgeranyl-1,4-benzoquinone |
| DMPBQ | 2,3-dimethyl-5-phytyl-1,4-benzoquinone |
| DNA | deoxyribonucleic acid |
| DW | dry weight |
| EDTA | ethylenediaminetetraacetic acid |
| EST | expressed sequence tags |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | Environmental Protection Agency |
| *E*-value | expectation value |
| F | field trial |
| $F_2$ | second filial generation |
| FARRP | Food Allergy Research and Resource Program |
| FDA | Food and Drug Administration |
| FMV | figwort mosaic virus |
| FOIA | Freedom of Information Act |
| FW | fresh weight |
| g | gram |
| G | guanine |
| GC | growth chamber |
| GenBank | National Institutes of Health genetic sequence database |
| GH | glasshouse |
| h | hour |

| | |
|---|---|
| ha | hectare |
| HGA | homogentisic acid |
| HGGT | homogentisate geranylgeranyl transferase |
| HmgA | homogentisate dioxygenase |
| HmgB | fumarylacetoacetate hydrolase |
| HmgC | maleylacetoacetate isomerase |
| HPP | 4-hydroxyphenylpyruvate |
| HPPD | native *p*-hydroxyphenylpyruvate dioxygenase |
| HPT | homogentisate phytyltransferase |
| HRAC | Herbicide Resistance Action Committee |
| HST | homogentisate solanyltransferase |
| HU | hemagglutinating unit |
| IgE | immunoglobulin E |
| ILSI | International Life Sciences Institute |
| kb | kilobase pairs |
| kDa | kilodalton |
| kg | kilogram |
| l | liter |
| lb | pound |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| mg | milligram |
| MGGBQ | 2-methyl-6-geranylgeranyl-1,4-benzoquinone |
| min | minute |
| ml | milliliter |
| MOA | mode of action |
| MPBQ | 2-methyl-6-phytyl-1,4-benzoquinone |
| mRNA | messenger ribonucleic acid |
| MSBQ | 2-methyl-6-solanyl-1,4-benzoquinone |
| MPBQ MT | MPBQ methyltransferase |
| *N* | sample size |
| N/A | not applicable |
| NCBI | National Center for Biotechnology Information |
| ND | no data |
| NDF | neutral detergent fiber |
| ng | nanogram |
| NOAEL | no-observed-adverse-effect level |
| NOS | nopaline synthase |
| nr/nt | non-redundant nucleotide |
| NS | nonselective |
| OECD | Organisation for Economic Co-operation and Development |
| ORF | open reading frame |
| ori | origin of replication |
| *P* | probability |
| PAGE | polyacrylamide gel electrophoresis |
| PAT | phosphinothricin acetyltransferase |
| *pat* | phosphinothricin acetyltransferase gene |
| PB | preplant burndown |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PDP | phytyldiphosphate |
| PE | pre-emergent |
| pg | picogram |
| PR | preplant residual |
| PS | post-emergent |
| PVDF | polyvinylidene flouride |
| RefSeq | NCBI Reference Sequence Collection |
| *repA* | replication gene from *Pseudomonas aeruginosa* plasmid VS1 |
| $R_1$, $R_2$ | substituent group (in a chemical structure) |
| RNA | ribonucleic acid |
| RSD | relative standard deviation |
| S | Svedberg unit |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SEM | atandard error of the mean |
| 35S enhancer | transcriptional enhancer region of the cauliflower mosaic virus |
| SGF | simulated (mammalian) gastric fluid |
| SIF | simulated (mammalian) intestinal fluid |
| SMP | synthetic minimal plant (promoter) |
| *spec* | spectinomycin resistance gene |
| spp. | species (plural) |
| 35S promoter | promoter region of the cauliflower mosaic virus |
| SSC | sodium chloride–sodium citrate |
| subsp. | subspecies |
| syn | synonym |
| T | thymine |
| $T_0$, $T_1$, $T_2$, $T_3$, etc. | $T_0$ is the designation used for the original transformed plant, SYHT0H2, and $T_1$, $T_2$, $T_3$, etc., refer to successive self-pollinated generations of SYHT0H2 soybean |
| TAM | L-tyrosine aminotransferase |
| TC | tocopherol cyclase |
| T-DNA | transferred DNA |
| TIU | trypsin inhibitor unit |
| TMT | tocopherol methyltransferase |
| TMV | tobacco mosaic virus |
| tris | 2-amino-2(hydroxymethyl)-1,3-propanediol |
| TYRA | L-tyrosine aminotransferase |
| U.S. | United States (of America) |
| U.S.C. | United States Code |
| USDA | United States Department of Agriculture |
| v. | version |
| var. | variety |
| v/v | volume to volume |
| *virG* | regulatory gene from *Agrobacterium tumefaciens* |
| WSSA | Weed Science Society of America |
| wt. | weight |
| w/v | weight to volume |
| °C | degrees Celsius |
| °F | degrees Fahrenheit |
| ♀ | female |
| µg | microgram |

| | |
|---|---|
| µmol | micromole |
| ® | registered trademark |
| × | cross, cross-pollination |
| ⊗ | self-pollination |

**Soybean Growth Stages**

| | |
|---|---|
| Vegetative: | |
| VC | unrolled unifoliate leaves |
| V1 | one set of unfolded trifoliate leaves |
| V2–V*n* | 2 through *n* fully developed trifoliate leaf nodes |
| Reproductive: | |
| R1 | beginning of flowering |
| R2 | full flowering |
| R3 | beginning of pod development |
| R4 | full pod |
| R5 | beginning seed |
| R6 | full seed |
| R7 | beginning of seed maturity |
| R8 | full maturity |

**Amino Acids**

| | |
|---|---|
| Ala, A | alanine |
| Arn, N | asparagine |
| Asp, D | aspartic acid |
| Cys, C | cysteine |
| Glu, E | glutamic acid |
| Gly, G | glycine |
| His, H | histidine |
| Ile, I | isoleucine |
| Leu, L | leucine |
| Lys, K | lysine |
| Met, M | methionine |
| Phe, F | phenylalanine |
| Pro, P | proline |
| Ser, S | serine |
| Thr, T | threonine |
| Trp, W | tryptophan |
| Tyr, Y | tyrosine |
| Val, V | valine |

## I. Rationale for Development of SYHT0H2 Soybean

Crops improved through modern biotechnology have brought significant benefits to United States (U.S.) agriculture in the form of improved yields, pest management, and crop quality. Continued innovation in this area will benefit growers, consumers, and the environment.

Syngenta Crop Protection AG (Syngenta) and Bayer CropScience AG (BCS) have co-developed a transgenic soybean cultivar that is tolerant to herbicides that inhibit plant *p*-hydroxyphenyl-pyruvate dioxygenase (HPPD), such as mesotrione, and tolerant to applications of glufosinate-ammonium herbicide. Soybean derived from transformation Event SYHT0H2 was developed through *Agrobacterium*-mediated transformation to stably incorporate the genes *avhppd-03* and *pat* into the soybean genome. The gene *avhppd-03* encodes the enzyme *p*-hydroxyphenyl-pyruvate dioxygenase (AvHPPD-03) derived from oat *(Avena sativa)*. 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 enzyme phosphinothricin acetyltransferase (PAT) which, when produced in plants, acetylates L-phosphinothricin, the active form of glufosinate-ammonium herbicide, resulting in post-emergence tolerance.

### I.A. Basis of the Request for a Determination of Nonregulated Status

Under the authority of the Plant Protection Act (7 U.S.C. 7701 *et seq.*) and the regulations under 7 CFR 340, the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) regulates importation, interstate movement, and environmental release of organisms and products altered or produced through genetic engineering that are plant pests or which there is reason to believe are plant pests. Subject to regulation is any organism that has been altered or produced through genetic engineering if the donor organism, recipient organism, or vector or vector agent belongs to any taxa designated under 7 CFR 340.2 and meets the definition of a plant pest, or is unclassified, or its classification is unknown; any product that contains such an organism; and any other organism or product altered or produced through genetic engineering that the Administrator determines is a plant pest or has reason to believe is a plant pest.

The vector agent used to produce SYHT0H2 soybean, *pat,* and some of the regulatory sequences used to drive expression of *pat* and *avhppd-03* are from organisms listed under 7 CFR 340.2. Although the vector agent, *Agrobacterium tumefaciens,* is a plant pathogen, the transformation process that created SYHT0H2 soybean used a disarmed strain. The gene encoding PAT, which confers tolerance to glufosinate-ammonium, was derived from *Streptomyces viridochromogenes* and codon-optimized for plant expression. In addition, regulatory sequences from figwort mosaic virus, cauliflower mosaic virus, Cestrum yellow leaf curling virus, tobacco mosaic virus, and *A. tumefaciens* were introduced during the production of SYHT0H2 soybean. The gene *pat* and the regulatory sequences *per se* do not impart plant pest properties. No nucleotide sequences from *A. tumefaciens, S. viridochromogenes,* or the plant viruses imparting plant pest properties were transferred to SYHT0H2 soybean.

The regulations (7 CFR 340.6) provide that any person may petition APHIS to seek a determination that an article should not be regulated. Syngenta and BCS herein present data and

justification for a determination of nonregulated status for SYHT0H2 soybean based on an absence of plant pest potential.

### I.B. Benefits of SYHT0H2 Soybean

The extensive use of glyphosate-based weed-control programs in corn, cotton, and soybean over the past 15 years and the accompanying increase in conservation tillage farming have resulted in the selection of resistant weeds and a shift in weed populations to species that are inherently more tolerant to glyphosate, thus making them more difficult to control (Owen 2008). Twenty-three glyphosate-resistant weed species have been identified globally, 13 of which are found in the U.S. (Heap 2012). In addition, with the increased use of glyphosate-only weed-control programs, weeds that previously were not an agronomic problem are becoming more prevalent and difficult to control. These weed shifts are occurring predominantly, but not exclusively, with difficult-to-control broadleaf weeds. Some examples are *Ipomoea, Amaranthus,* and *Commelina* species (Culpepper 2006). Cultivation of SYHT0H2 soybean will provide growers with an opportunity to use glufosinate-ammonium and HPPD-inhibitor herbicides, such as mesotrione, for control of problematic weeds in soybean production systems. The glufosinate- and mesotrione-tolerance traits can also be integrated into existing glyphosate-tolerant soybean programs by crossing SYHT0H2 soybean with a glyphosate-tolerant cultivar.

Mesotrione is a systemic, translocated herbicide with soil residual activity that is used for control of predominantly dicot weed species in a number of crops, including corn. Glufosinate-ammonium is a contact herbicide that is applied to crops post-emergence and has no soil residual activity. Glufosinate-ammonium controls a broad spectrum of monocot and dicot weed species. Glufosinate-ammonium and mesotrione have distinct herbicidal modes of action, both of which differ from that of glyphosate. The use of herbicide mixtures and alternation of herbicides with different modes of action are cornerstones of an integrated weed management program.

### I.C. Submissions to Other Regulatory Agencies

Syngenta and BCS are pursuing regulatory approvals for SYHT0H2 soybean cultivation in the U.S. and Canada, and may seek cultivation approvals in other countries in the future. SYHT0H2 soybean falls within the scope of the U.S. Food and Drug Administration's (FDA's) policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA 1992). Syngenta and BCS will soon initiate the consultation process for SYHT0H2 soybean with FDA. Regulatory approvals that facilitate global trade in soybean commodities will be sought in the following countries: Mexico, Colombia, Japan, Republic of Korea, Republic of China, People's Republic of China, Philippines, Indonesia, Thailand, Singapore, Australia/New Zealand, Republic of South Africa, the Russian Federation, and the European Union. Cultivation or importation approvals in other countries will be sought on an as-needed basis.

Crop metabolism and residue studies have been conducted to support establishment of a tolerance for mesotrione residues in or on soybean as required under the Federal Insecticide, Rodenticide, and Fungicide Act. A tolerance petition and label amendment application for post-emergence use of mesotrione on soybean will be submitted to the U.S. Environmental Protection Agency (EPA). Tolerances for pre-emergent use of mesotrione in or on soybean have already

been established by EPA (40 CFR 180.571). Tolerances for residues of glufosinate-ammonium in or on soybean have already been established by EPA (40 CFR 180.473).

### I.D. References


Culpepper SA. 2006. Glyphosate-induced weed shifts. *Weed Technology* 20:277–281.

FDA. 1992. Foods for human consumption and animal drugs, feeds, and related products: Foods derived from new plant varieties; policy statement. U.S. Food and Drug Administration. *Federal Register* 57:22984.

Heap I. 2012. *The International Survey of Herbicide Resistant Weeds.* http://www.weedscience.org/In.asp (accessed June 11, 2012).

Owen MD. 2008. Weed species shifts in glyphosate-resistant crops. *Pest Management Science* 64:377–387.

## II. The Biology of Soybean

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 U.S., Brazil, Argentina, China, and India (Soy Stats 2012).

### II.A. Overview of Soybean Biology

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, center of origin and diversity, identification, reproductive biology, crosses, and ecology.

### II.B. Recipient Soybean Line

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 40° and 42° north latitude (see Figure II-1). 'Jack' has white flowers, gray 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.

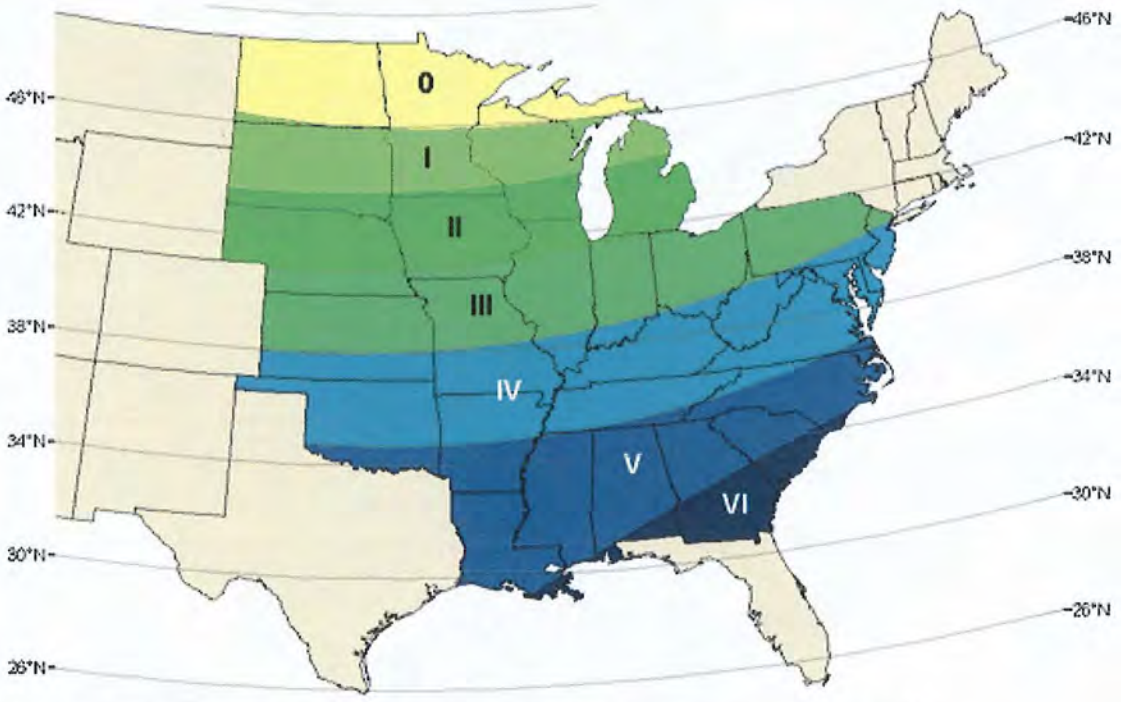


**Figure II-1. Zones of adaptation for soybean maturity groups in the continental U.S. (Zhang *et al.* 2007)**

## II.C. References


Nickell CD, Noel GR, Thomas DJ. 1990. Registration of 'Jack' soybean. *Crop Science* 30:1365.

OECD. 2000. *Consensus Document on the Biology of* Glycine max *(L.) Merr. (soybean).* Series on the Harmonization of Regulatory Oversight in Biotechnology. No. 15. ENV/JM/MONO(2000)9. Paris, France: Organisation for Economic Co-operation and Development. 22 pp. http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2000)9&doclanguage=en.

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## III. Development of SYHT0H2 Soybean

This section describes the method by which soybean was transformed to produce herbicide-tolerant soybean plants, the development of Event SYHT0H2 soybean, and production of test and control seed lots for use in the studies described in this petition.

### III.A. Description of the Transformation Method

*Agrobacterium tumefaciens*–mediated transformation of immature seed of soybean variety 'Jack' was used to produce herbicide-tolerant plants (Hwang *et al.* 2008, Que *et al.* 2008). By this method, genetic elements between 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 binary transformation plasmid pSYN15954 (described in Section IV), 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.

### III.B. Development of Event SYHT0H2 Soybean

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 III-1.

All shipments and field releases of SYHT0H2 soybean in the U.S. were carried out under USDA notifications, which are listed in Appendix A.

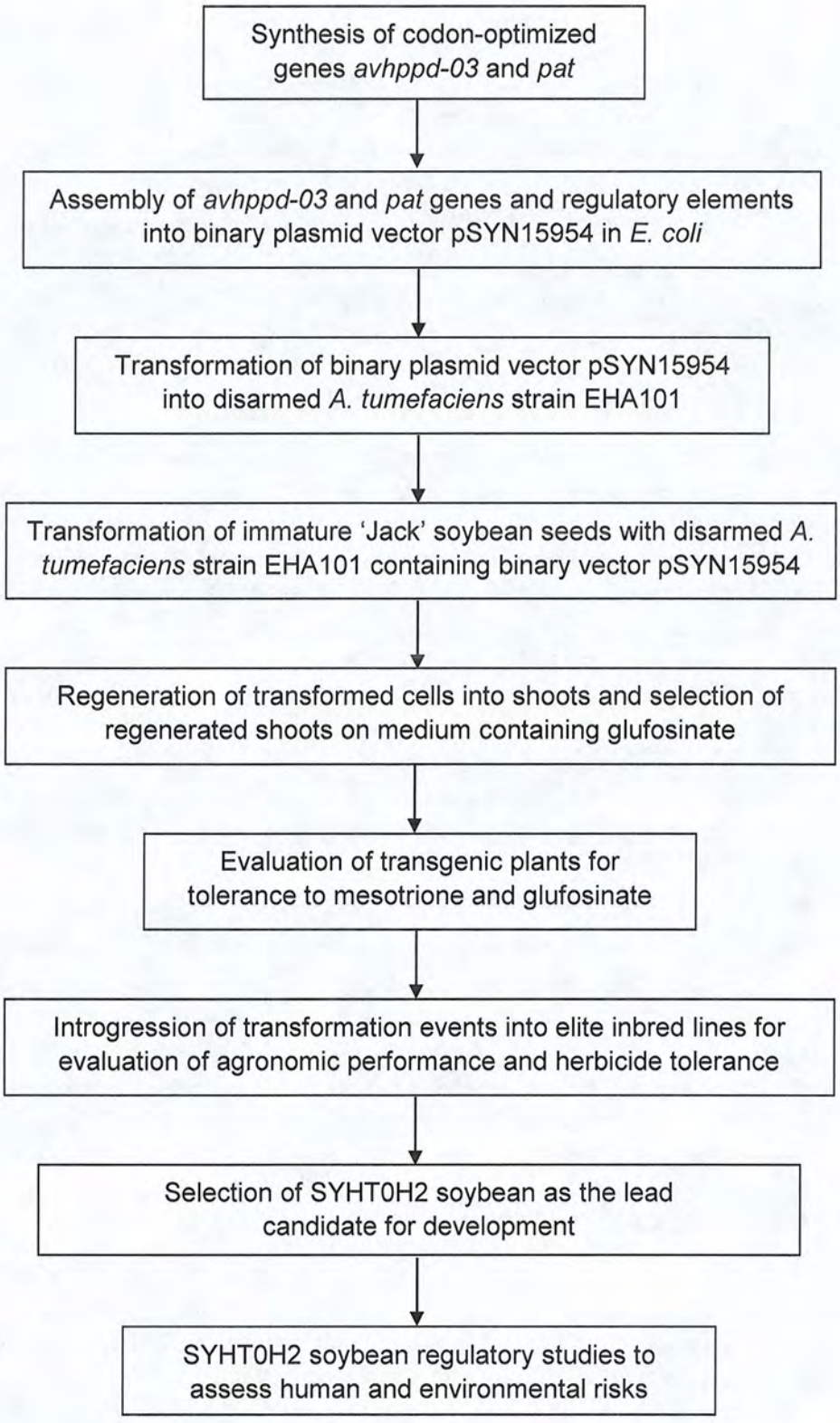


**Figure III-1. Steps in the development of SYHT0H2 soybean**

### III.C. Production of Test and Control Seed

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 III-2 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 used.

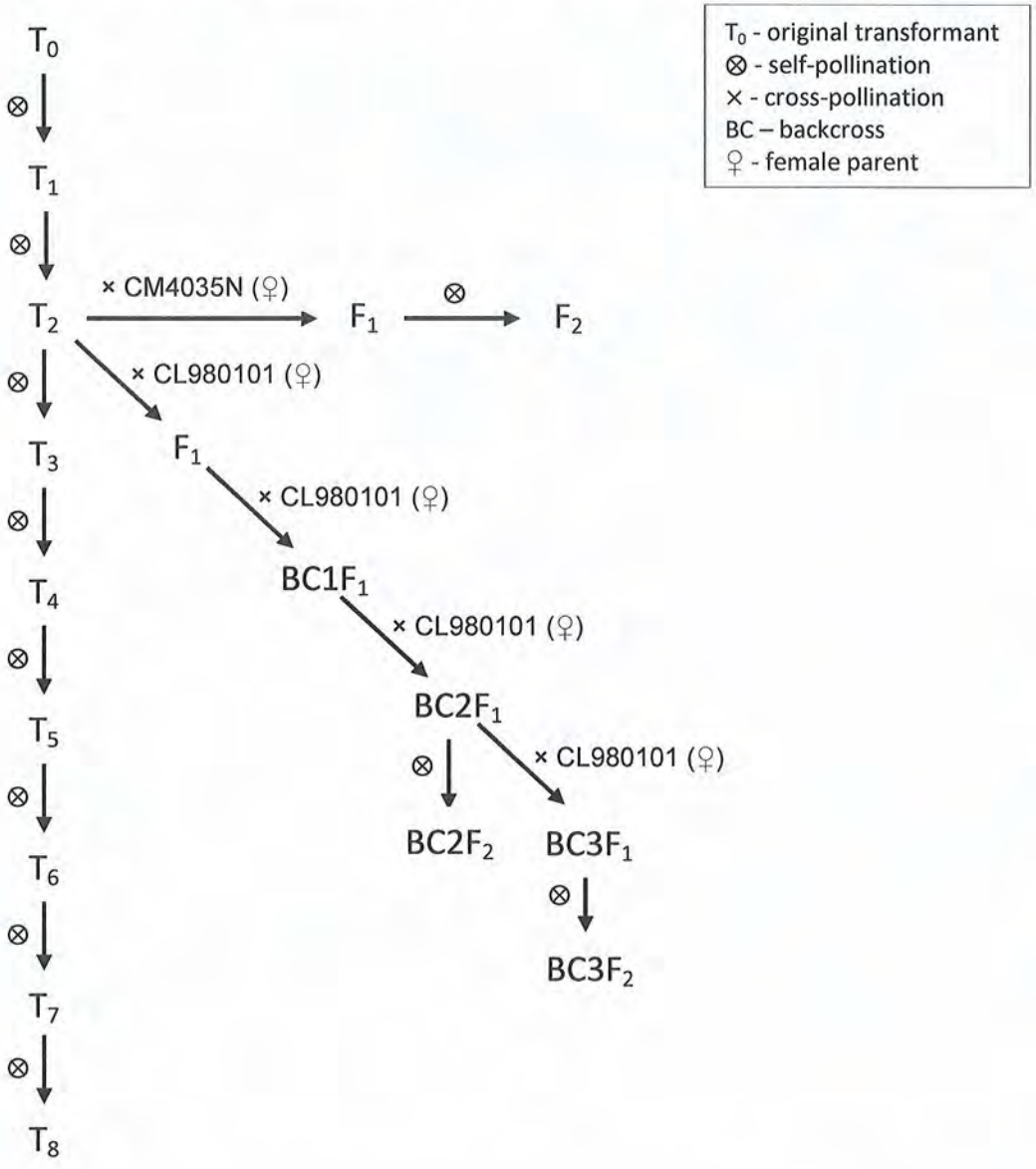


**Figure III-2. Pedigree of the SYHT0H2 plant materials used in regulatory studies**

The transformation recipient line was 'Jack' soybean. CM4035N and CL980101 are commercial nontransgenic soybean lines.

### III.D. Quality Control of Test and Control Materials

All test and control seed lots were analyzed by real-time PCR for the presence of the SYHT0H2 T-DNA and adventitious DNA from other soybean transformation events. All SYHT0H2 soybean seed lots were confirmed to contain the genes *avhppd-03* and *pat,* based on nucleotide sequence. Control seed lots were found not to contain any SYHT0H2 DNA. None of the test and control seed lots contained detectable nucleotide sequences that would indicate contamination with DNA from other regulated events under development at Syngenta or deregulated events for which testing methodology was available.

### III.E. References

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## IV. Donor Genes and Regulatory Sequences

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. A map of the transformation plasmid is shown in Figure IV-1, and each genetic element in the transformation plasmid is described in Table IV-1.

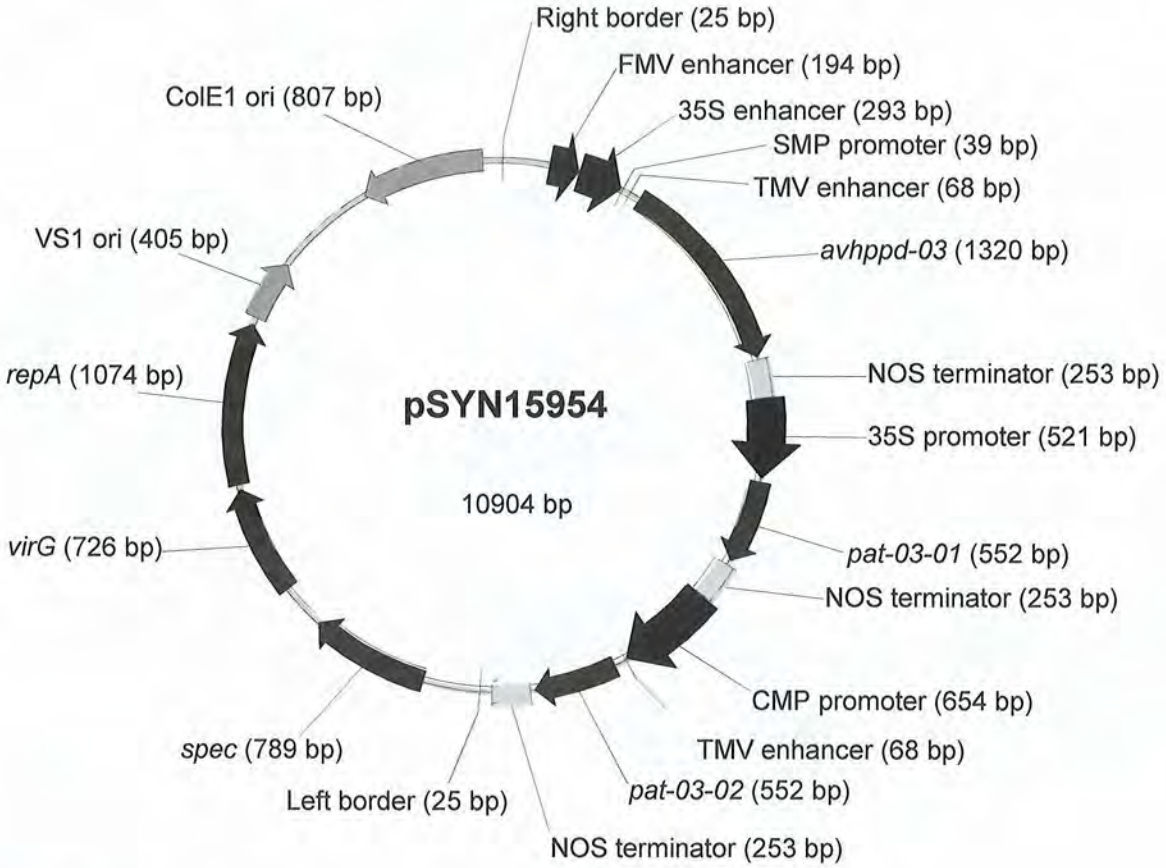


**Figure IV-1. Plasmid map for the vector pSYN15954**

**Table IV-1. Description of the genetic elements in vector pSYN15954**

| 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. |
| 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. |

*(continued)*

| Genetic element | Size (bp) | Position | Description |
|---|---|---|---|
| ***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). |
| Intervening sequence | 77 | 5494 to 5570 | Noncoding intervening sequence with restriction sites used for cloning. |

*(continued)*

| Genetic element | Size (bp) | Position | Description |
|---|---|---|---|
| **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 *Escherichia 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 acide 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). |

## IV.A. References


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## V. Genetic Characterization of Event SYHT0H2 Soybean

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.

Parts V.A. through V.F., below, describe the design, results, and conclusions of each genetic characterization study. Details of the materials and methods used in these studies are provided in Appendix B, and the general conclusions of the genetic characterization studies are summarized in Part V.G.

### V.A. 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. 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.

### V.B. 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.

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–

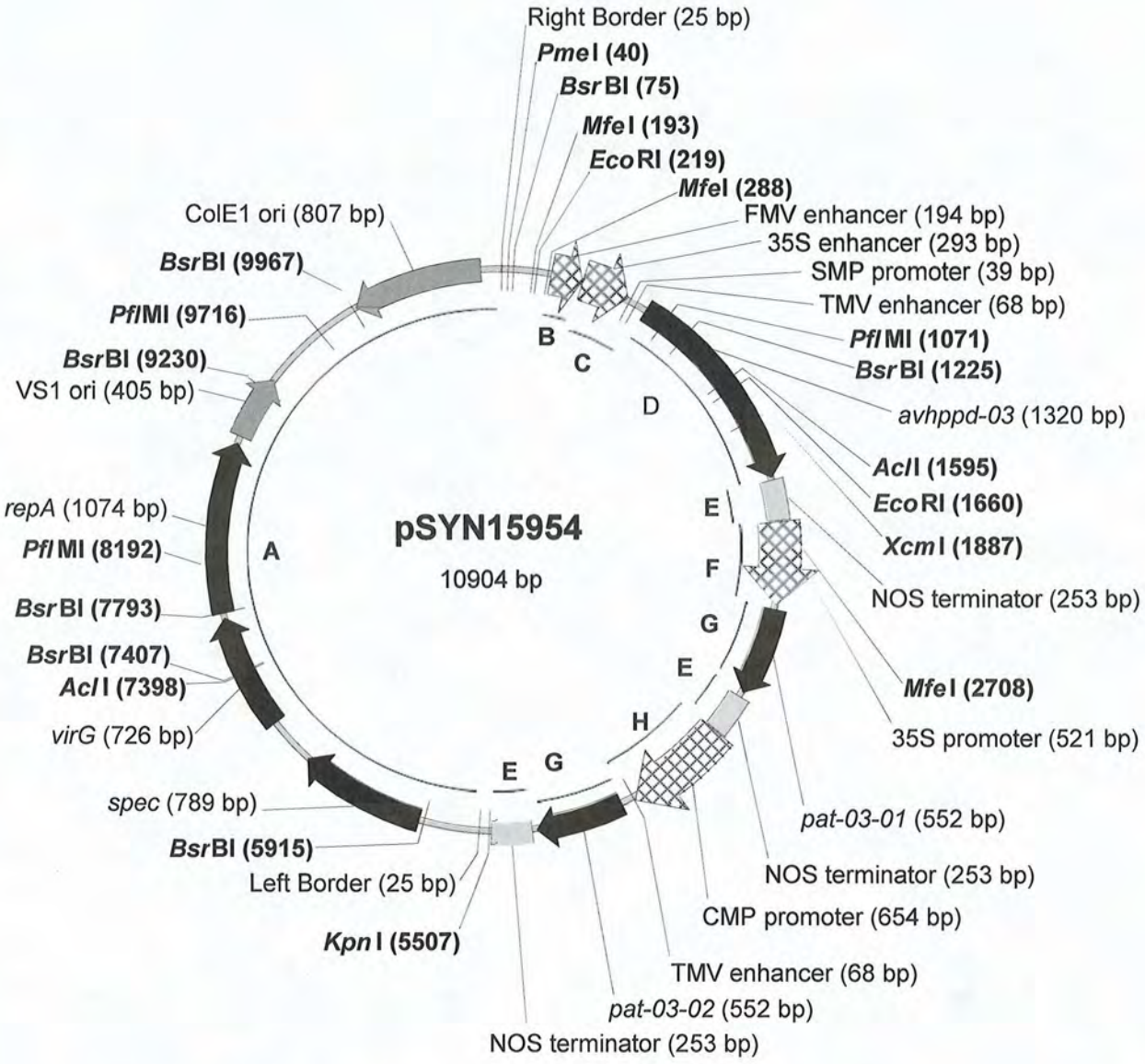
specific probe, (7) an FMV enhancer–specific probe, and (8) a plasmid pSYN15954 backbone sequence probe. Each functional-element-specific probe except the *pat*-specific probe covered every base of the functional element present in the plasmid pSYN15954 T-DNA. Because the pSYN15954 T-DNA included two *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 V-1 is a map of plasmid pSYN15954 showing the locations of the restriction endonuclease sites and probe annealing sites. Figure V-2 is a map showing the locations of the restriction sites and probes in the SYHT0H2 soybean insert.

Table V-1 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 in Part V.A., 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 V-1. Map of plasmid pSYN15954 showing the restriction sites and probes used in Southern blot analyses**

Restriction enzymes and sites are indicated by bold type.

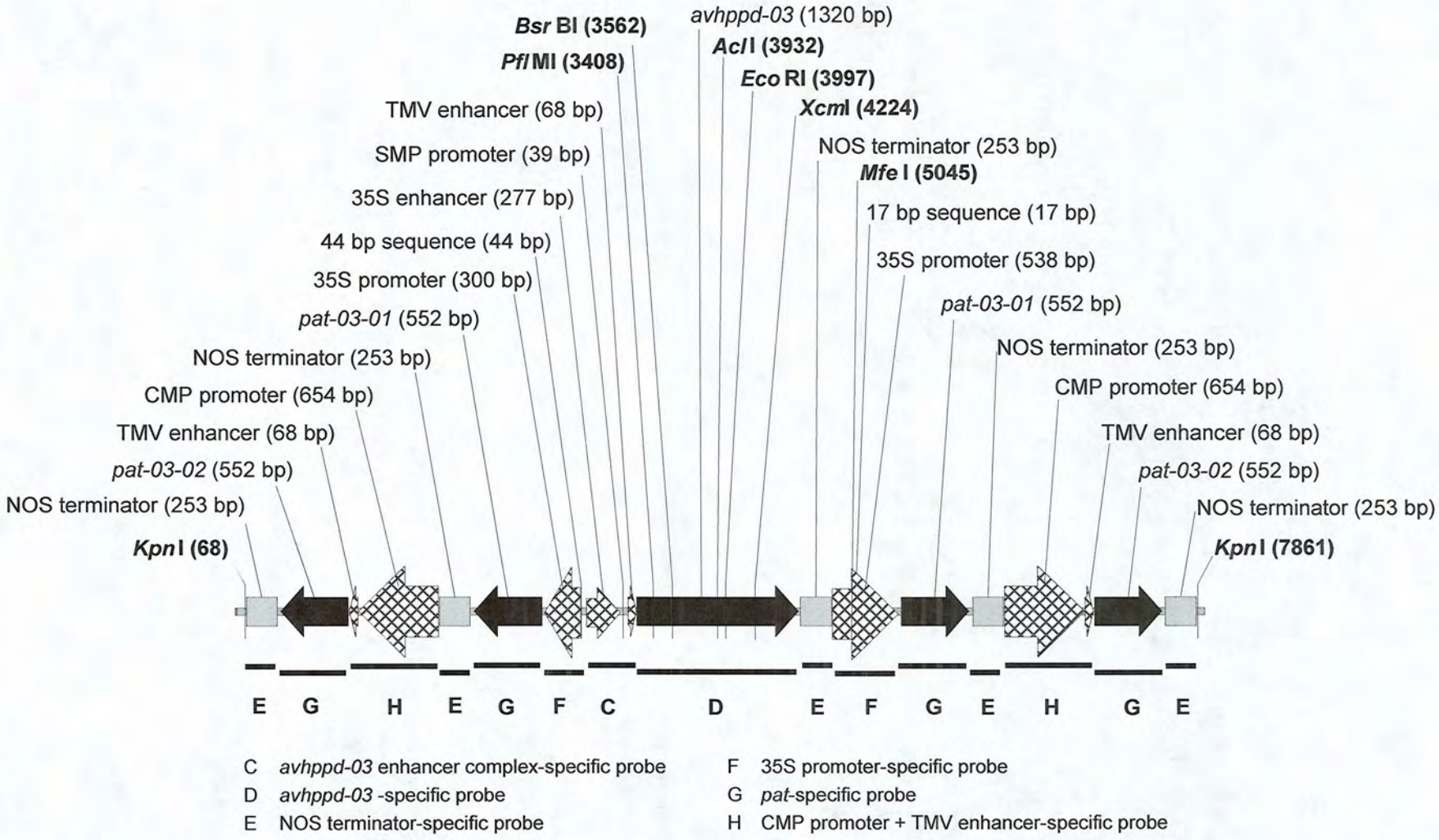



**Figure V-2. Map of the SYHT0H2 DNA insert showing the restriction sites and probes used in Southern blot analyses.**
Restriction enzymes and sites are indicated by bold type.

**Table V-1. Expected and observed hybridization bands in Southern blot analyses for copy number of functional elements and absence of plasmid backbone sequence**

| 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-4A, 3 | 2 | >3.9, >4.0 | ~7.6, ~10 |
| | | positive control | V-4A, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Eco*RI | SYHT0H2 $T_4$ | V-4B, 3 | 2 | >4.0, >3.9 | ~4.8, ~8.3 |
| | | positive control | V-4B, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Kpn*I + *Bsr*BI | SYHT0H2 $T_4$ | V-4C, 3 | 2 | ~3.5, ~4.3 | ~3.5, ~4.3 |
| | | positive control | V-4C, 5 | 1 | ~5.5 | ~5.4[b] |
| *avhppd-03* enhancer complex | *Eco*RI | SYHT0H2 $T_4$ | V-5A, 3 | 2 | >4.0, >3.9 | ~4.8, ~8.2 |
| | | positive control | V-5A, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Xcm*I | SYHT0H2 $T_4$ | V-5B, 3 | 2 | >4.2, >3.7 | ~4.3, ~5.7 |
| | | 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 | ~5.3[b] |
| 35S promoter | *Eco*RI | SYHT0H2 $T_4$ | V-6A, 3 | 2 | >4.0, >3.9 | ~4.8, ~8.3 |
| | | positive control | V-6A, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Xcm*I | SYHT0H2 $T_4$ | V-6B, 3 | 2 | >4.2, >3.7 | ~4.3, ~5.7 |
| | | positive control | V-6B, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Kpn*I + *Bsr*BI | SYHT0H2 $T_4$ | V-6C, 3 | 2 | ~3.5, ~4.3 | ~3.5, ~4.3 |
| | | positive control | V-6C, 5 | 1 | ~5.5 | ~5.4[b] |
| CMP promoter + TMV enhancer | *Eco*RI | SYHT0H2 $T_4$ | V-7A, 3 | 2 | >4.0, >3.9 | ~4.8, ~8.3 |
| | | positive control | V-7A, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Mfe*I | SYHT0H2 $T_4$ | V-7B, 3 | 2 | >5.0, >2.9 | ~5.2, ~6.2 |
| | | 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 | ~5.4[b] |
| NOS terminator | *Acl*I | SYHT0H2 $T_4$ | V-8A, 3 | 2 | >3.9, >4.0 | ~7.6, ~10 |
| | | positive control | V-8A, 5 | 1 | ~5.5 | ~5.3[b] |
| | *Eco*RI | SYHT0H2 $T_4$ | V-8B, 3 | 2 | >4.0, >3.9 | ~4.8, ~8.3 |
| | | positive control | V-8B, 5 | 1 | ~5.5 | ~5.5 |
| | *Kpn*I + *Bsr*BI | SYHT0H2 $T_4$ | V-8C, 3 | 2 | ~3.5, ~4.3 | ~3.5, ~4.3 |
| | | positive control | V-8C, 5 | 1 | ~5.5 | ~5.5 |

*(continued)*

| 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-9A, 3 | 0 | N/A | N/A |
| | | positive control | V-9A, 5 | 1 | ~5.5 | ~5.5 |
| | *Pfl*MI | SYHT0H2 $T_4$ | V-9B, 3 | 0 | N/A | N/A |
| | | positive control | V-9B, 5 | 1 | ~5.5 | ~5.5 |
| | *Kpn*I + *Bsr*BI | SYHT0H2 $T_4$ | V-9C, 3 | 0 | N/A | N/A |
| | | positive control | V-9C, 5 | 1 | ~5.5 | ~5.4[b] |
| Plasmid backbone | *Acl*I | SYHT0H2 $T_4$ | V-10A, 3 | 0 | N/A | N/A |
| | | positive control | V-10A, 5 | 1 | ~5.4 | ~1.9, ~3.5, ~5.5[b] |
| | N/A | positive control | V-10A, 7 | 1 | ~5.4 | ~5.5[b] |
| | *Pfl*MI | SYHT0H2 $T_4$ | V-10B, 3 | 0 | N/A | N/A |
| | | positive control | V-10B, 5 | 1 | ~5.4 | ~5.7[b] |
| | *Kpn*I | SYHT0H2 $T_4$ | V-10C, 3 | 0 | N/A | N/A |
| | | positive control | V-10C, 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.

### V.B.1. 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 V-3 (at the end of Section V.B.).

In the analysis of genomic DNA digested with *Eco*RI, two bands of approximately 4.9 and 8.3 kb were observed in the lane containing DNA from SYHT0H2 $T_4$ soybean (Table V-1; Figure V-3A, Lane 3). These bands were absent from the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-3A, 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 V-3A, Lane 5).

In the analysis of genomic DNA digested with *Mfe*I, one band of approximately 6.2 kb was observed in the lane containing DNA from SYHT0H2 $T_4$ soybean (Table V-1; Figure V-3B, Lane 3). This band was absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-3B, 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 V-3B, Lane 5).

In the analysis of genomic DNA digested with *Kpn*I, one band of approximately 7.8 kb was observed in the lane containing DNA from SYHT0H2 $T_4$ soybean (Table V-1; Figure V-3C, Lane 3). This band was absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-3C, 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 V-3C, 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*.

### V.B.2. 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 V-4 (at the end of Section V.B.).

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 V-1; Figure V-4A, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-4A, 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 V-4A, 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 V-1; Figure V-4B, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-4B, 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 V-4B, 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 V-1; Figure V-4C, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-4C, 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 V-4C, 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*.

### V.B.3. 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 V-5 (at the end of Section V.B.).

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 V-1; Figure V-5A, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-5A, 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 V-5A, 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 V-1; Figure V-5B, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-5B, 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 V-5B, 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 V-1; Figure V-5C, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-5C, 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 V-5C, 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.

#### V.B.4. 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 V-6 (at the end of Section V.B.).

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 V-1; Figure V-6A, Lane 3). These bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure V-6A, 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 V-6A, 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 V-1; Figure V-6B, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-6B, 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 V-6B, 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 V-1; Figure V-6C, Lane 3). These bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure V-6C, 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 V-6C, 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.

#### V.B.5. 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 V-7 (at the end of Section V.B.).

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 V-1; Figure V-7A, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-7A, 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 V-7A, 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 V-1; Figure V-7B, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-7B, 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 V-7B, 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 V-1; Figure V-7C, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-7C, 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 V-7C, 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.

### V.B.6. 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 V-8 (at the end of Section V.B.).

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 V-1; FigureV-8A, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-8A, 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 V-8A, 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 V-1; FigureV-8B, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-8B, 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 V-8B, 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 V-1; Figure V-8C, Lane 3). These bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure V-8C, 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 V-8C, 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.

### V.B.7. 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 V-9 (at the end of Section V.B.).

As discussed in Section V.A., 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 V-1; Figures V-9A through V-9C, Lane 3) or in the lanes containing DNA from nontransgenic 'Jack' soybean (Figures V-9A through V-9C, 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 V-9A through V-9C, Lane 5), as expected.

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

### V.B.8. Absence of Plasmid Backbone Sequence

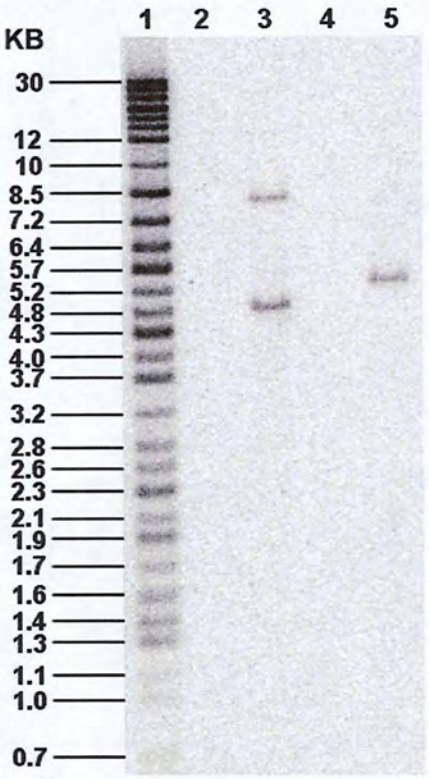
The results of the Southern blot analyses of SYHT0H2 genomic DNA with the pSYN15954 plasmid-backbone-specific probe are shown in Figure V-10 (at the end of Section V.B.).

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 V-1; Figures V-10A through V-10C, Lane 3) or in the lanes containing DNA from nontransgenic 'Jack' soybean (Figures V-10A through V-10C, 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 V10-B and V-10C, 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 V-10A, 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 V-10A, Lane 7).

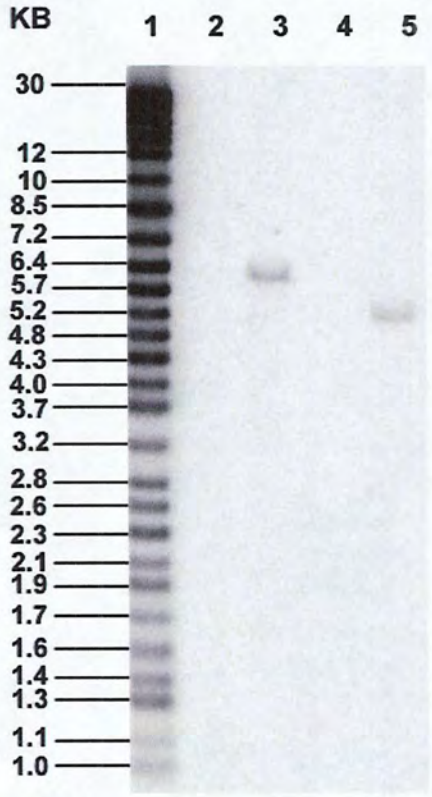
These results demonstrate that SYHT0H2 soybean does not contain any backbone sequences from the transformation plasmid pSYN15954.

(A) *Eco*RI



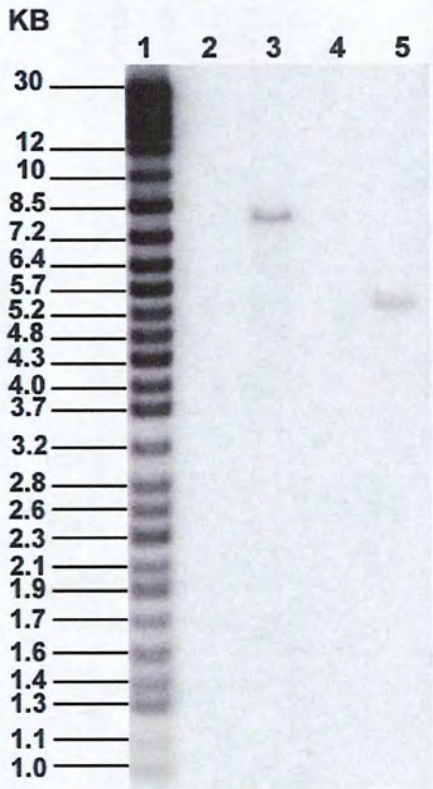

Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(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)

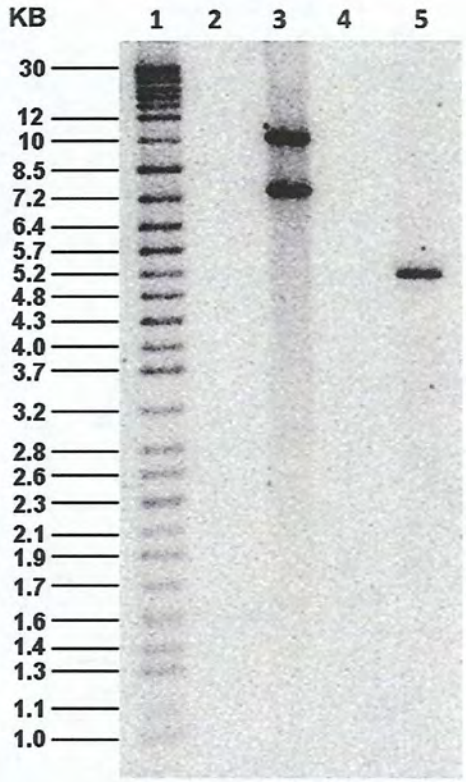
(C) *Kpn*I




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)

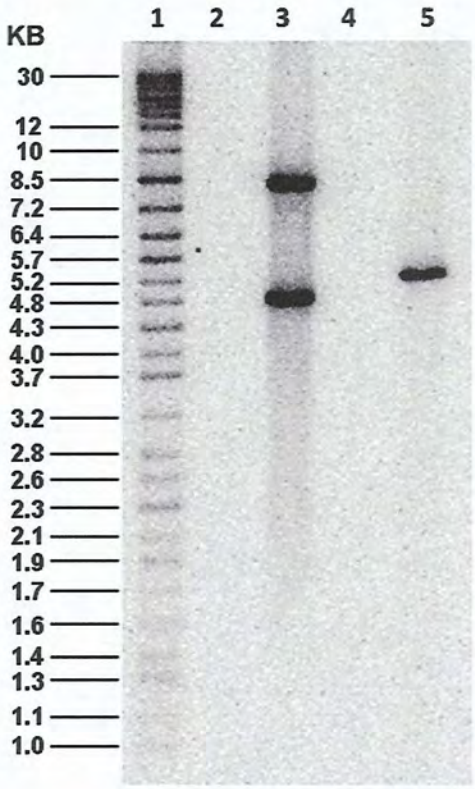
**Figure V-3. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *avhppd-03*–specific probe and the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I**

(A) *AclI*



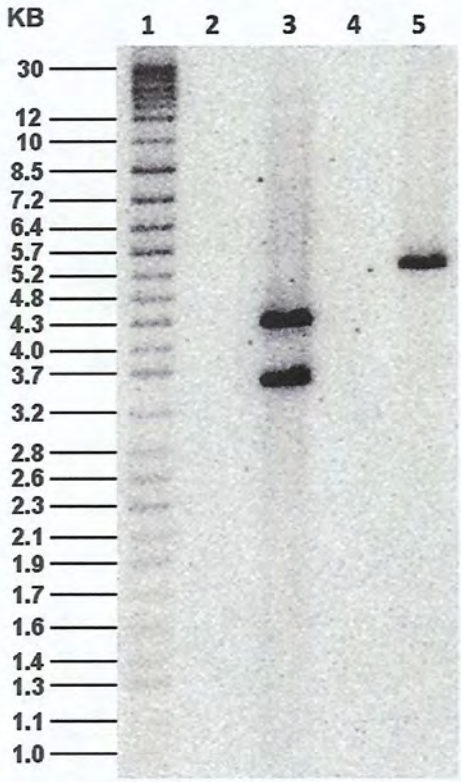

Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *AclI*
Lane 4 = 'Jack' digested with *AclI*
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *KpnI* + *PmeI* plus 'Jack' digested with *AclI*)

(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 *KpnI* + *PmeI* plus 'Jack' digested with *Eco*RI)

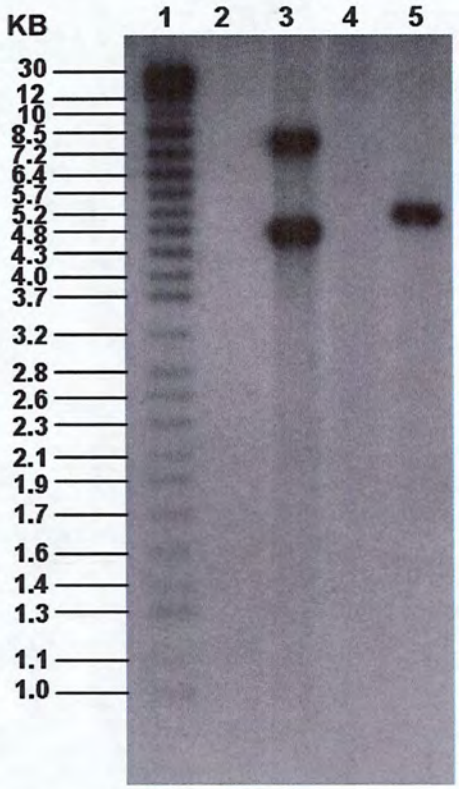
(C) *KpnI* + *Bsr*BI




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

**Figure V-4. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *pat*-specific probe and the restriction enzymes AclI, *Eco*RI, and *KpnI* + *Bsr*BI**

(A) *Eco*RI

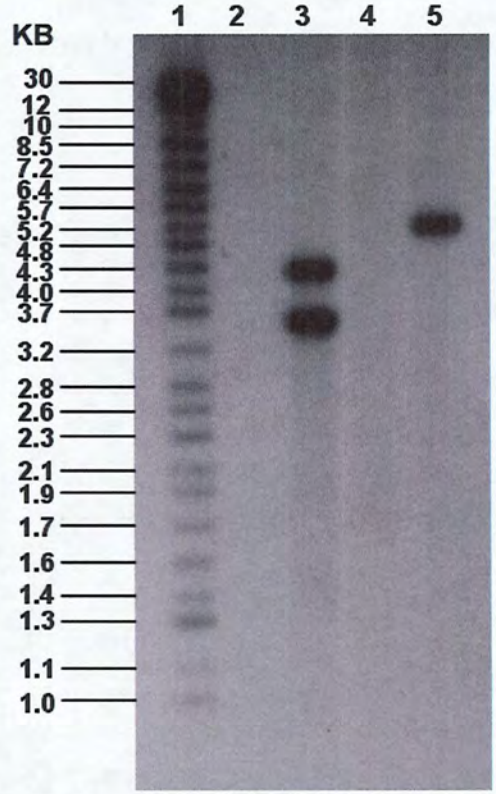



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(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)

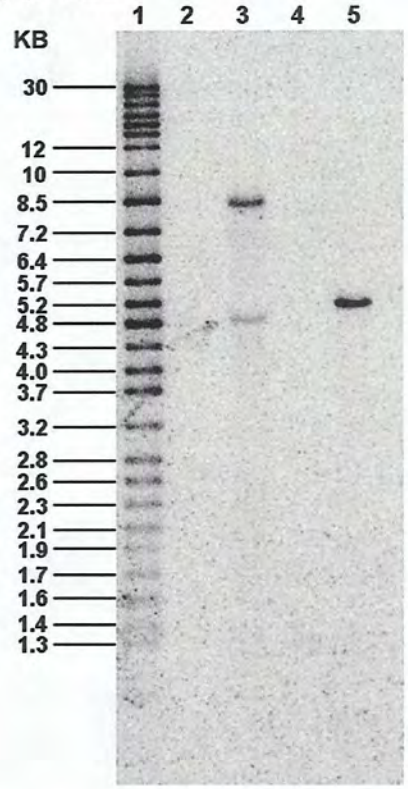
(C) *Kpn*I + *Bsr*BI




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)

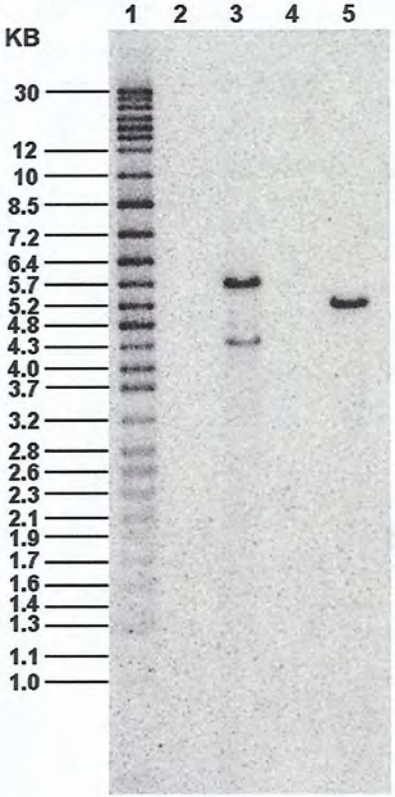
**Figure V-5. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the *avhppd-03* enhancer complex–specific probe and the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI**

(A) *Eco*RI



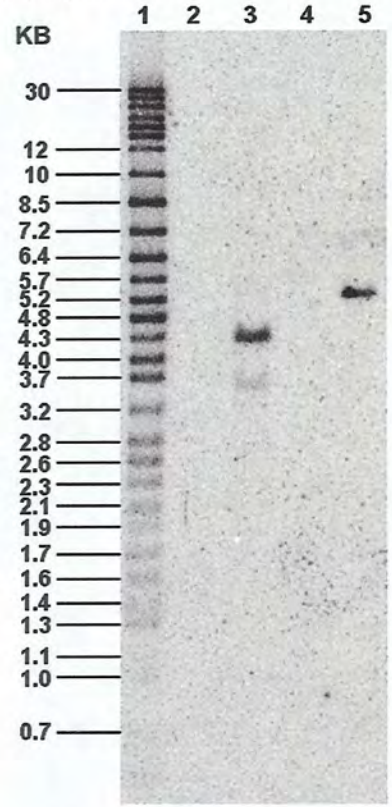

Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(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)

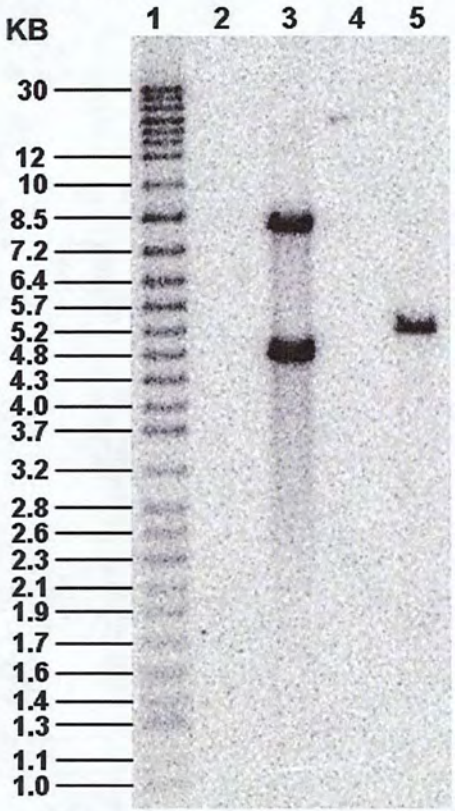
(C) *Kpn*I + *Bsr*BI




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)

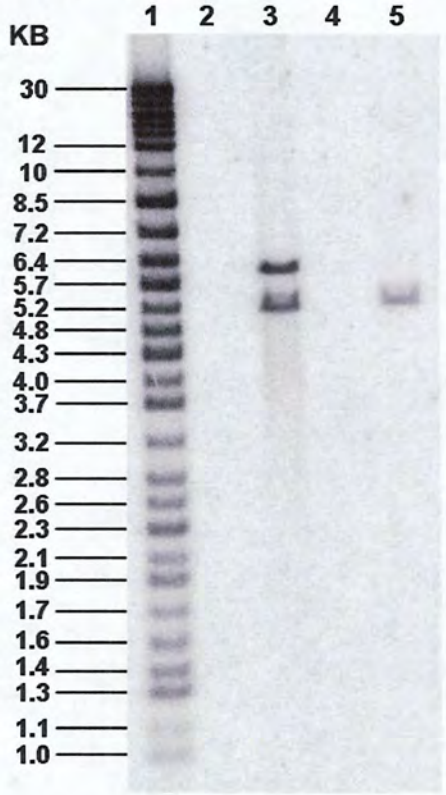
**Figure V-6. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 35S promoter–specific probe and the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI**

(A) *Eco*RI



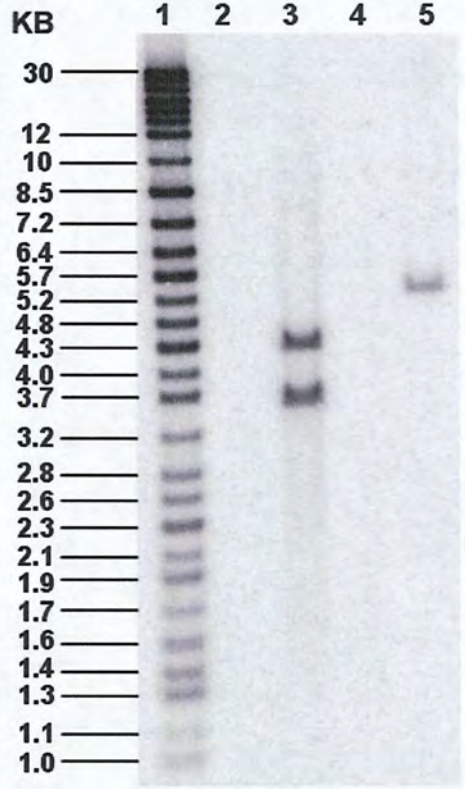

Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *Eco*RI
Lane 4 = 'Jack' digested with *Eco*RI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with *Eco*RI)

(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)

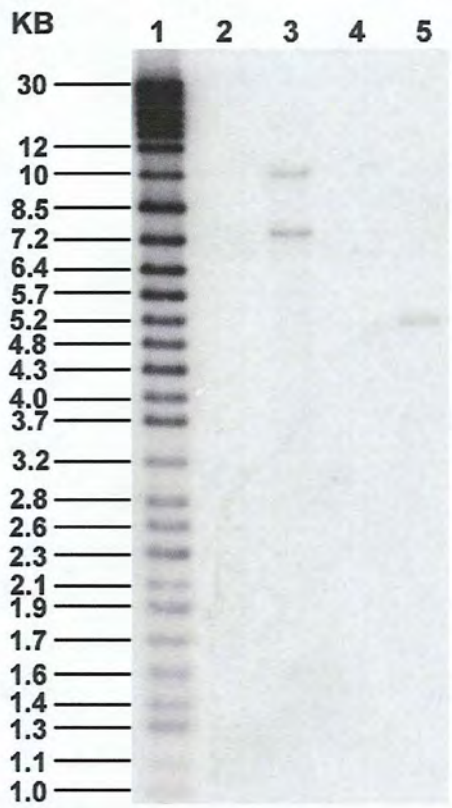
(C) *Kpn*I + *Bsr*BI




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 V-7. 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) *AclI*

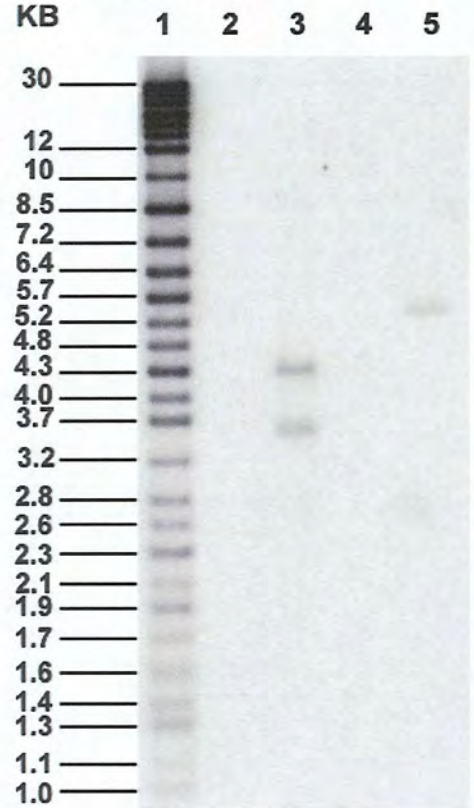



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with AclI
Lane 4 = 'Jack' digested with AclI
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *Kpn*I + *Pme*I plus 'Jack' digested with AclI)

(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)

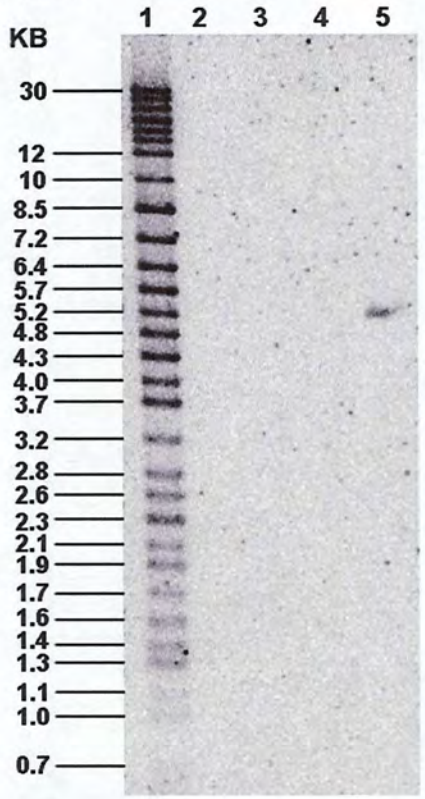
(C) *Kpn*I + *Bsr*BI




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)

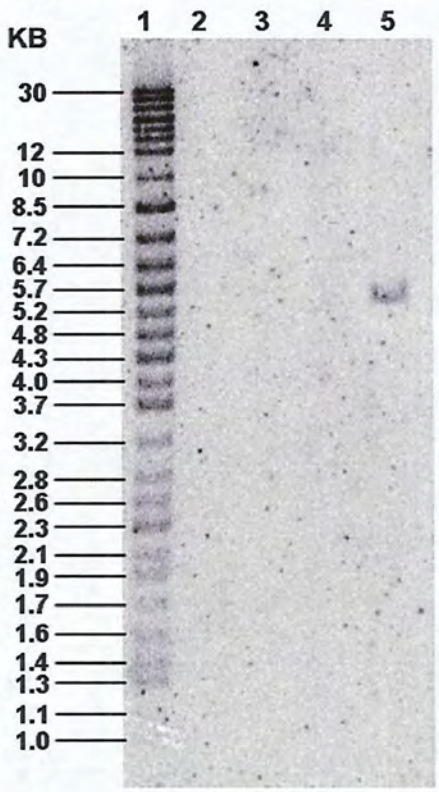
**Figure V-8. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the NOS terminator–specific probe and the restriction enzymes AclI, *Eco*RI, and *Kpn*I + *Bsr*BI**

(A) *AclI*



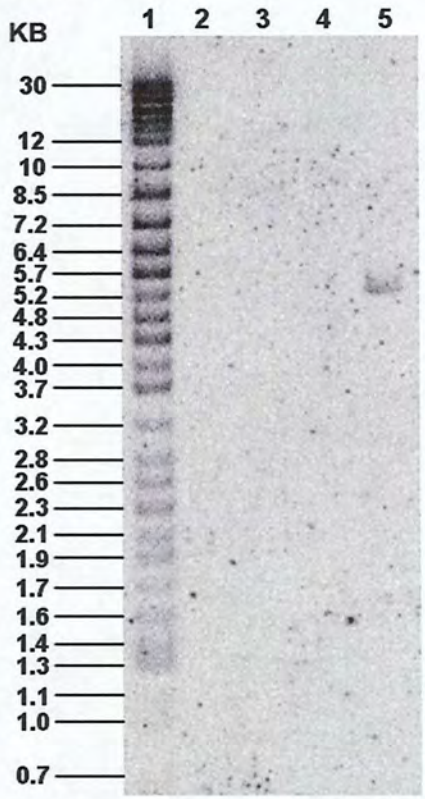

Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *AclI*
Lane 4 = 'Jack' digested with *AclI*
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *KpnI* + *PmeI* plus 'Jack' digested with *AclI*)

(B) *PflMI*




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

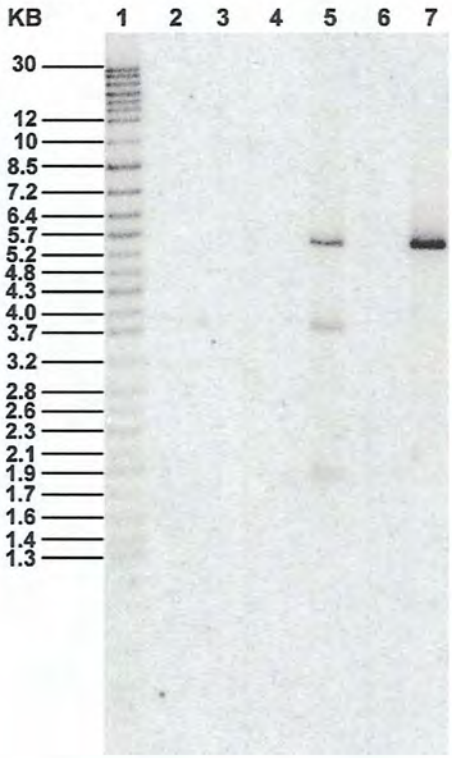
(C) *KpnI* + *BsrBI*




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

**Figure V-9. Functional element copy number Southern blot analysis of SYHT0H2 soybean with the FMV enhancer–specific probe and the restriction enzymes *AclI*, *PflMI*, and *KpnI* + *BsrBI***

(A) *AclI*

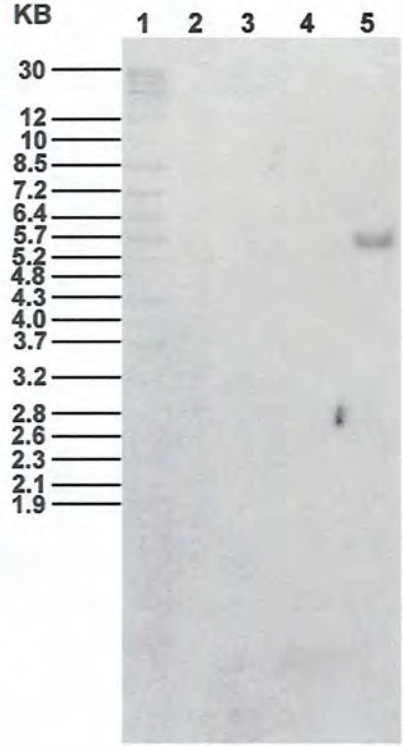



Lane 1 = molecular-weight markers
Lane 2 = blank
Lane 3 = SYHT0H2 T4 digested with *AclI*
Lane 4 = 'Jack' digested with *AclI*
Lane 5 = positive control (14.87 pg of pSYN15954 digested with *KpnI* + *PmeI* plus 'Jack' digested with *AclI*)
Lane 6 = blank
Lane 7 = positive control without 'Jack' (14.87 pg of pSYN15954 digested with *KpnI* + *PmeI*)

(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 *KpnI* + *PmeI* plus 'Jack' digested with *Pfl*MI)

(C) *KpnI*




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

**Figure V-10. Southern blot analysis of SYHT0H2 soybean with the pSYN15954 plasmid-backbone-specific probe and the restriction enzymes *AclI*, *Pfl*MI, and *KpnI***

## V.C. Genetic Stability of SYHT0H2 Soybean Over Three Generations

Southern blot analyses were performed to demonstrate the genetic stability of the SYHT0H2 insert over three generations. 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 III-2) 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 V-11 shows the locations of the T-DNA–specific probes and restriction enzymes in the T-DNA region of the SYHT0H2 transformation plasmid pSYN15954. Figure V-12 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 V-2 shows the expected and observed numbers and sizes of the hybridization bands, and Figure V-13 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 *Eco*RI, the lanes containing DNA from SYHT0H2 $T_4$, $T_5$, and $T_6$ soybean (Table V-2; Figure V-13A, 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 V-2; Figure V-13B, 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 V-2; Figure V-13C, 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 V-13A 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.

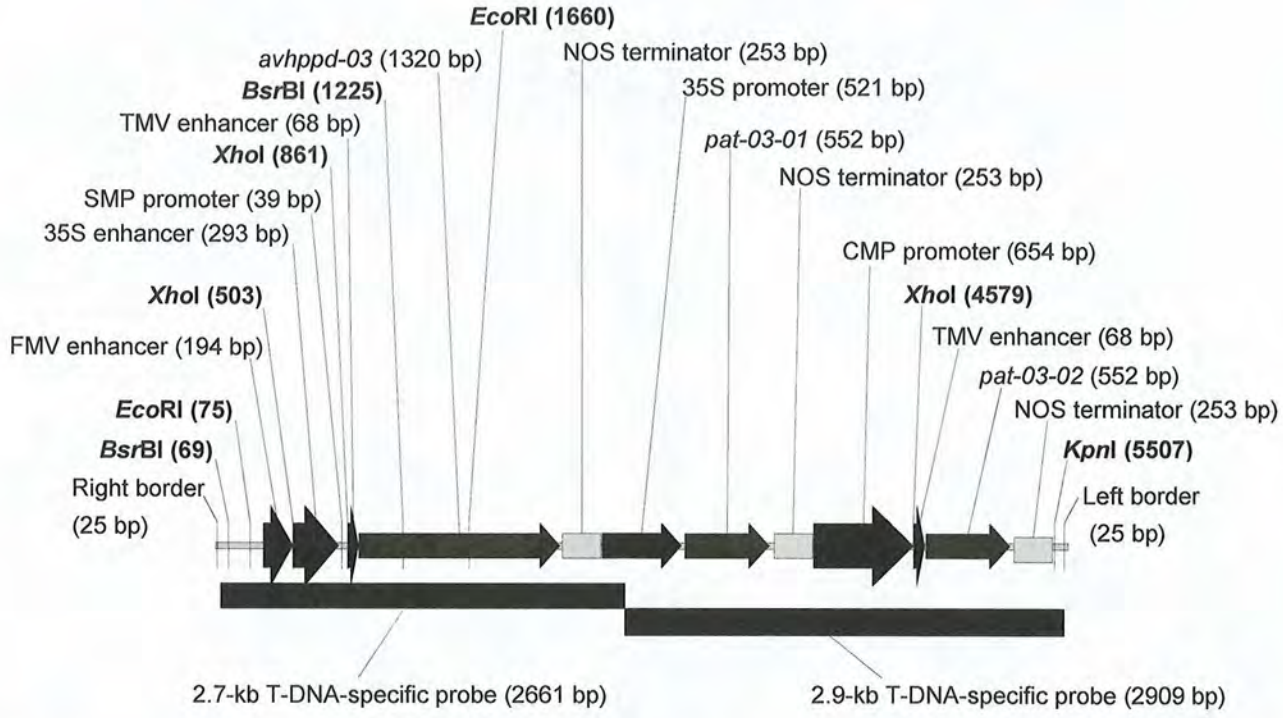



**Figure V-11. Locations of the 2.7- and 2.9-kb T-DNA–specific probes and the restrictions sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in the pSYN15954 transformation plasmid**

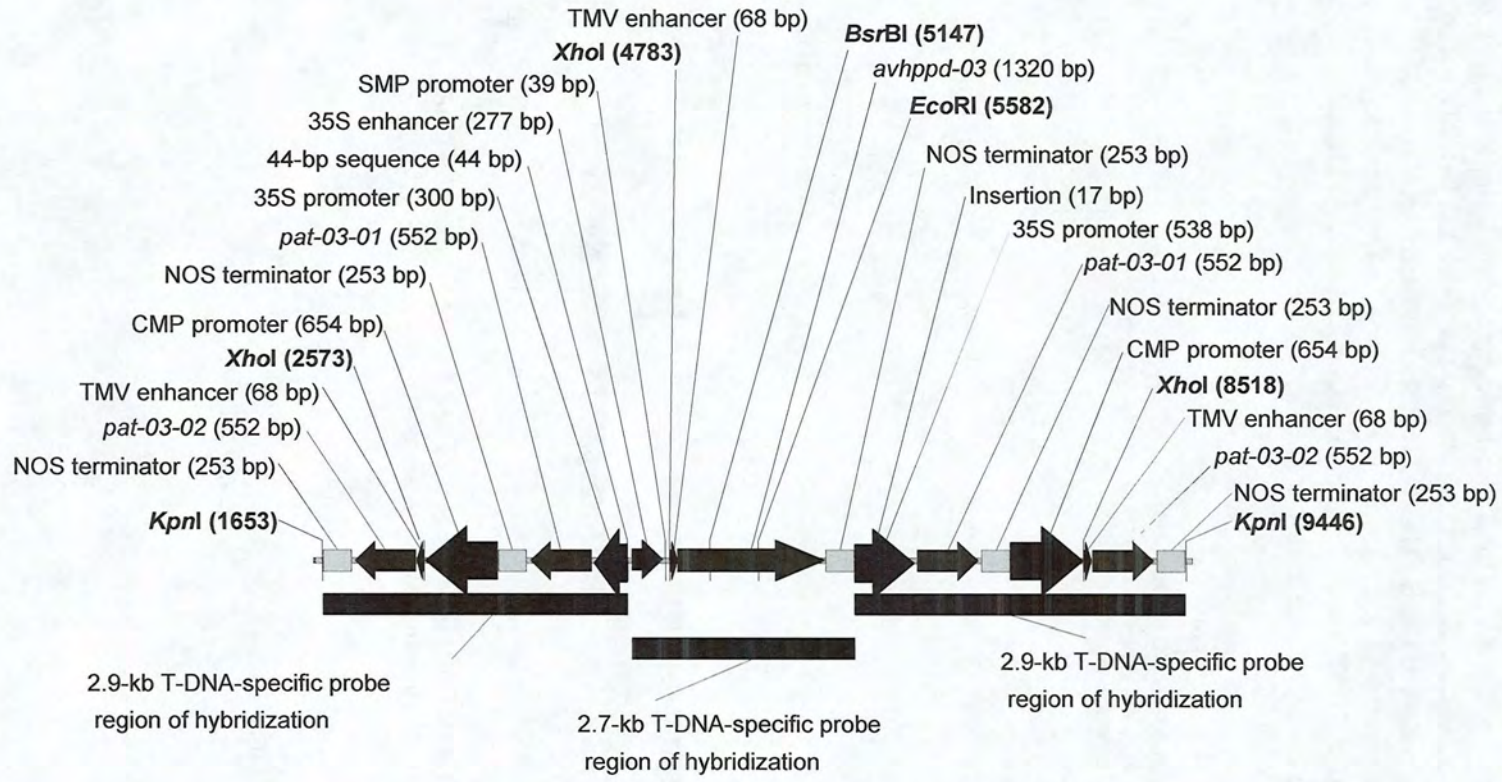
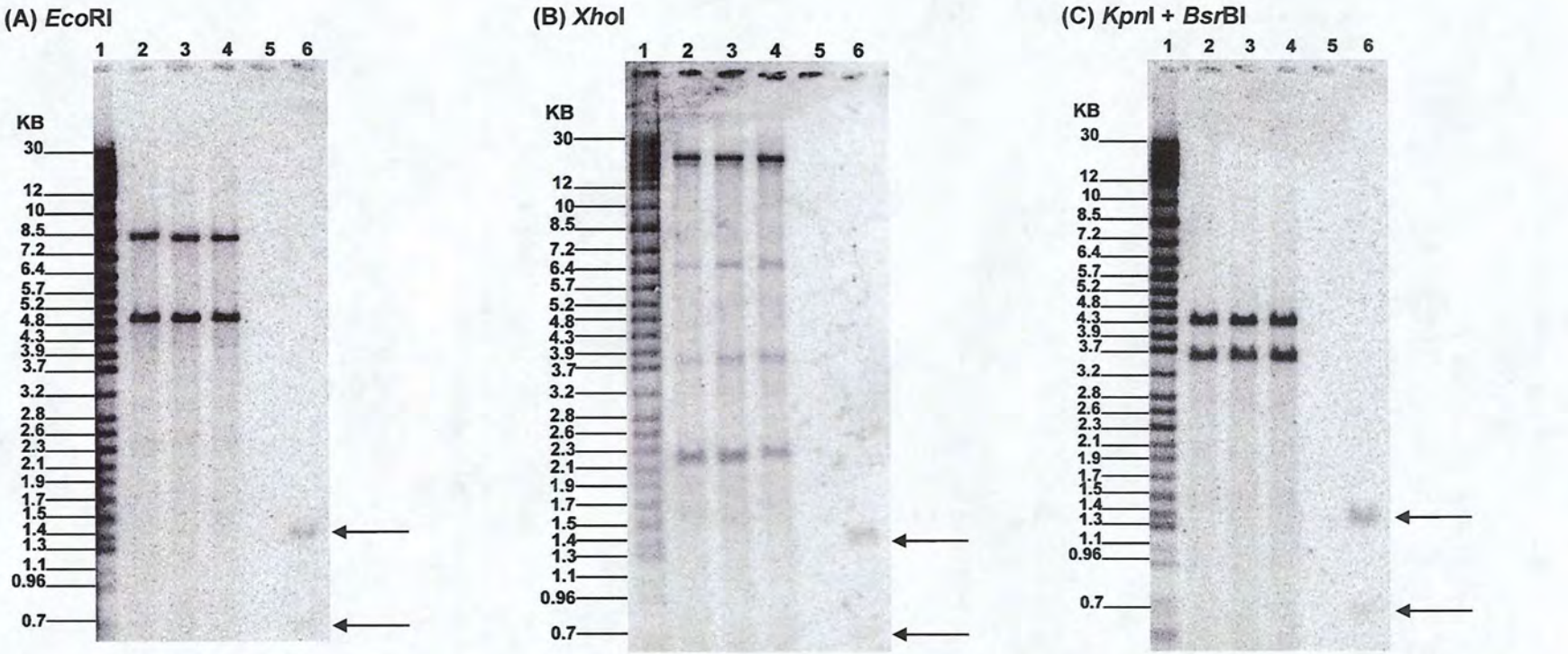



**Figure V-12. Locations of the 2.7- and 2.9-kb T-DNA–specific-probes and the restriction sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in the SYHT0H2 soybean insert**

**Table V-2. 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 | 2 | ~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 | 2 | ~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 of each of the elements in the soybean genome) plus 'Jack' digested with the indicated enzyme.




Lane 1 = molecular-weight markers
Lane 2 = SYHT0H2 T4 digested with *Eco*RI
Lane 3 = SYHT0H2 T5 digested with *Eco*RI
Lane 4 = SYHT0H2 T6 digested with *Eco*RI
Lane 5 = 'Jack' digested with *Eco*RI
Lane 6 = positive control (0.89 pg of CMP promoter–specific and 1.80 pg of *avhppd-03*–specific DNA fragments plus 'Jack' digested with *Eco*RI)

Lane 1 = molecular-weight markers
Lane 2 = SYHT0H2 T4 digested with *Xho*I
Lane 3 = SYHT0H2 T5 digested with *Xho*I
Lane 4 = SYHT0H2 T6 digested with *Xho*I
Lane 5 = 'Jack' digested with *Xho*I
Lane 6 = positive control (0.89 pg of CMP promoter–specific and 1.80 pg of *avhppd-03*–specific DNA fragments plus 'Jack' digested with *Xho*I)

Lane 1 = molecular-weight markers
Lane 2 = SYHT0H2 T4 digested with *Kpn*I + *Bsr*BI
Lane 3 = SYHT0H2 T5 digested with *Kpn*I + *Bsr*BI
Lane 4 = SYHT0H2 T6 digested with *Kpn*I + *Bsr*BI
Lane 5 = 'Jack' digested with *Kpn*I + *Bsr*BI
Lane 6 = positive control (0.89 pg of CMP promoter–specific and 1.80 pg of *avhppd-03*–specific DNA fragments plus 'Jack' digested with *Kpn*I + *Bsr*BI)

**Figure V-13. 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*B**

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

## V.D. Mendelian Inheritance of the DNA Insert

Three generations of Event SYHT0H2 soybean were individually analyzed for the presence of *avhppd-03* and *pat* by real-time PCR analysis (Ingham *et al.* 2001). 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 analyzed in this study ($F_2$, $BC2F_2$, and $BC3F_2$, as shown in the pedigree diagram, Figure III-2); 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 V-3 and for *pat* in Table V-4. The critical value for rejection of the hypothesis of segregation according to Mendelian inheritance at $\alpha = 0.05$ 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 V-3. 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 |
| $\chi^2$ | 1.83 | | 0.87 | | 0.15 | |

**Table V-4. 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 |
| $\chi^2$ | 1.83 | | 0.87 | | 0.15 | |

### V.E. Sequence Analysis to Determine whether the SYHT0H2 Insert Interrupted a Known Soybean Gene

PCR analysis (as described in Appendix B) was used to determine (1) the genomic sequences flanking the 5′ and 3′ end of the SYHT0H2 insert and (2) the genomic sequence in nontransgenic 'Jack' soybean at the point of integration of the SYHT0H2 insert. Sequence analysis of the SYHT0H2 insertion site demonstrated that 15 bp of soybean genomic sequence were deleted when the SYHT0H2 insert integrated into the soybean genome. In addition, 7 bp present in the 3′ flanking region adjacent to the SYHT0H2 insert did not align to the sequence of the nontransgenic soybean genome at the insertion site. Such insertions are often observed during *A. tumefaciens*–mediated transformation and have been called "filler" DNA (Chilton and Que 2003, Windels *et al.* 2003, Tzfira *et al.* 2004).

The genomic flanking sequences were screened for similarity with DNA sequences found in public databases. This comparison provided an indication of whether the SYHT0H2 insert disrupted any known endogenous soybean gene. Sequence similarity analyses were performed with the Basic Local Alignment Search Tool for Nucleotides (BLASTN) program, v. 2.2.19 (Altschul *et al.* 1997). The sequences were compared with the DNA sequences in the latest version of the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (nr/nt) database (NCBI 2012) and with sequences in the latest version of the PlantGBD Viridiplantae expressed sequence tags (EST) database (PlantGDB 2012).

The NCBI nr/nt database contains all nucleotide sequences from the National Institutes of Health genetic sequence database (GenBank), the NCBI Reference Sequence Collection (RefSeq), the European Molecular Biology Laboratory, and the DNA Data Bank of Japan, together with nucleotide sequences derived from the three-dimensional structures described in the Brookhaven Protein Data Bank. GenBank is an archival repository of all sequences, whereas the RefSeq database is a non-redundant set of reference standards that includes chromosomes, complete genomic molecules (organelle genomes, viruses, and plasmids), intermediate assembled genomic contigs, curated genomic regions, ribonucleic acids (RNAs), messenger RNAs (mRNAs), and proteins. The nr/nt database does not contain any EST sequences, sequence tagged sites, genome sequence survey sequences, or phase 0, 1, or 2 high-throughput genome sequences. The nr/nt database was updated on March 14, 2012 and contained over 15 million unique sequences.

The PlantGBD Viridiplantae EST database contains the GenBank EST sequences for the Viridiplantae taxon (green plants), generated from sequences extracted from the NCBI EST database (Boguski *et al.* 1993) and classified as belonging to the Viridiplantae taxon through parsing conducted by PlantGDB (Duvick *et al.* 2008). The PlantGBD Viridiplantae EST database was updated on January 26, 2012 and contained over 21 million sequences.

BLASTN analyses were performed on the 1000-bp soybean genomic sequences flanking the 5′ and 3′ ends of the SYHT0H2 insert. The searches identified all alignments to sequences in the NCBI nr/nt and PlantGBD Viridiplantae EST databases with search results yielding an expectation value (*E*-value) of 10 or lower. (Lower *E*-values indicate lower probabilities that sequence similarities occurred by chance.) The parameters for the BLASTN analyses are given in Appendix B (Section B.9.).

The results of the BLASTN analyses of the genomic sequences flanking the SYHT0H2 insert indicated that the insert does not disrupt any known endogenous soybean gene. Details of each BLASTN analysis are described below.

### V.E.1. BLASTN Analyses Using the NCBI nr/nt Database

The BLASTN analyses using the NCBI nr/nt database resulted in numerous alignments to the soybean genomic sequences flanking the SYHT0H2 insert. All alignments were examined, and none of them provided evidence that a known soybean gene was interrupted by the SYHT0H2 insert. Alignments to known endogenous soybean genes would be expected to have the lowest *E*-values in the BLASTN analyses. The ten results with the lowest *E*-values (and therefore the greatest sequence similarity) are discussed below. Analysis of the remaining alignments with higher *E*-values did not change the interpretation of the data and therefore are not discussed in this petition.

In the BLASTN analysis of the soybean genomic sequence flanking the 5′ end of the insert, two of the ten alignments with the lowest *E*-values ($E = 2.1$) were to unannotated soybean genomic sequences (accession nos. AC235173.1 and EF533702.1). The lowest *E*-values (0.13) were for alignments to nucleotide sequences from *Oryza sativa* (rice) (accession nos. AC136229.3 and AC130609.2) and *Mus musculus* (mouse) (accession no. AC093445.4). The five remaining sequences aligned to unannotated genomic sequences from pig, rat, human, and mouse.

In the BLASTN analysis of the soybean genomic sequence flanking the 3′ end of the insert, the ten lowest *E*-values were for alignments to genomic sequences from *Glycine max* and *Glycine soja* (wild soybean) ($E = 1 \times 10^{-66}$ to $3 \times 10^{-64}$). One alignment ($E = 5 \times 10^{-66}$, length = 144 nucleotides) was to intron sequence from a gene encoding *O*-acetylserine(thiol)lyase in *G. soja* (accession no. EF535995.1) (Zhang *et al.* 2008). Another alignment ($E = 7 \times 10^{-65}$) was to a *G. max* sequence predicted to be a protein complex responsible for biogenesis of lysosome-related organelles (accession no. XM_003544873.1) (Falcón-Pérez *et al.* 2002). The remaining eight of the ten highest alignments were to unannotated genomic sequence from *G. max*. Most of the alignments analyzed were to the same region of the 3′ genomic sequence flanking the insert, suggesting that the sequence in this region is repetitive. None of the alignments to soybean genomic sequences were immediately adjacent to the genome–insert junction. Furthermore, none of these alignments corresponded to genes for which alignments were found in the 5′ flanking sequence.

### V.E.2. BLASTN Analyses Using the PlantGDB Viridiplantae EST Database

BLASTN analyses using the PlantGDB Viridiplantae EST database resulted in numerous alignments to the soybean genomic sequences flanking the SYHT0H2 insert. All alignments were examined, and none of them provided evidence that a known soybean gene was interrupted by the SYHT0H2 insert. The ten results with the lowest *E*-values (a measure of the probability that matches between sequences occurred by chance) are discussed below. Analysis of the remaining alignments with higher *E*-values did not change the interpretation of the data and therefore are not discussed in this petition.

In the BLASTN analysis of the soybean genomic sequence flanking the 5′ end of the insert, none of the alignments were to soybean sequences. The lowest *E*-value was for mRNA sequence from

*Zingiber officinale* (ginger) ($E = 0.009$). The remaining highest alignments ($E = 2.3$) were to mRNA sequences from Swingle citrumelo *(Citrus paradisi* × *Poncirus trifoliata),* cowpea *(Vigna unguiculata),* and meadow fescue (*Festuca pratensis).*

In the BLASTN analysis of the soybean genomic sequence flanking the 3′ end of the insert, all of the ten lowest *E*-values were for alignments to soybean mRNA sequences. All alignments were to the same region of the 3′ genomic sequence flanking the insert, suggesting that the sequence in this region is repetitive. It has been noted that gene prediction programs often erroneously label repetitive regions as genes (Bennetzen *et al.* 2004), resulting in inclusion in EST databases of repetitive regions misannotated as genes. None of the alignments to soybean mRNA sequences were immediately adjacent to the genome–insert junction, and no corresponding alignments were found in the 5′ flanking sequence.

### V.F. Analysis of Putative Open Reading Frames

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 encoding AvHPPD-03 and PAT) and 1 putative ORF at the 5′ junction.

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 $1 \times 10^{-5}$ 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 analyzing 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.

### V.G. Summary of the Genetic Characterization of Event SYHT0H2 Soybean

Genetic characterization studies demonstrated that SYHT0H2 soybean contains, at a single locus within the soybean genome, 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. It does not contain any extraneous DNA fragments of these functional elements elsewhere in the SYHT0H2

soybean genome, and it does not contain the the FMV enhancer or plasmid backbone sequence from transformation plasmid pSYN15954.

Nucleotide sequence analysis determined that the SYHT0H2 insert consists of two inverted and truncated copies of the pSYN15954 T-DNA centered on the right border proximal regions. The 5′ copy lacks the right border, the entire *avhppd-03* cassette, part of the 35S promoter, and the left border. The 3′ copy lacks the right border, the FMV enhancer, 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 the gene *avhppd-03* is located between the two copies. Finally, a 17-bp DNA insertion is located in the 35S promoter of the 3′ copy, the last 15 bp of which duplicate the sequence just upstream of this insertion. The results of the Southern blot analyses are consistent with the results of the nucleotide sequence analysis.

Sequence analysis of the SYHT0H2 insertion site demonstrated that 15 bp of soybean genomic sequence were deleted when the SYHT0H2 insert integrated into the soybean genome, and that 7 bp present in the 3′ flanking region adjacent to the SYHT0H2 insert did not align to the sequence of the nontransgenic soybean genome at the insertion site. BLASTN analyses comparing the soybean genomic sequence flanking the SYHT0H2 insert with sequences in public databases indicated that the insert does not disrupt any known endogenous soybean gene.

The observed segregation ratios for *avhppd-03* and *pat* in three generations of SYHT0H2 soybean plants were as expected for a gene inherited according to Mendelian principles. The data indicate that the insert is inherited as a single locus in the soybean nuclear genome. These data and the results of Southern blot analyses of three generations of SYHT0H2 soybean indicate that the transgenic locus is stably inherited during conventional breeding.

Bioinformatic analysis of the DNA sequence in the SYHT0H2 soybean DNA insert and at the insert-to-genomic-DNA junctions was used to identify putative open reading frames identified 47 putative ORFs in the T-DNA sequence (excluding the 5 ORFs encoding AvHPPD-03 and PAT) and 1 putative ORF at the 5′ junction. Of the 47 putative unintended ORFs identified, bioinformatic analysis ruled out the potential for expression of 46. None of the ORF sequences showed relevant biological similarity to any known or putative allergen or toxin.

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## VI. Characterization and Safety of the AvHPPD-03 Protein

The AvHPPD-03 protein produced in SYHT0H2 soybean has been characterized and tested to determine its potential for causing adverse effects in humans and livestock. This process included assessments of the protein's origin and function, mode of action, physicochemical properties, enzymatic activity, and potential toxicity or allergenicity. The concentrations of AvHPPD-03 in soybean tissues also were determined for use in the risk assessment. Details of the materials and methods used in the studies described in this section are provided in Appendices C and D.

### VI.A. Identity and Function of the AvHPPD-03 Protein

Herbicides that competitively inhibit endogenous plant HPPD 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 to 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 VI-1 (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.

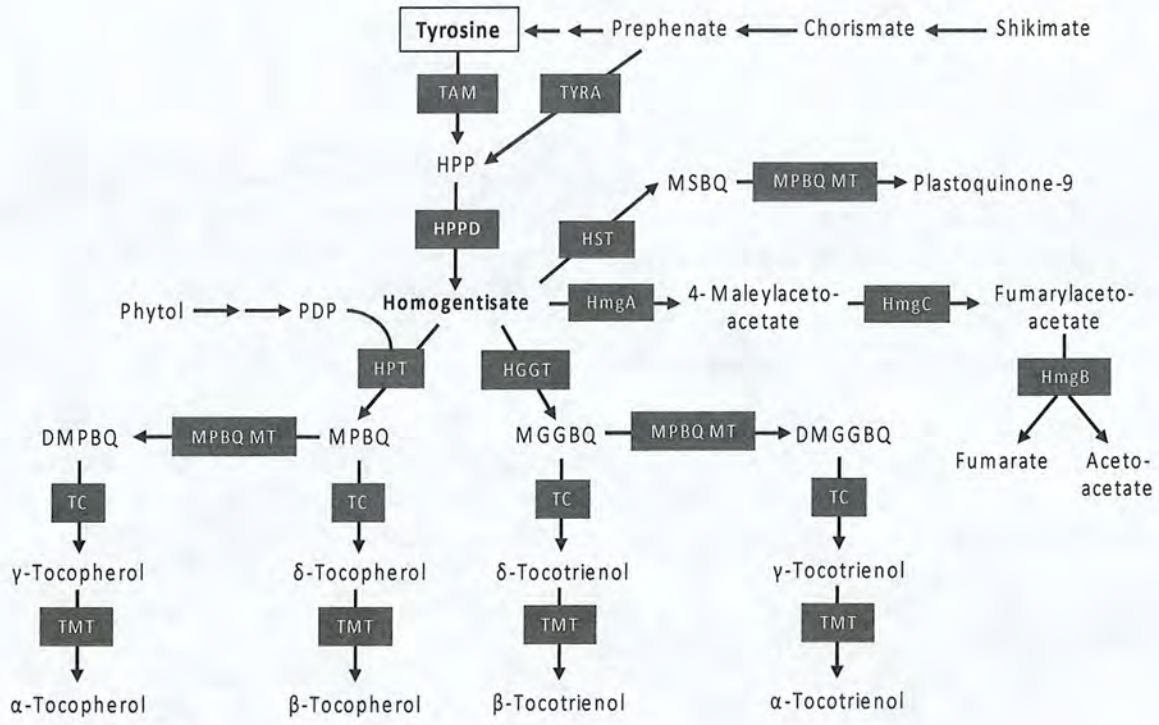


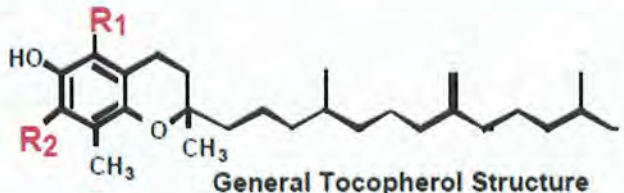

**Figure VI-1. Tyrosine catabolic pathway including HPPD metabolism**

**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

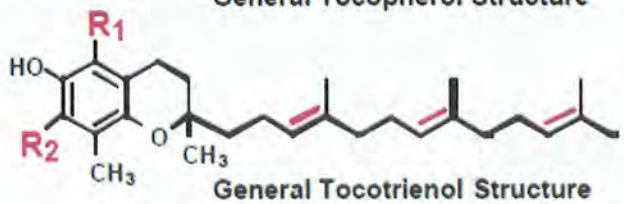
**Enzymes:**
TAM = L-tyrosine aminotransferase
TYRA = chorismate mutase-prephenate dehydrogenase
HPPD = *p*-hydroxyphenylpyruvate dioxygenase
HST = homogentisate solanyltransferase
MPBQ MT = MPBQ methyltransferase
HmgA = homogentisate dioxygenase
HmgB = fumarylacetoacetate hydrolase
HmgC = maleylacetoacetate 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 VI-2). 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 VI-2. 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.

### VI.B. Levels of AvHPPD-03 Protein in SYHT0H2 Soybean Tissues

The concentrations of AvHPPD-03 in various SYHT0H2 plant tissues were quantified via enzyme-linked immunosorbent assay (ELISA). The tissues analyzed were leaves (at four growth stages), roots (at two growth stages), forage, and seed. All growth stages referenced in this petition are as defined in Pedersen (2009). Tissues were collected from SYHT0H2 soybean and a nontransgenic, near-isogenic (control) soybean 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 VI-1). 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, performance of the herbicide-tolerance trait has been demonstrated in replicated efficacy field trials (see Appendix E).

**Table VI-1. 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.

### VI.C. Characterization and Equivalence of Plant-Produced and Microbially Produced AvHPPD-03 Protein

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. 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 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 VI-3). 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 VI-4 and VI-5). 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 VI-6).

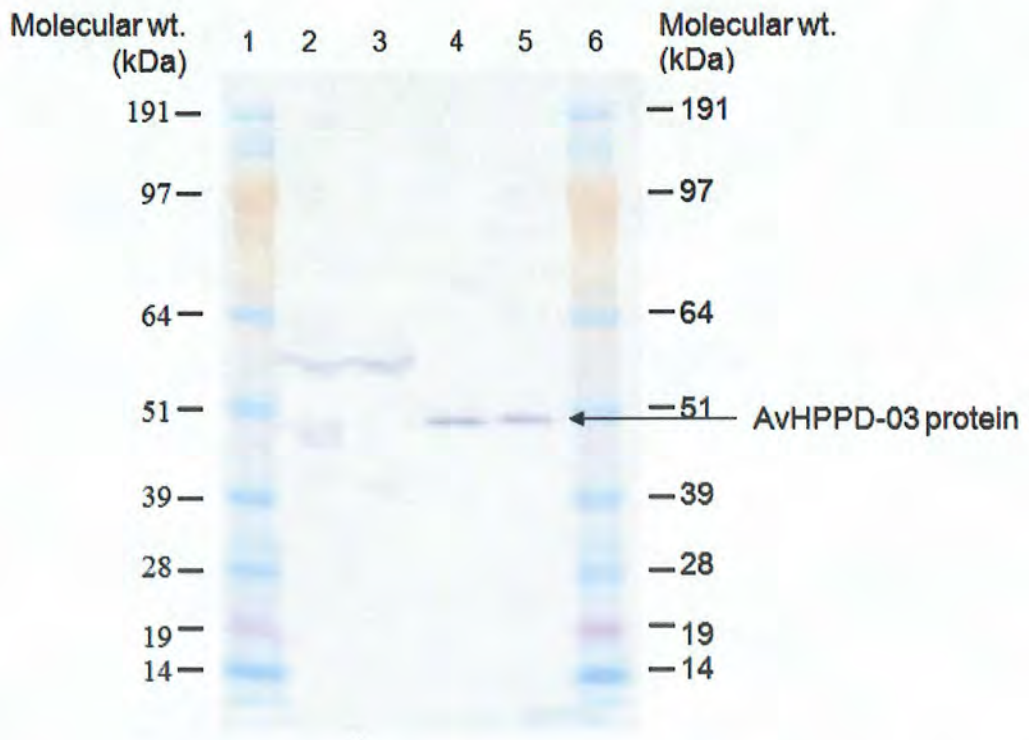



**Figure VI-3. Western blot analysis of plant-produced and microbially produced AvHPPD-03**

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 VI-4. 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 VI-5. 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 VI-6. *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 analyzed to ensure that no post-translational glycosylation of the protein had occurred *in planta*; *E. coli* cannot produce glycosylated proteins. As shown in Figure VI-7, 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 VI-2). 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.

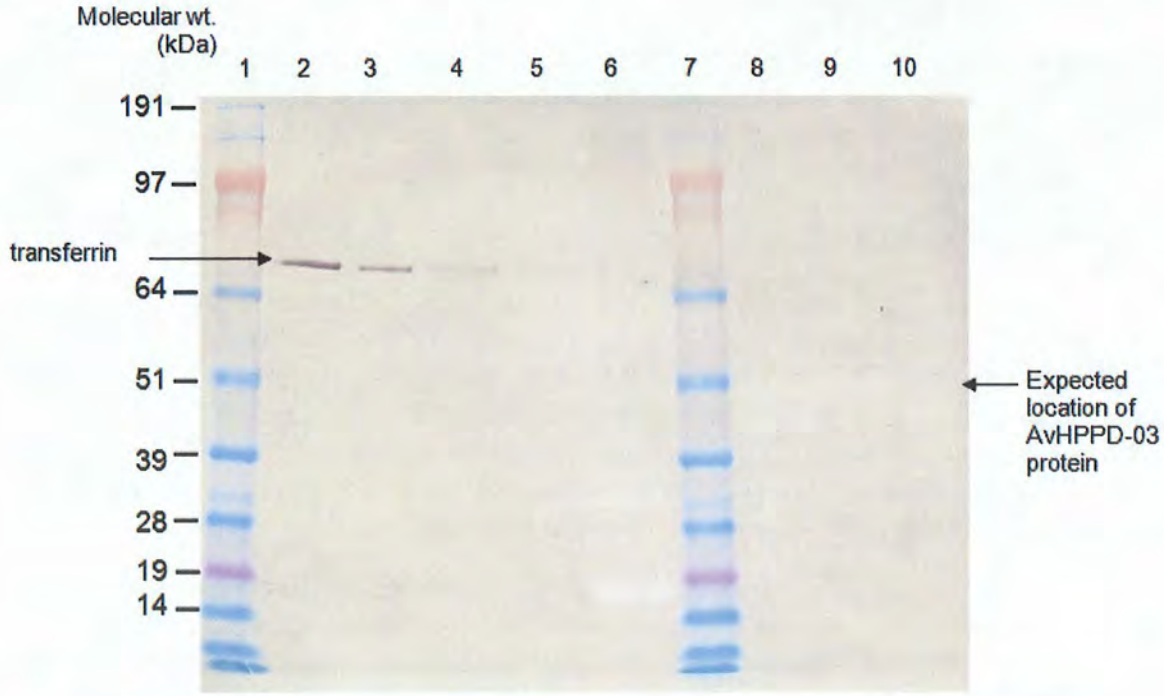



**Figure VI-7. Glycosylation analysis of plant-produced and microbially produced AvHPPD-03**

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 VI-2. 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.

### VI.D. Assessment of AvHPPD-03 Toxicity

The potential toxicity of the AvHPPD-03 protein in SYHT0H2 soybean was evaluated through (1) 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 and (2) a study of the acute oral toxicity of AvHPPD-03 in mice.

#### VI.D.1. Analysis of Amino Acid Sequence Similarity of AvHPPD-03 and Known Toxins

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 HPPDs 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 $1.20 \times 10^{-175}$ 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 VI-3.

**Table VI-3. 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). As discussed in Section IV.A., 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.

### VI.D.2. 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).

A microbially produced test substance containing AvHPPD-03 (72.2% purity w/v) 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, as described in Section VI.C. 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.

#### VI.D.3. 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.

### VI.E. Assessment of AvHPPD-03 Allergenic Potential

Although virtually all food allergens are proteins, only a few of the many proteins found in foods are allergenic, and the probability that a novel protein will become a food allergen is small. Because there is no single definitive test to predict food allergenicity in humans, a weight-of-evidence approach was used to assess the potential allergenicity of AvHPPD-03. This approach is consistent with the recommendations of the Codex Alimentarius Commission (2009). The following types of characterization data were considered for AvHPPD-03 in the weight-of-evidence assessment:

- source
- amino acid sequence similarity to known allergenic proteins
- susceptibility to digestive enzymes
- susceptibility to heat inactivation
- glycosylation status
- relative abundance in the commodity crop

A separate assessment for the allergenic potential of SYHT0H2 soybean with regard to endogenous soybean allergens was also conducted (see Section IX).

#### VI.E.1. Oat as the Source Organism for 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).

### VI.E.2. Analysis of Amino Acid Sequence Similarity of AvHPPD-03 and Known or Putative Allergens

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). 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.

### VI.E.3. 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). 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 (see Section VI.C). 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.

#### VI.E.3.a. *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 VI-8), indicating that AvHPPD-03 was rapidly and completely digested by pepsin.

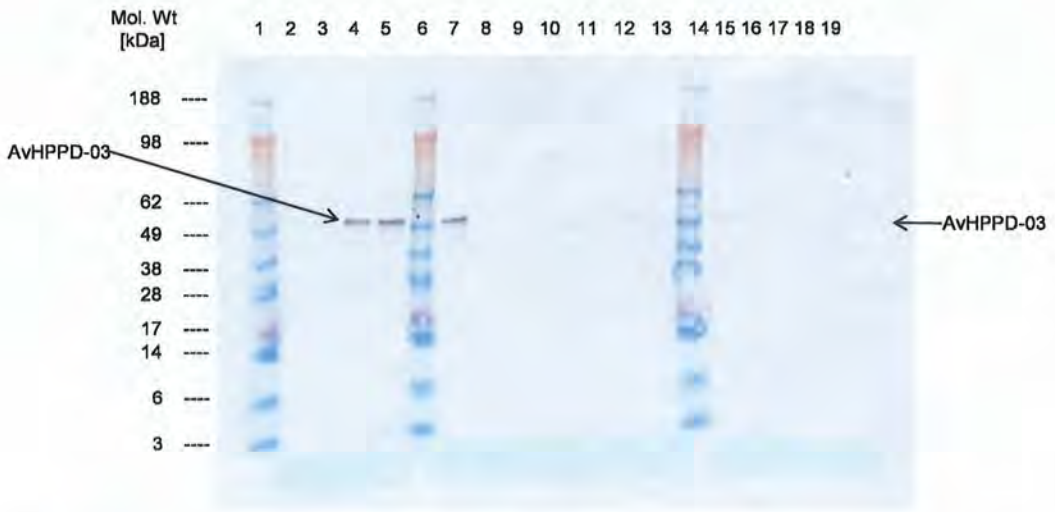



**Figure VI-8. Immunoreactivity analysis by Western blot of AvHPPD-03 following digestion in SGF**

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).

### VI.E.3.b. *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. 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 VI-9), indicating that AvHPPD-03 was completely digested by intestinal proteases within 5 minutes.

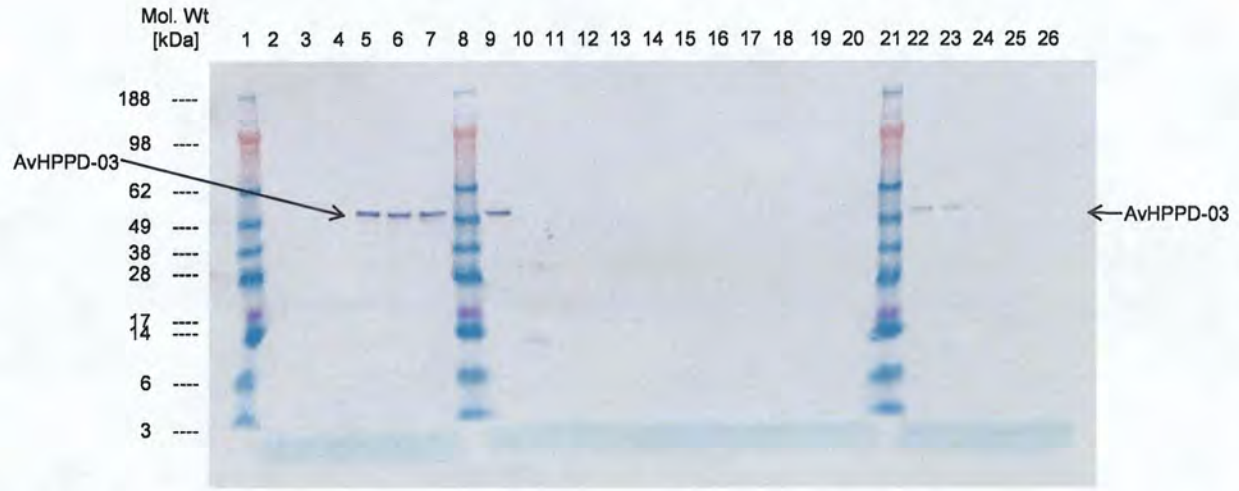



**Figure VI-9. Immunoreactivity analysis by Western blot of AvHPPD-03 following digestion in SIF**

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.

### VI.E.4. Effect of Temperature on the AvHPPD-03 Protein

The effects of temperature on the immunoreactivity and enzymatic activity of AvHPPD-03 were investigated. 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.

### VI.F. Conclusions on AvHPPD-03 Protein Safety

A substantial body of evidence exists to support the safety of the AvHPPD-03 protein, including its status as a safely consumed endogenous protein in oat. HPPD proteins, as a group of biochemically and structurally related proteins, are ubiquitous in commonly consumed food plants and animals, and the level of AvHPPD-03 in SYHT0H2 soybean is low. AvHPPD-03 is not acutely toxic in mice, and bioinformatic analyses demonstrated that AvHPPD-03 has no significant amino acid sequence similarity to known toxins or allergens. The weight of evidence from bioinformatic analysis and assays for digestibility, heat inactivation, and glycosylation status supports the conclusion that AvHPPD-03 is unlikely to be a food allergen. Therefore, it is concluded that AvHPPD-03 is not likely to pose a risk to the health of humans or other mammals through consumption of SYHT0H2 soybean.

### VI.G. References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## VII. Characterization and Safety of the PAT Protein

The PAT protein produced in SYHT0H2 soybean has been characterized and tested to determine its potential for causing adverse effects in humans and livestock. This process included an assessment of the protein's origin and function, mode of action, physicochemical properties, enzymatic activity, and potential toxicity or allergenicity. The concentrations of PAT in soybean tissues also were determined for use in the risk assessment. Details of the materials and methods used in the studies described in this section are provided in Appendices C and D.

### VII.A. Identity and Function of the 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).

### VII.B. Levels of PAT Protein in SYHT0H2 Soybean Tissues

The concentrations of PAT protein in various SYHT0H2 plant tissues were quantified via ELISA. The tissues analyzed 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 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 VII-1). 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, performance of the herbicide-tolerance trait has been demonstrated in replicated efficacy field trials, as summarized in Appendix E.

**Table VII-1. 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 |

### VII.C. Existing Safety Data and History of Safe Exposure

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. 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 U.S. (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 VII-2 (ILSI 2011). A list of transgenic crops containing PAT that have been approved for food and feed use globally is shown in Table VII-3. 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 VII-2. 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* |
| | DAS-Ø6275-8 | | *S. hygroscopicus* |
| | DAS-59122-7 | | *S. viridochromogenes* |

*(continued)*

| Species | Events or crosses | Alternate designations | Source of PAT gene |
|---|---|---|---|
| | 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* |
| | 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 VII-3. 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[b] |
|---|---|---|---|---|---|---|---|
| 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 |
| 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. |

*(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[b] |
|---|---|---|---|---|---|---|---|
| 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.

### VII.D. Characterization and Equivalence of the Plant-Produced and Microbially Produced PAT Proteins

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.

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 VII-1. 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 VII-2 and VII-3). The *N*-terminal sequencing analysis revealed that the plant-produced PAT lacked the *N*-terminal methionine (Figure VII-4).

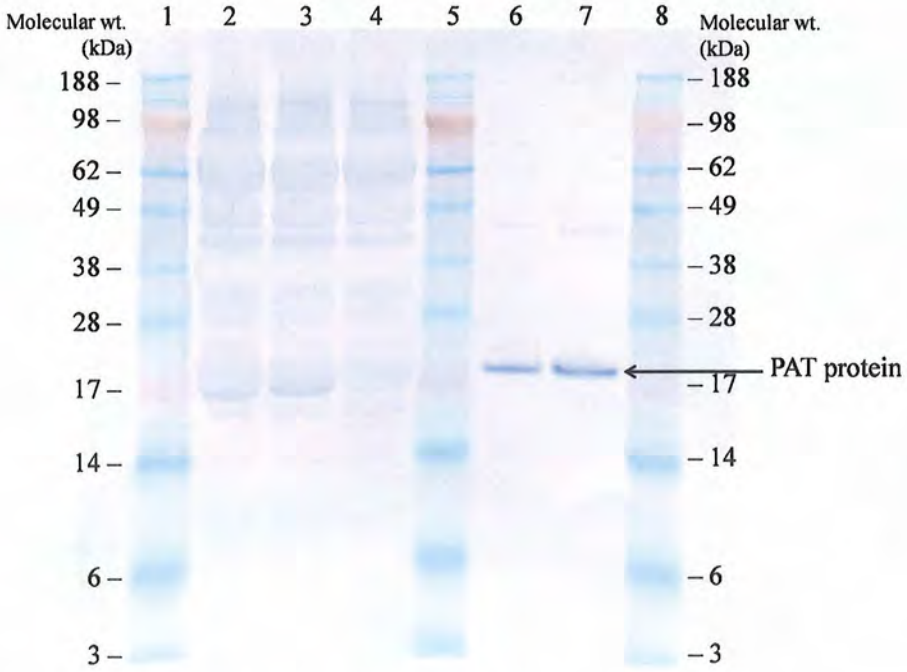


**Figure VII-1. Western blot analysis of plant-produced and microbially produced PAT**

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 VII-2. 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 VII-3. 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 VII-4. *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 analyzed 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 VII-5, 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 VII-4).

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.

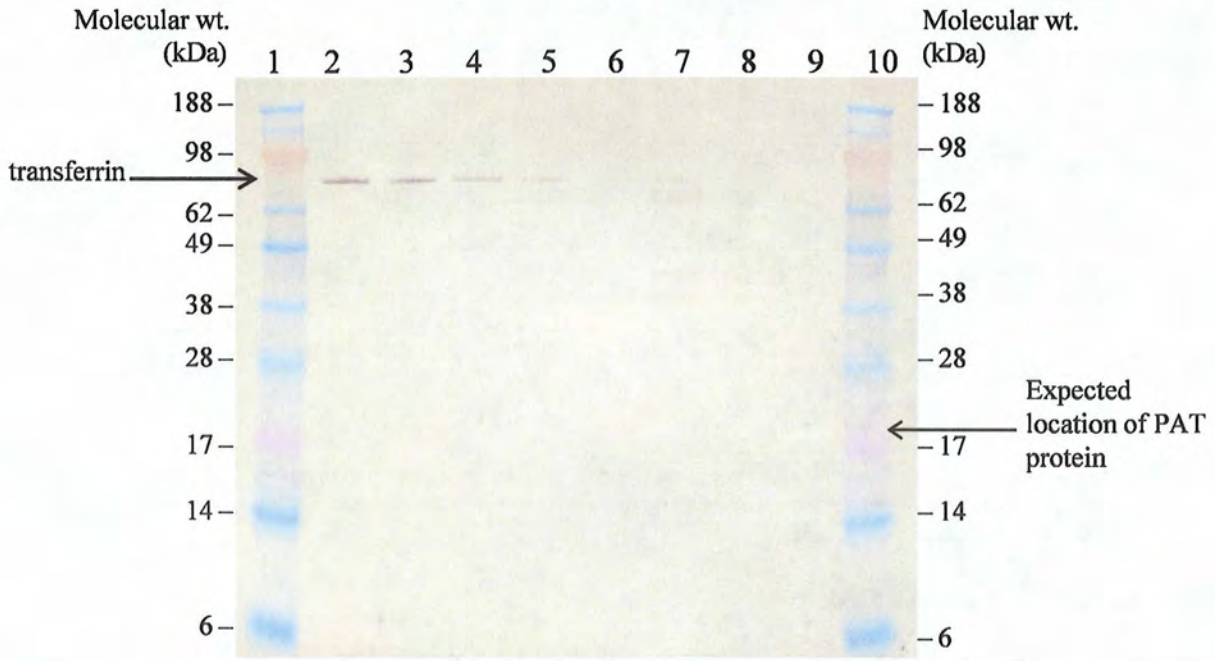


**Figure VII-5. Glycosylation analysis of plant-produced and microbially produced PAT**

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: 1000 ng soybean trypsin inhibitor (negative control)

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 VII-4. 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.

### VII.E. Assessment of PAT Toxicity

The coding sequence of the gene *pat* is derived from a common soil microbe, *S. viridochromogenes*, that is not known to be a pathogen (Kutzner 1981). Acetyltransferase proteins have not been described as toxic to humans or animals and are likely to occur frequently in nature. No adverse health effects have been related to these enzymes.

#### VII.E.1. Analysis of Amino Acid Sequence Similarity of PAT and Known Toxins

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. The results of this updated bioinformatic analysis support previous analyses, including those reported by Hérouet *et al.* (2005).

#### VII.E.2. Acute Toxicity of PAT Protein in Mice

The microbially produced PAT test substance was used to assess acute toxicity in mice (Hérouet *et al.* 2005). OF1 mice (9 weeks old) received an intravenous injection of PAT in 0.9% saline

solution at a dose of 1 or 10 mg/kg b.w., and a negative control group received the dosing vehicle only. The dose volume was 10 ml/kg b.w. This route of administration results in a high degree of bioavailability of the administered test substance and is therefore a very conservative test of acute toxicity. No mortality or systemic effects were observed at either dose level 4 hours or 15 days after administration, demonstrating that PAT was not acutely toxic to mice.

#### VII.E.3. 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.

### VII.F. Assessment of PAT Allergenic Potential

A weight-of-evidence approach (as described in Section VI for AvHPPD-03) was used to assess the potential allergenicity of PAT. The following types of characterization data were considered: source, amino acid sequence similarity to known allergenic proteins, susceptibility to digestive enzymes, susceptibility to heat inactivation, glycosylation status, and relative abundance in the commodity crop (described in Section VII.B, above).

#### VII.F.1. Analysis of Amino Acid Sequence Similarity of PAT and Known or Putative Allergens

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). 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.

#### VII.F.2. Digestive Fate of PAT Protein

The susceptibility of PAT to proteolytic degradation was evaluated in simulated mammalian gastric fluid containing pepsin and in simulated mammalian intestinal fluid containing pancreatin (a mixture of intestinal proteases including trypsin, chymotrypsin, carboxypeptidase, and elastase). The time points used in both analyses were 0, 0.5, 2, 5, 10, 20, 30, and 60 minutes, and the samples were analyzed 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.

### VII.F.3. 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.

### VII.F.4. Conclusions on PAT Allergenicity Potential

The weight of evidence indicates that PAT is unlikely to be a food allergen, because it is derived from a source organism that contains no known allergens; it is not significantly similar in amino acid sequence to any known allergens; and, as expressed in SYHT0H2 soybean, it shows no glycosylation. Furthermore, PAT is produced at very low levels in soybean seed, its enzyme activity is completely inactivated at 55°C, and it is rapidly degraded in simulated mammalian gastric and intestinal fluids. Together, this evidence shows that PAT is not similar to known allergens, that little or no dietary exposure to intact PAT protein would occur in humans or other mammals via consumption of SYHT0H2 soybean, and that PAT is unlikely to be allergenic.

### VII.G. Conclusions on PAT Protein Safety

The safety of the PAT protein was previously established, and additional, updated information is provided in this petition. This summary of safety assessment conclusions is based on existing PAT safety data summarized by Hérouet *et al.* (2005), new bioinformatic studies, and a new study demonstrating the equivalence of the microbially produced PAT test substance to PAT produced in SYHT0H2 soybean. PAT has a long history of safe use in transgenic food crops and a very specific and well-characterized mode of action; it is not acutely toxic, and it has no characteristics consistent with potential allergenicity. Updated bioinformatic comparisons showed no significant amino acid sequence similarity between PAT and any known toxins or allergens. It is concluded that PAT does not pose a risk to the health of humans or other mammals through consumption of SYHT0H2 soybean.

### VII.H. References

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## VIII. Compositional Assessment of SYHT0H2 Seed and Forage

The major soybean commodity products are seed, oil, and meal. A bushel (60 lb) of soybeans yields about 48 lb of protein-rich meal and 11 lb of oil (ASA 2012). Unprocessed soybeans are not suitable for food, and their use for animal feed remains limited, because they contain antinutritional factors such as trypsin inhibitors and lectins. However, adequate heat processing inactivates these factors. Soybean oil is used for human consumption and is a source of glycerol, fatty acids, sterols, and lecithin. Whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans, full-fat soy flour, and traditional soy foods (e.g., miso, soy milk, soy sauce, and tofu). Soybean 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. This chapter describes a study conducted to measure and compare key nutrients and antinutrients in forage and seed from SYHT0H2 and conventional soybean.

### VIII.A. 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 U.S. during 2010. The test plants were SYHT0H2 soybean in the genetic background 'Jack' and the control plants were nontransgenic, near-isogenic soybean variety 'Jack.' Six nontransgenic commercial soybean varieties were also included in the study as references to establish a range of typical values for the components analyzed. The test, control, and reference entries are listed in Table VIII-1.

**Table VIII-1. Identification of test, control, and reference soybean varieties**

| Entry identification | Entry description | Variety | Relative maturity |
|---|---|---|---|
| SYHT0H2 | test | 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 VIII-1. The soil type, previous year's crop, and planting date for each location are listed in Table VIII-2. These trials were planted under USDA notification 10-064-116n.

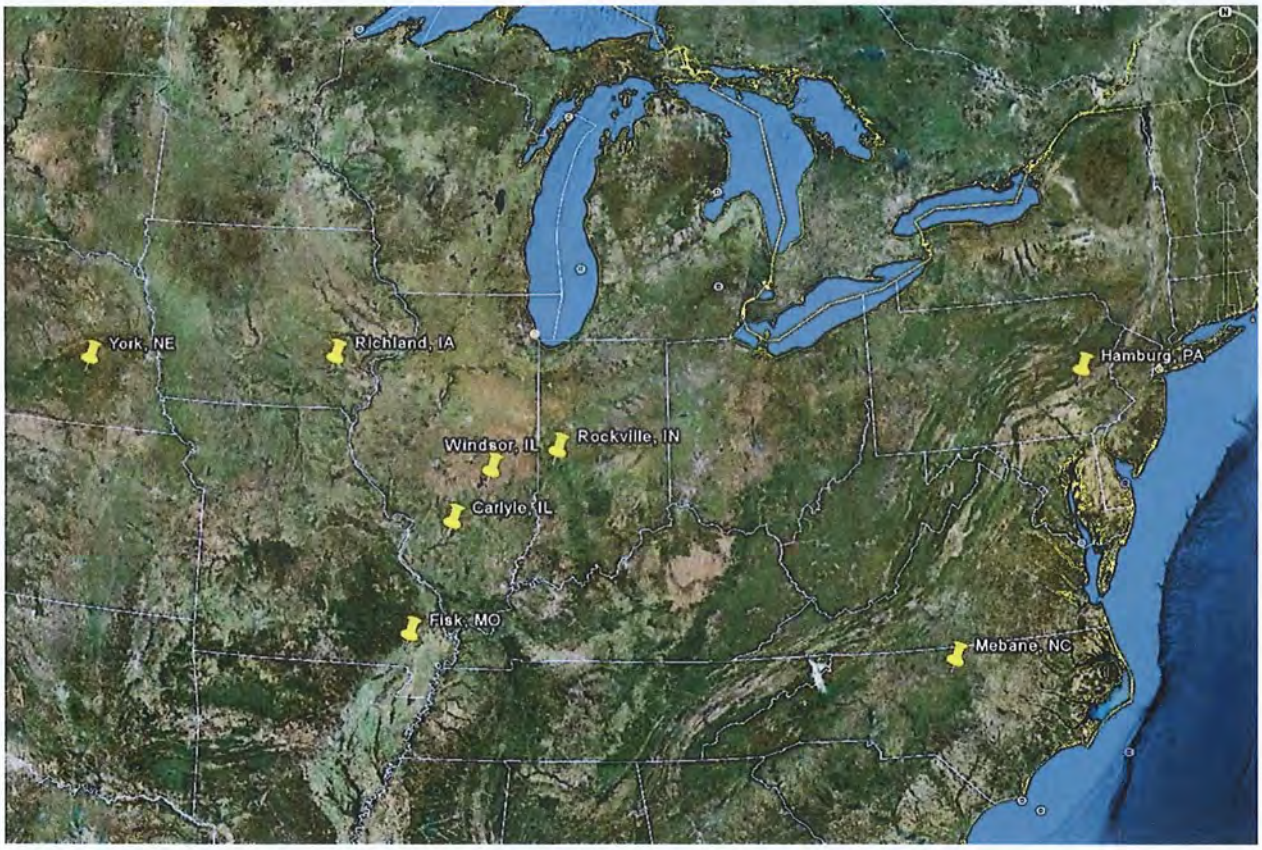


**Figure VIII-1. Satellite view of composition trial locations in the U.S.**
The location designated is the city nearest to the field plots.

**Table VIII-2. 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 analyzed are listed in Table VIII-3. 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 VIII-3. 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.

### VIII.B. 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 components 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).

### VIII.C. Compositional Analysis Results

Sections VIII.C.1 and VIII.C.2 describe the compositional analysis results for SYHT0H2 soybean forage and seed and compares them with the results for the nontransgenic, near-isogenic control soybean, as well as the reference-variety and ILSI database ranges. The conclusions from the compositional analysis are presented in Section VIII.C.3.

#### VIII.C.1. Forage

Across-location means and statistics for the proximate components of forage are shown in Table VIII-4. 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 VIII-4. Proximate composition of forage from SYHT0H2 and 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.

### VIII.C.2. 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.

#### VIII.C.2.a. Proximates, Minerals, and Vitamins

As shown in Tables VIII-5 and VIII-6, 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 VIII-7, 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 VIII-8. 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 VIII-5. Proximate composition of seed from SYHT0H2 soybean and 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 VIII-6. Mineral composition of seed from SYHT0H2 soybean and 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 |
| $N = 192$ | Range | 2050–3860 | 48.0–110 | 1820–3090 | 4200–8570 | 13800–24700 |
| 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 VIII-7. Vitamin composition of seed from SYHT0H2 soybean and 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 |
| $N = 192$ | Range | <LOQ–0.104 | 0.253–1.02 | 0.270–0.532 | 0.224–0.680 | 0.106–0.886 |
| 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 VIII-8. Vitamin E composition of seed from SYHT0H2 soybean and 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 | – | – | – | – |
| *N* = 192 | Range | 0.0115–0.0771 | <LOQ–0.00779 | 0.127–0.236 | 0.0320–0.112 | <LOQ | <LOQ | <LOQ | <LOQ |
| 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 $P < 0.05$.

### VIII.C.2.b. 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 VIII-9. 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 VIII-2.

Of the 22 fatty acids analyzed, 13 were below the LOQ in all replicates of SYHT0H2 soybean; the results for the remaining nine fatty acids are shown in Table VIII-10. 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 VIII-11, 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.

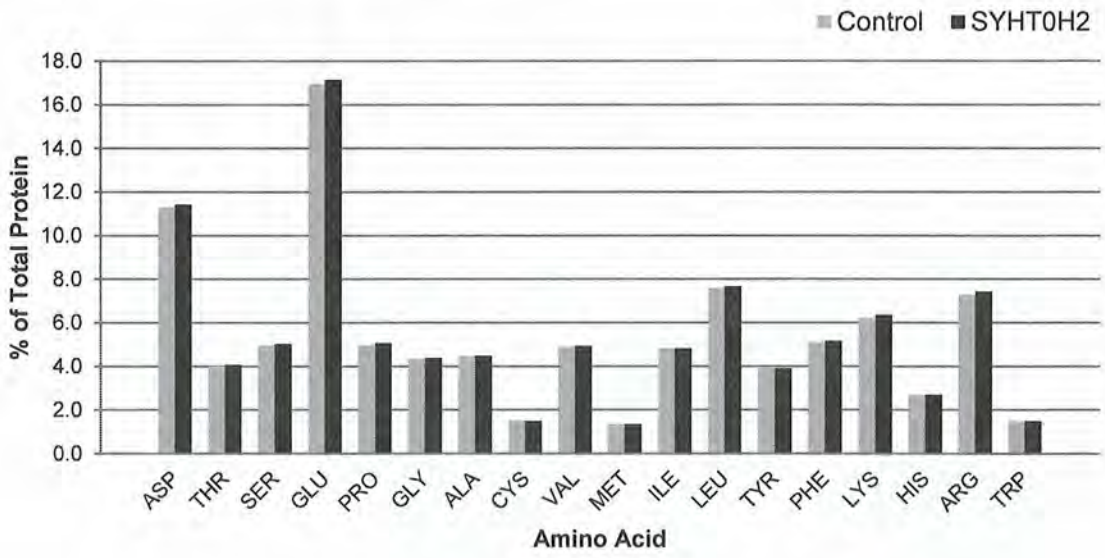


**Figure VIII-2. Amino acid profiles in SYHT0H2 and control soybean seed**

**Table VIII-9. Amino acid composition of seed from SYHT0H2 soybean and 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 VIII-10. Fatty acid composition of seed from SYHT0H2 soybean and control soybean (% 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 VIII-11. Antinutrient composition of seed from SYHT0H2 soybean and 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.

### VIII.C.3. 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.

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## IX. Safety and Nutritional of Assessment of SYHT0H2 Soybean and Derived Soybean Products

The safety of SYHT0H2 soybean and its nutritional comparability to conventional, nontransgenic soybean were assessed through consideration of the compositional assessment described in Section VIII, the safety assessments of the AvHPPD-03 and PAT proteins described in Sections VI and VII, the results of a broiler chicken feeding study, and an endogenous allergen assessment of SYHT0H2 soybean. The latter two studies are described in this section.

### IX.A. Nutritional Assessment of SYHT0H2 Soybean

As discussed in Section VIII, 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.

#### IX.A.1. Broiler Chicken Feeding Study

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. 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.

#### IX.A.2. Conclusions of the Nutritional Assessment of SYHT0H2 Soybean

No biologically important differences in the levels of key nutritional components of forage and seed were observed between SYHT0H2 soybean plants and conventional soybean varieties. In addition, no adverse effects were observed on broiler chickens consuming diets prepared with SYHT0H2 soybean meal in comparison with broiler chickens consuming diets prepared with nontransgenic soybean meal. Therefore, it is concluded that SYHT0H2 soybean and soybean meal processed from raw SYHT0H2 soybeans are nutritionally and compositionally comparable to raw and processed soybean from conventional varieties, and that SYHT0H2 soybean is expected to provide adequate nutrition as part of formulated diets delivered to growing livestock.

### IX.B. Safety Assessment of SYHT0H2 Soybean

As discussed in Sections VI and VII, both AvHPPD-03 and PAT have specific, well-understood modes of action, and both are homologous with proteins in many species to which humans and animals are exposed daily without concern. AvHPPD-03 is derived from a common food crop (oat). PAT has been safely used and consumed in commercially available transgenic crops and has a permanent U.S. EPA tolerance exemption in all crops under 40 CFR 174.522. AvHPPD-

03 and PAT do not share significant amino acid similarity to known toxins and are not acutely toxic in mice. The evidence presented in Sections VI and VII also indicates that AvHPPD-03 and PAT are unlikely to be human allergens. Furthermore, the potential for dietary exposure of humans or other animals to these proteins is negligible, based on their very low levels in SYHT0H2 soybean seed and further reductions in concentration that would occur during processing of soybean seed fractions used in food products and animal feeds. The safety of SYHT0H2 soybean was further assessed in an endogenous allergen study, described below.

#### IX.B.1. Endogenous Allergen Assessment of SYHT0H2 Soybean

Soybean is one of the most commonly implicated sources of food allergy. As part of the overall human safety assessment, SYHT0H2 soybean seed was assessed to determine whether its endogenous allergen content differed from that of a nontransgenic, near-isogenic control soybean (variety 'Jack') or commercially available nontransgenic soybean reference varieties (NE0800097 and NB04024376). Two methods were used to assess endogenous allergen content: (1) Western blotting for qualitative evaluation of human serum immunoglobulin E (IgE) antibody binding to soybean proteins and (2) mass spectrometry for quantitative assessment of the expression of 12 characterized soybean allergen proteins.

Five soybean-reactive human sera were used to assess the patterns of IgE antibody binding to soybean proteins in a Western blot assay. For each of the five sera, SYHT0H2 soybean was compared with the control soybean and two soybean reference varieties. The numbers of bound proteins in extracts of SYHT0H2, control, and reference-variety soybeans were similar. No proteins were present in SYHT0H2 soybean that were not also present in the control soybean, and the banding pattern for SYHT0H2 soybean was most similar to that of the control soybean. The variations observed in IgE binding to SYHT0H2 soybean were consistent with the overall level of variation observed for the other soybean varieties.

Mass spectrometry was used to measure the concentrations of 12 known soybean allergens in the seed of SYHT0H2 soybean, a nontransgenic, near-isogenic control soybean, and 17 soybean reference varieties. The 12 allergens were four glycinin subunits (G1, G2, G3, and G4), glycinin precursor, beta-conglycinin alpha subunit and alpha subunit 2, beta-conglycinin beta subunit, Kunitz trypsin inhibitors 1 and 3, Gly m Bd 28K, and 34 kDa maturing seed protein. To provide an estimate of each allergen's natural range of concentration in conventional soybean, the data for the 17 reference-variety soybeans were used to calculate a tolerance interval for each allergen representing 90% of the observed range in concentrations. In addition, for comparative purposes, a measure of the total allergen content of each soybean variety was calculated as the sum of the concentrations of all 12 allergens.

The concentrations of allergens in SYHT0H2 soybean were similar to those in the nontransgenic control soybean; for 7 of the 12 allergens, the standard errors of the mean allergen concentrations overlapped. For the remaining 5 allergens, the concentration was higher in SYHT0H2 than in the control soybean for 2 allergens and lower for 3 allergens. For 10 allergens, the mean concentrations in SYHT0H2 soybean fell within the tolerance intervals for the reference-variety soybeans. For the remaining 2 allergens, the mean concentration in SYHT0H2 soybean was below the tolerance interval for one and above it for the other; in the latter case, the mean concentration was higher in the control soybean than in SYHT0H2 soybean. The summed

allergen content of SYHT0H2 soybean was similar to that of the control soybean and was within the range of the summed allergen concentrations for the reference-variety soybeans, as shown in Figure IX-1.

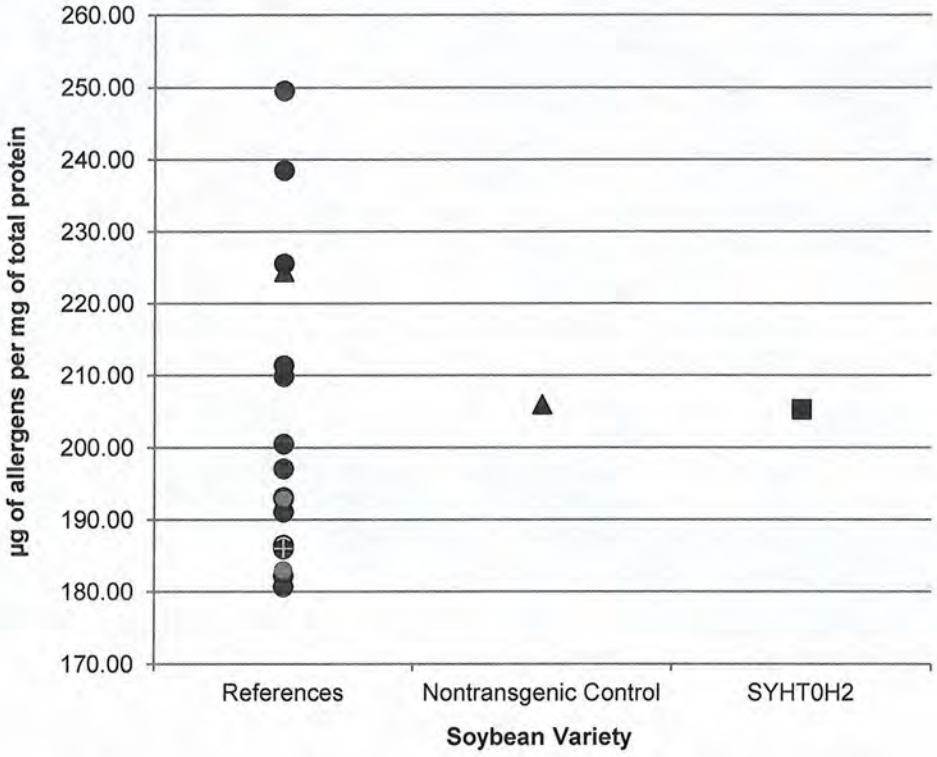


**Figure IX-1. Summed mean concentrations of 12 allergens in each soybean variety**

In summary, the results of the Western blot and mass spectrometric analyses indicate that SYHT0H2 soybean was similar to the nontransgenic, near-isogenic control soybean and to the reference-variety soybeans with respect to IgE binding of human sera to soybean seed proteins, and that SYHT0H2 soybean was most similar to its nontrangenic, near-isogenic counterpart with respect to seed content of known soybean allergens. Taken together, these results indicate that SYHT0H2 soybean is comparable to and as safe as conventional soybean varieties and will not pose greater health risks to soybean-allergic consumers than does nontransgenic soybean.

### IX.B.2. Conclusions of the Safety Assessment of SYHT0H2 Soybean

The proteins AvHPPD-03 and PAT were shown to be nontoxic and unlikely to be allergenic, and the potential for dietary exposure of humans and other animals to these proteins is low. In addition, the allergenicity of SYHT0H2 soybean seed proteins is expected to be comparable to that of nontransgenic soybean. Therefore, it is concluded that SYHT0H2 soybean is as safe as conventional soybean.

### IX.C. SYHT0H2 Nutrition and Safety Conclusions

The data and information presented in this petition support the conclusions that SYHT0H2 soybean is compositionally and nutritionally comparable to and as safe as conventional soybean and that no adverse health effects will result from exposure to either AvHPPD-03 or PAT present in SYHT0H2 soybean.

### IX.D. References


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## X. Phenotypic and Agronomic Characteristics

Field, growth-chamber, and glasshouse studies were conducted to determine whether reproductive, growth, or survival characteristics of SYHT0H2 soybean differed from those of conventional soybean. Replicated field trials were conducted to assess plant growth properties, reproductive capability, survival, seed dispersal, and interactions with environmental stressors. A growth-chamber study measured seed germination and dormancy characteristics, and a glasshouse study examined pollen viability and morphology. Unintended changes in these characteristics could indicate altered plant fitness and pest potential of SYHT0H2 soybean.

These studies employed standard designs and included the nontransgenic, near-isogenic soybean variety 'Jack' as a control. Some studies employed additional nontransgenic commercial soybean varieties as references. The phenotypic characteristics evaluated and the metrics employed are shown in Table X-1.

**Table X-1. Phenotypic characteristics evaluated in SYHT0H2 soybean[a]**

| Characteristic | Variables[1] | Timing[2] | Description |
|---|---|---|---|
| Germination, dormancy, and emergence | germination and dormancy (GC) | after 5, 8, and 13 days | percentages of normal germinated, abnormal germinated, and dormant seed |
| | germination and soil emergence (F) | 14 days after planting, VC–V2 | count of emerged plants |
| Vegetative growth | seedling vigor (F) | VC–V2 | visual estimate of average emerged plant vigor from 1 to 3; 1 = excellent (tall plants with large leaves), 2 = average, and 3 = poor (short plants with small leaves) |
| | plant height (F) | R5–R6 | distance in centimeters from the soil surface to the uppermost node on the main stem from 10 randomly selected plants |
| | lodging (F) | R8 (pre-harvest) | visual estimate of lodging severity from 0 to 9; 0 = no lodging, 1 = 10% of plants lying flat, and 9 = 90% of plants lying flat |
| Reproductive growth | days to 50% flowering (F) | R1–R2 | number of days from the planting date to the date on which approximately 50% of the plants in a plot were flowering |
| | pollen viability (GH) | R1–R2 | percentage of viable pollen cells |
| | pollen morphology (GH) | R1–R2 | observations on cell shape and measured diameter in micrometers of 10 randomly selected cells |
| | flower color (F) | R1–R2 | color of flowers: purple, white, or mixed |
| | days to maturity (F) | R8 | number of days from the planting date to the maturity date |
| | seed test weight (F) | R8 | weight in pounds per bushel of harvested threshed seed |
| | seed moisture (F) | R8 | moisture percentage of harvested shelled seed |
| | yield (F) | R8 | harvested shelled seed yield in pounds per plot; corrected to ~13% moisture and converted to bushels per acre |

*(continued)*

| Characteristic | Variables[1] | Timing[2] | Description |
|---|---|---|---|
| Plant survival | final stand count (F) | R8 (pre-harvest) | number of plants surviving |
| Seed dispersal | pod shattering (F) | R8 (pre-harvest) | visual estimate of shattering severity from 0 to 9; 0 = no shattering and 9 = complete shattering |
| Ecological interactions | insect feeding, disease presence, severe weather (F) | monthly | qualitative observations |

[1]GC = growth-chamber study, F = field trial, and GH = glasshouse study.
[2]Soybean vegetative (V) and reproductive (R) growth stages as defined by Pedersen (2009).

### X.A. Seed Germination and Dormancy

Enhanced germination or seed dormancy can indicate the potential for a plant to be a pest. Most weed seeds undergo periods of dormancy (Ross and Lembi 1985), which contributes to weed persistence. Dormancy mechanisms, including hard seed, vary by species and are generally complex processes. Soybean seeds rarely display dormancy, and soybeans emerge as volunteers only under certain, limited environmental conditions. To evaluate the effects of the introduced genes *avhppd-03* and *pat* on dormancy potential and germination characteristics, these characteristics were compared between seed of SYHT0H2 soybean, the nontransgenic, near-isogenic control soybean variety 'Jack', and three commercial reference varieties of soybean in a growth-chamber study.

The design of this study was modified from the design described in the Association of Official Seed Analysts Rules for Testing Seeds (AOSA 2010a) by the addition of four temperature regimes, to assess germination and dormancy under suboptimal conditions. The test, control, and reference entries are listed in Table X-2. Seed testing was conducted under six temperature regimes:

- Constant temperatures: 10°C, 25°C, 30°C
- Alternating temperatures: 10°C/20°C, 10°C/30°C, 20°C/30°C

Eight replicates of 50 seeds of each entry were tested at each temperature regime. The seeds were not chemically treated and were cleaned prior to study initiation. On Day 0, the seeds were rolled in moistened germination towels and placed in an unlighted, temperature-controlled growth chamber. For the alternating-temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for 8 hours per cycle.

**Table X-2. Identification of test, control, and reference substances**

| Entry identification | Entry description | Variety | Relative maturity |
|---|---|---|---|
| SYHT0H2 | test | 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 |

The seeds were examined on days 5, 8, and 13 except for the seeds tested at 25°C or 20°C/30°C, which were examined on days 5 and 8 (the industry standard for germination testing). The seeds were categorized according to the following criteria:

- normally germinated (exhibiting normal development of all the essential structures, including the root, hypocotyl, and epicotyl)
- abnormally germinated (lacking a well-developed root, hypocotyl, or epicotyl or exhibiting deep lesions or mechanical damage)
- dead (not germinated, visibly deteriorated, and soft to the touch)
- firm swollen (visibly swollen and firm to the touch)
- hard (not having imbibed water and firm to the touch)

On day 5, normally germinated seeds were counted and discarded. On day 8, germinated and dead seeds were counted and discarded. On day 13 (or on day 8 for the seeds tested at 25°C or 20°C/30°C), germinated and dead seeds were counted and removed, and the remaining firm swollen and hard seeds were subjected to the tetrazolium test (AOSA 2010b) for evaluation of viability and categorized as "viable firm swollen," "viable hard," or "dead" (nonviable firm swollen and hard seeds). Seeds that had not germinated but remained viable beyond the incubation period (viable firm swollen and viable hard seed) were considered to have dormancy potential. Seeds that were contaminated or damaged by bacteria or fungi were removed at each evaluation, to avoid contamination of the remaining seeds.

The germination response data for each temperature regime were combined across replicates. The data for SYHT0H2 and the nontransgenic control soybean were assembled into a $2 \times 2$ contingency table of normally germinated seed versus all "not normally germinated" seed, which included seed categorized as abnormally germinated, viable firm swollen, viable hard, or dead. Fisher's exact test was used to compare the germination responses of the SYHT0H2 and control soybean at each temperature regime. Statistical significance was based on an alpha of 0.05. The results are summarized in Table X-3; rates of normal germination are reported as percentages, and the raw data are reported for each category of "not normally germinated" seed ($N = 400$ for each entry at each temperature regime).

**Table X-3. Soybean seed germination (*N* = 400 per entry except as noted)[1]**

| Temp (°C) | Entry | Normally germinated | | Abnormally germinated | Viable firm swollen | Viable hard | Dead |
|---|---|---|---|---|---|---|---|
| | | % | *P* | | | | |
| 10 | SYHT0H2 | 98.75 | 1.000 | 0 | 0 | 0 | 1.25 |
| | Control | 99.00 | | 0 | 0 | 0.50 | 0.50 |
| | Reference varieties | 99.75–100 | | 0 | 0–0 | 0–0 | 0–0.25 |
| 25 | SYHT0H2 | 98.00 | 0.812 | 1.25 | 0 | 0.25 | 0.5 |
| | Control | 97.50 | | 1.25 | 0 | 0.50 | 0.75 |
| | Reference varieties | 91.75–99.00 | | 1.00–5.25 | 0–0 | 0–0 | 0–0.25 |
| 30 | SYHT0H2 | 99.50 | 1.000 | 0 | 0 | 0 | 0.50 |
| | Control | 99.25 | | 0 | 0 | 0 | 0.75 |
| | Reference varieties | 98.00–99.75 | | 0 | 0–0 | 0–0 | 0.25–2.00 |
| 10/20 | SYHT0H2 | 99.50 | 1.000 | 0 | 0.25 | 0 | 0.25 |
| | Control | 99.50 | | 0 | 0.25 | 0 | 0.25 |
| | Reference varieties | 98.50–99.75 | | 0 | 0–0.25 | 0–0 | 0.0–1.25 |
| 10/30 | SYHT0H2 | 99.25 | 0.624 | 0 | 0 | 0.25 | 0.50 |
| | Control | 99.75 | | 0 | 0 | 0 | 0.25 |
| | Reference varieties[2] | 98.50–100 | | 0 | 0–0 | 0–0 | 0–1.50 |
| 20/30 | SYHT0H2 | 96.25 | 0.128 | 2.25 | 0 | 0 | 1.50 |
| | Control | 98.25 | | 1.25 | 0 | 0 | 0.50 |
| | Reference varieties | 91.25–98.75 | | 1.00–4.25 | 0–0 | 0–0 | 0–4.50 |

[1]For the three reference varieties, the range of means is reported.
[2]*N* = 398.

The rate of normal germination did not differ significantly between SYHT0H2 and the nontransgenic control soybean under any of the temperature regimes and was at least 96%. The frequencies of abnormally germinated, viable firm swollen, viable hard, and dead SYHT0H2 soybean seed were comparable to those observed for the nontransgenic control and reference-variety seed. A total of 3 viable firm swollen or viable hard SYHT0H2 soybean seeds were observed, compared with 5 for the nontransgenic control. These results indicate that germination characteristics of SYHT0H2 soybean do not differ from those of conventional soybean and that SYHT0H2 soybean shows no indications of enhanced dormancy potential.

### X.B. Pollen Viability and Morphology

As a measure of potentially enhanced reproductive capability, pollen cell viability and morphology were compared between SYHT0H2 soybean, the nontransgenic, near-isogenic control soybean, and three commercial reference varieties of soybean. The entries were the same as those used in the seed germination and dormancy assessments (shown in Table X-2, above). Thirty plants of each entry were grown in an environmentally controlled glasshouse under a 16- and 8-hour light/dark cycle with daytime temperatures ranging from 21°C to 30°C and nighttime

temperatures from 21°C to 27°C. Flowers were collected from 10 randomly selected plants at the R2 to R3 growth stages over a period of 13 days until at least 15 flowers were obtained from each plant. Pollen was collected and immediately fixed for microscopic examination in a 70% (v/v) ethanol solution. The fixed pollen samples were stained with Lugol's solution (iodine–potassium iodide), which readily binds to starch in viable cells (Pedersen *et al.* 2004).

The percentages of viable and nonviable pollen cells were computed after examination of at least 100 randomly selected cells by light microscopy under 80X to 128X magnification. Pollen grains that were deeply stained, spherical, and turgid (and not burst or injured) were classified as morphologically normal and viable. Pollen grains that were weakly stained (little or no color) were classified as nonviable. Morphology was assessed by visual examination of all grains in the field of view. Cell size was determined as the mean diameter of 10 randomly selected pollen grains under 180X magnification. Student's *t*-test was used to compare the mean percent viability and cell diameter between pollen from SYHT0H2 and control soybean. Statistical significance was based on an alpha 0.05.

The results are shown in Table X-4, which also shows the ranges of the mean values for the three reference varieties. SYHT0H2 and control soybean pollen did not differ significantly in viability or size, and no differences in cell morphology were observed. The mean viability and diameter of SYHT0H2 soybean pollen fell within the ranges for the reference varieties.

**Table X-4. Soybean pollen viability and diameter (*N* = 10 per entry)[1]**

| | Viability (%) | | | Diameter (µm) | | |
|---|---|---|---|---|---|---|
| **Entry** | **Mean** | **SD** | ***P*** | **Mean** | **SD** | ***P*** |
| SYHT0H2 | 98.53 | 0.28 | 0.926 | 22.75 | 0.34 | 0.694 |
| Nontransgenic control | 98.51 | | | 22.81 | | |
| Reference varieties | 97.4–99.4 | | | 20.2–25.4 | | |

[1]SD = standard deviation.

### X.C. Field Agronomic Trials

Field trials were conducted to assess whole-plant growth and agronomic characteristics during the 2010 growing season at eight locations in the U.S. and during the 2010–2011 growing season at ten locations in Argentina. The test substance was SYHT0H2 soybean seed in the genetic background 'Jack' ($T_6$ generation), and the control substance was seed of the nontransgenic, near-isogenic soybean 'Jack.' The locations selected were representative of where soybean is commercially grown and were suitable for planting 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 trial plots were planted in a randomized complete block design with four replicate plots per entry. The plots were either four or 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 (including weed) control. Observations and measurements were taken from rows 2 and 3 of each plot.

The phenotypic characteristics evaluated in these field trials were those routinely examined by soybean breeders and are listed in Table X-1 (above). In addition, the plots were monitored for naturally occurring ecological stressors, including insect damage, disease incidence, and abiotic stress. Stressor observations were made every four weeks after the plants reached the V2 growth stage, and visual estimates were recorded on a scale of 0 to 9 (0 = no stress; 9 = very high stress). Collectively, these observations were suitable for identifying potential differences in competiveness in the environment between SYHT0H2 soybean and the nontransgenic control soybean. The data for six of the characteristics (early and final stand counts, height, test weight, seed moisture, and seed yield) were subjected to analysis of variance (ANOVA) across locations using a mixed-effects model:

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

where $Y_{ijk}$ is the observed response for genotype $i$ in location $j$ block $k$, $U$ is the overall mean, $G_i$ is the fixed effect of genotype, $L_j$ is the random effect of location, $B(L)_{jk}$ is the random effect of block within location, $GL_{ij}$ is the random effect of the location-by-genotype interaction, and $e_{ijk}$ is the residual error. 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 data for the other six characteristics were not statistically analyzed, because they did not satisfy the assumptions for ANOVA. Flower color is a categorical variable. The measurements of seedling vigor, days to flowering, lodging, pod shattering, and days to maturity had very limited ranges of values, particularly within locations, and would not provide a valid estimate of experimental error. An across-location mean was computed for each of these characteristics. Observations of naturally occurring ecological stressors and their impacts on the test and control plants were qualitative and were not quantified.

### X.C.1. Field Trials Conducted in the U.S.

A satellite view of the U.S. trial locations planted in 2010 is shown in Figure X-1. The soil type, previous year's crop, and trial planting date for each location are listed in Table X-5. These trials were planted under USDA notification 10-064-116n.

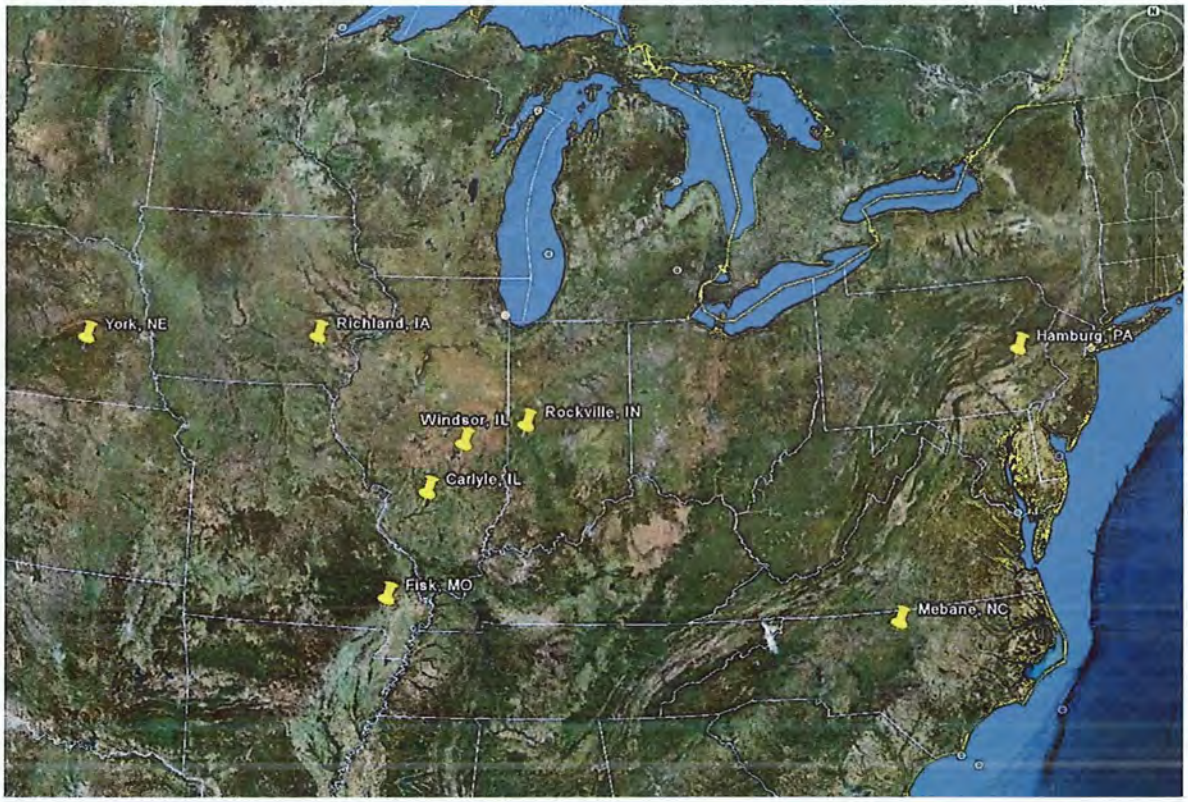


**Figure X-1. Satellite view of agronomic trial locations in the U.S.**
The locations indicated are the cities nearest to the field plots.

**Table X-5. Field location soil types, cropping histories, and planting dates**

| 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 results for the six phenotypic characteristics that were analyzed by ANOVA are summarized in Table X-6. Yield measured in pounds per plot was mathematically converted to bushels per acre. None of these characteristics differed significantly between SYHT0H2 and control soybean. The results for the remaining six phenotypic characteristics are summarized in Table X-7. The percent differences between the mean values for SYHT0H2 and control soybean did not exceed 2.5% except for a 15% difference in pod shattering, which was lower for SYHT0H2 than for control soybean. The mean pod shattering ratings for both entries were very low (<1 on a scale of 0 to 9), and decreased shattering potential does not indicate increased plant pest potential.

The observations of naturally occurring ecological stressors and their impacts on the trial entries at each location are summarized in Table X-8. No differences in the type, incidence, or severity of insects or disease were observed between the SYHT0H2 and control soybean plots, and no differences in plot responses to these biotic stressors or to severe or unusual climatic events were observed.

**Table X-6. Results of ANOVA comparisons between characteristics of SYHT0H2 and control soybean in agronomic field trials in the U.S. (*N* = 32)**

| Characteristic | Mean (range) SYHT0H2 | Mean (range) Control | SEM | % Difference | *P* |
|---|---|---|---|---|---|
| Early stand count | 158 (106–198) | 156 (77–185) | 9.2 | 1.2 | 0.703 |
| Final stand count | 145 (110–183) | 144 (72–178) | 6.7 | 0.7 | 0.918 |
| Height (cm) | 91.1 (48–126) | 88.7 (55–127) | 7.22 | 2.7 | 0.453 |
| Test weight (lb/bu) | 56.4 (51–58.6) | 56.7 (50.9–59) | 0.57 | –0.5 | 0.505 |
| Seed moisture (%) | 9.00 (5.4–12.9) | 8.96 (5.5–13.4) | 0.722 | 0.4 | 0.803 |
| Seed yield (lb/plot)[a] | 3.77 (1.54–5.8) | 3.52 (1.64–5.8) | 0.360 | 7.1 | 0.203 |
| Seed yield (bu/ac)[b] | 36.5 (14.9–56.1) | 34.1 (15.9–56.1) | N/A | 7.0 | N/A |

[a]Measured value corrected to 13% moisture.
[b]Value computed from pounds per plot. N/A = not applicable (ANOVA not conducted).

**Table X-7. U.S. field trial results for soybean characteristics not subjected to ANOVA (*N* = 32)**

| Characteristic | Mean (range) SYHT0H2 | Mean (range) Control | % Difference |
|---|---|---|---|
| Seedling vigor rating | 1.66 (1–3) | 1.63 (1–3) | 1.8 |
| Days to 50% flowering | 42.0 (30–67) | 42.5 (31–67) | –1.2 |
| Flower color | white | white | N/A[a] |
| Lodging rating | 1.63 (0–8) | 1.59 (0–9) | 2.5 |
| Pod shattering | 0.344 (0–4) | 0.406 (0–3) | –15.3 |
| Days to maturity | 97.6 (71–123) | 97.3 (71–123) | 0.3 |

[a]Not applicable.

Table X-8. Qualitative observations from U.S. trials of plot interactions with biotic and abiotic stressors

| Location | Both SYHT0H2 and control soybean | SYHT0H2 only | Control only |
|---|---|---|---|
| Carlyle, IL | Minimal damage from bean leaf beetles and green clover worms at V6–R1, whiteflies at R4, grasshoppers at R6. Minimal frogeye leaf spot at R6. | No abiotic stress. | Mild stress in one plot due to excess water. |
| Fisk, MO | Minimal damage from salt marsh caterpillars and grasshoppers at V2–R6, stink bugs at R3 and R8. No disease or abiotic stress. | — | — |
| Hamburg, PA | Mild to moderate herbicide damage from Basagran (ai: bentazon) at V2–V6. Minimal damage from whiteflies and leafhoppers at R1–R2. Minimal to mild powdery mildew at R3. Minimal to mild damage from drought at R6–R7. | — | — |
| Mebane, NC | Fields clean, no insect damage or disease stress. Mild drought stress at R6. | — | — |
| Richland, IA | Minimal damage from Japanese beetles, bean leaf beetles, and green clover worm at V8–V9. Minimal to mild brown and cercospora leaf spot at V8–V9, minimal frogeye leaf spot at V8–R6. Minimal stress from soil compaction at V8–V9. | — | — |
| Rockville, IN | Minimal damage from bean leaf beetles at V2–R7, grasshoppers at R3–R7. Minimal septoria leaf spot at R3–R7, frogeye leaf spot at R3. Minimal stress from wind at V2. | — | — |
| Windsor, IL | Minimal damage from soybean loopers at R5. No disease or abiotic stress. | — | — |
| York, NE | Fields clean; no insect damage, disease, or abiotic stress. | — | — |

### X.C.2. Field Trials Conducted in Argentina

A satellite view of the Argentine trial locations planted for the 2010–2011 season is shown in Figure X-2. The soil type, previous year's crop, and trial planting date for each location are listed in Table X-9.

The results for the six phenotypic characteristics that were analyzed by ANOVA are summarized in Table X-10. Significant reductions in early stand count, final stand count, plant height, and yield for SYHT0H2 soybean were observed. The cause of the 17.9% reduction in early stand count is unknown, and the significant reductions in final stand count and yield stem from the low early stand count. The average 4-cm reduction in plant height for SYHT0H2 soybean in the Argentina trials is of no consequence from an agronomic or plant pest potential perspective.

The results for the remaining six phenotypic characteristics are summarized in Table X-11. The differences between the mean values for SYHT0H2 and control soybean were less than 10% except for a 32% difference in pod shattering, which was higher for SYHT0H2 soybean than for the control soybean. The mean pod shattering ratings for both entries were low (<2 on a scale of 0 to 9). As shown in Table X-12, no differences in the type, incidence, or severity of insects or disease were observed between the SYHT0H2 and control soybean plots, and no differences in plot responses to these biotic stressors or to severe or unusual climatic events were observed.

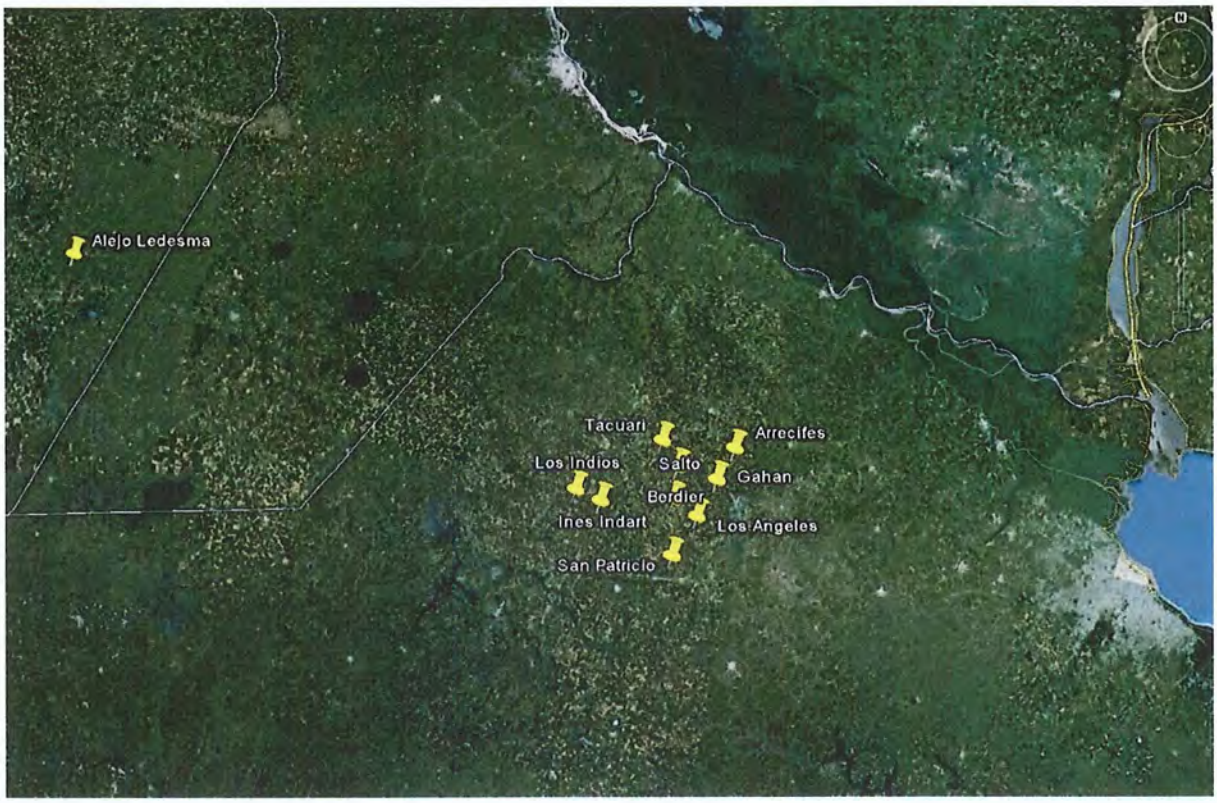


**Figure X-2. Satellite view of agronomic trial locations in Argentina**
The locations indicated are the cities nearest to the field plots.

**Table X-9. Argentina field location soil types, cropping histories, and planting dates**

| Location | Soil type | Previous crop | Planting date (2010) |
|---|---|---|---|
| Alejo Ledesma, Cordoba | sandy loam | peanut | December 23 |
| Arrecifes, Buenos Aires | silt loam | soybean | December 15 |
| Berdier, Buenos Aires | silt loam | soybean | December 26 |
| Gahan, Buenos Aires | silty clay loam | soybean | December 14 |
| Ines Indart, Buenos Aires | silt loam | soybean | December 21 |
| Los Angeles, Buenos Aires | silt loam | soybean | December 14 |
| Los Indios, Buenos Aires | silt loam | soybean | December 11 |
| Salto, Buenos Aires | silt loam | maize | December 13 |
| San Patricio, Buenos Aires | silt loam | fallow | December 21 |
| Tacuari, Buenos Aires | silt loam | maize | December 15 |

**Table X-10. Results of ANOVA comparisons between characteristics of SYHT0H2 and control soybean in agronomic field trials in Argentina (*N* = 40)**

| Characteristic | Mean (range) SYHT0H2 | | Control | | SEM | % Difference | *P* |
|---|---|---|---|---|---|---|---|
| Early stand count | 150.9* | (96–175) | 181.5 | (113–202) | 4.11 | –17.9 | <0.001 |
| Final stand count | 143.8* | (82–170) | 171.5 | (84–196) | 4.52 | –16.2 | <0.001 |
| Height (cm) | 63.3* | (40.7–89.8) | 67.4 | (48.6–89.8) | 3.54 | –6.1 | <0.001 |
| Test weight (lb/bu) | 72.7 | (70.2–5.7) | 72.6 | (70.3–75.6) | 0.39 | 0.1 | 0.628 |
| Seed moisture (%) | 12.5 | (10.2–16.0) | 12.5 | (10.1–15.9) | 0.63 | 0 | 0.398 |
| Seed yield (lb/plot)[a] | 3.70* | (2.01–5.84) | 3.92 | (1.79–5.93) | 0.308 | –5.6 | 0.013 |
| Seed yield (bu/ac)[b] | 29.9 | (16.3–46.1) | 31.7 | (14.7–46.6) | N/A | –5.7 | N/A |

[a]Measured value corrected to 13% moisture.
[b]Value computed from pounds per plot. N/A = not applicable (ANOVA not conducted).
*Significantly different from control soybean at $P < 0.05$.

**Table X-11. Argentina field trial results for soybean characteristics not subjected to ANOVA (*N* = 32)**

| Characteristic | Mean (range) SYHT0H2 | Control | % Difference |
|---|---|---|---|
| Seedling vigor rating | 2.23 (2-3) | 2.03 (1-3) | 9.9 |
| Days to 50% flowering | 35.4 (32-39) | 35.1 (31-39) | 0.9 |
| Flower color | white | white | N/A[a] |
| Lodging rating | 1.18 (0-3) | 1.15 (0-3) | 2.6 |
| Pod shattering | 1.25 (0-3) | 0.95 (0-3) | 31.6 |
| Days to maturity | 99.0 (88-111) | 99.7 (87-108) | –0.7 |

[a]Not applicable.

**Table X-12. Qualitative observations from Argentina trials on plot interactions with biotic and abiotic stressors**

| Location | Both SYHT0H2 and control soybean | SYHT0H2 only | Control only |
|---|---|---|---|
| Alejo Ledesma | Minimal damage from thrips, colospis, and red root caterpillar at V2 and thrips at R5. Minimal frogeye leaf spot at R5, mild brown leaf spot, leaf blight, and frogeye leaf spot at R6. Mild water stress at R6. | — | — |
| Arrecifes | Minimal damage from thrips and stalk borer at V2, thrips and soybean looper at R4, grasshopper at R6. Minimal brown leaf spot at R4, mild brown and frogeye leaf spot and soybean mosaic virus at R6, mild anthracnose and leaf blight at R8. No abiotic stress. | Minimal fusarium at V2 and bacterial blight at R4. | Minimal leaf blight and soybean mosaic virus at R4. |
| Berdier | Mild damage from thrips at V2, minimal damage from grasshopper, red root caterpillar, and bean shoot borer at R3 and R6. Minimal brown leaf spot at R3, moderate leaf spot and downy mildew at R6, minimal leaf spot and anthracnose at R8. Moderate damage from strong winds at V2. | — | — |

| Location | Both SYHT0H2 and control soybean | SYHT0H2 only | Control only |
|---|---|---|---|
| | | | *(continued)* |
| Gahan | Mild damage from thrips and stalk borer at V2, thrips and grasshopper at R3, grasshopper, soybean looper, and South American bollworm at R6. Minimal fusarium at V2, mild brown leaf spot and bacterial blight at R3, mild leaf spot and leaf blight at R6, mild leaf blight and anthracnose at R8. Minimal water stress at V2. | — | — |
| Ines Indart | Minimal damage from thrips at V2, thrips and soybean looper at R2, grasshopper, soybean looper, and South American bollworm at R6. Minimal brown leaf spot, bacterial blight, and soybean mosaic virus at R2, mild brown and frogeye leaf spot and leaf blight at R6, mild leaf blight and anthracnose at R8. Mild water stress resulting in soil compaction at V2. | — | Minimal fusarium at V2. |
| Los Angeles | Mild damage from thrips at V2 and R3, red root caterpillar, grasshopper, and South American bollworm at R6. Mild brown leaf spot and leaf blight at R3 and R6, leaf blight and anthracnose at R8. Minimal water stress at V2. | — | Mild fusarium at V2. |
| Los Indios | Minimal damage from thrips at V2 and R3, grasshopper, soybean looper, and South American bollworm at R6. Minimal brown leaf spot and bacterial blight at R3, mild brown leaf spot, downy mildew, and leaf blight at R6, mild leaf blight and anthracnose at R8. Minimal water stress at V2. | Minimal fusarium at V2. | Minimal damage from soybean looper at V2. |
| Salto | Mild damage from thrips at V2 and R3, minimal damage from soybean looper and South American bollworm at R6. Minimal fusarium at V2, brown leaf spot at R3, brown leaf spot, leaf blight, and frogeye leaf spot at R6, anthracnose and leaf blight at R8. Mild water stress at V2. | — | — |
| San Patricio | Mild damage from thrips, red spider mite, and red root caterpillar at V2. Minimal brown and frogeye leaf spot and downy mildew at R3, mild leaf blight, brown leaf spot, and downy mildew at R6, mild leaf blight and anthracnose at R8. No abiotic stress. | — | — |
| Tacuari | Minimal damage from thrips at V2, grasshopper and stalk borers at R3, and soybean looper and grasshopper at R6. Minimal brown leaf spot and bacterial blight at R3 and R6, mild leaf blight and anthracnose at R8. Mild water stress at V2. | Minimal soybean mosaic virus at R3 and R6. | Minimal damage from stalk borer at V2. |

### X.C.3. Conclusions from Agronomic Field Trials

Relative to control soybean a 16% to 18% reduction in stand count, a 4-cm reduction in plant height, and a 5.7% reduction in yield were observed for SYHT0H2 soybean in the Argentine trials. However, corresponding effects were not observed in the U.S. trials. In fact, average stand counts, plant height, and yield were higher for the SYHT0H2 soybean than for the nontransgenic control soybean, though not significantly so. The observed reductions in early and final stand counts in Argentina do not indicate a transformation-induced effect on seed germination, as germination rates did not differ between SYHT0H2 and control soybean seed in the growth-chamber study. Furthermore, seedling vigor of SYHT0H2 soybean was normal in both the U.S. and Argentine trials. Pod shattering was 32% higher in SYHT0H2 than control

soybean in the Argentine trials, but 15% lower in the U.S. trials. These inconsistent responses reflect natural biological variation.

### X.D. Assessment of Weediness Potential

Very few differences between SYHT0H2 and nontransgenic control soybean growth, reproductive, or survival characteristics were observed in field, growth chamber, and glasshouse studies. The statistically significant differences observed in the Argentine field trial were not observed in the U.S. field trial, indicating that they represented natural variability. None of these phenotypic differences indicate altered plant pest potential for SYHT0H2 soybean.

### X.E. References


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## XI. Potential Environmental Effects of SYHT0H2 Cultivation

The environmental impact of SYHT0H2 soybean cultivation is considered in the context of potential harm to wildlife, including species beneficial to agriculture, and the potential for the cultivar to become a weed.

### XI.A. Potential Impact on Wildlife

HPPD isozymes are ubiquitous throughout the plant and animal kingdoms and are essential to aerobic forms of life (Lee *et al.* 2008), with the exception of some Gram-positive bacteria (Gunsior *et al.* 2004). Wildlife exposure to AvHPPD-03 will occur through the consumption of SYHT0H2 soybean or through contact with or consumption of soil in which SYHT0H2 soybean is cultivated. Wildlife species potentially exposed to AvHPPD-03 via plant tissue or soil will have previously been exposed to proteins with similar structure and function. No harmful effects of such exposure are known.

Thirty-eight genetically modified cultivars expressing *pat*, including four soybean transformation events, are approved for environmental release in at least one country (ILSI 2011). PAT is normally produced in *Streptomyces* bacteria, which commonly occur in soil. Therefore, PAT or functionally similar proteins are ubiquitous in the environment. Wildlife species potentially exposed to PAT via SYHT0H2 soybean tissue or soil will have previously been exposed to enzymes with similar structure and function. No harmful effects of such exposure are known.

There are no material differences in crop composition or phenotype between SYHT0H2 and conventional soybean. Thus, there is no basis for concluding that cultivation of SYHT0H2 soybean will be more harmful to any threatened or endangered species than cultivation of conventional soybean.

### XI.B. Gene Flow

Soybean is a self-pollinating species. Soybean flowers are receptive to pollen for about 72 hours, and the anthers mature in the bud and pollinate the stigma of the same flower, resulting in a high percentage of self-pollination (OECD 2000). For cross-fertilization to occur, synchrony of both flowering and pollen dispersal are required. Numerous studies have examined cross-pollination rates in conventional soybean. Although cross-pollination has been observed at a rate as high as 7.74% (Abrams *et al.* 1978), most studies found rates were less than 1% (Caviness 1966). The cross-pollination rate generally decreases with increasing distance from the pollen source (e.g., Ray *et al.* 2003, Yoshimura *et al.* 2006, Abud *et al.* 2007). Cross-pollination is most likely facilitated by pollinators rather than wind.

Although *Glycine max* is a self-pollinating species, hybridization is possible with wild species in the subgenus *Soja*, which includes *Glycine soja* and *Glycine gracilis*. *G. soja* occurs in Korea, Taiwan, Japan, the Yangtze Valley, northeast China, and along the border between China and the former Soviet Union, and *G. gracilis* is found in northeast China (OECD 2000). *G. soja* and *G. gracilis* do not occur naturally in the U.S. or Canada. The genus *Glycine* also includes the subgenus *Glycine*, which contains 12 wild perennial species. These species have not been found in the U.S. or Canada. Furthermore, attempts to produce fertile hybrids between *G. max* and

species of the subgenus *Glycine* were unsuccessful; hybrids were created only with difficulty in a laboratory setting and were sterile.

Cultivation of SYHT0H2 soybean in the U.S. or Canada will not lead to the transfer of *avhppd-03* and *pat* to wild relatives of soybean. The USDA specifies that for production of foundation, registered, or certified soybean seed, the only isolation distance required is one sufficient to prevent mechanical mixture (USDA 2011). Theoretically, AvHPPD-03 or PAT could enter the soil as a result of horizontal gene flow of *avhppd-03* and *pat* leading to their expression in soil microorganisms. However, multiple reviews have concluded that there is minimal likelihood of horizontal gene transfer between transgenic plants and soil microorganisms (US EPA 2001, Conner *et al.* 2003, Keese 2008). Should *avhppd-03* or *pat* from SYHT0H2 soybean be integrated into a plasmid or chromosome of a bacterium, it is highly unlikely that AvHPPD-03 or PAT will be produced, because the codon use in *avhppd-03* and *pat* is optimized for expression in plants, not bacteria (see Section IV). Therefore, it is highly unlikely that AvHPPD-03 or PAT will be produced in soil via transformation of bacteria with genes from SYHT0H2 soybean.

### XI.C. Weediness Potential

Weediness of a crop plant could adversely affect both wild plant populations and the yields of other crops. The abundance and diversity of wild plants could be reduced if feral populations of SYHT0H2 soybean or hybrids of SYHT0H2 soybean with wild species establish and spread into semi-natural or natural habitats. Organisms that rely on these wild plants for food or shelter could also be harmed (e.g., Raybould and Wilkinson 2005). If SYHT0H2 soybean is more likely than conventional soybean to be a volunteer weed, the yields of other crops could be affected. Volunteers reduce crop yield directly through competition and indirectly by acting as "green bridges" for pests and pathogens (Longden 1993, Yarham and Gladders 1993).

Several characteristics make it unlikely for conventional soybean to form feral populations. Soybean is a highly domesticated crop that is not found in North America outside of cultivation. Soybean seeds rarely display dormancy characteristics and only under certain environmental conditions will seed overwinter and germinate in a subsequent crop as a volunteer weed (OECD 2000). Several features of soybean make it unlikely to form self-sustaining weedy populations in agriculture: it is easily controlled in subsequent crops by mechanical or chemical means; seed dispersal is limited because seeds are held inside pods; the seeds lack primary dormancy, so young plants are exposed to harsh winter conditions; and soybean is uncompetitive with perennial plants. A laboratory study has shown that germination and dormancy characteristics of SYHT0H2 seed are no different than those of conventional soybean seed (see Section X.A).

The introduced traits in SYHT0H2 soybean are not intended to affect the range or frequency of soybean outcrossing, and phenotypic data showed no indication that the genetic modification resulted in enhancement of reproductive characteristics of SYHT0H2 soybean compared with conventional soybean (see Section X.C).

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## XII. Impact on Agronomic Practices

Soybean is the second-largest crop in the U.S. by acres planted. In 2011, 75 million acres were planted, which yielded 3.1 billion bushels with a gross value of $35.8 billion (USDA 2012b). Average yield in 2011 was 41.5 bu/ac, for which growers received an average price of $12.50/bu. The ten highest-producing states in 2011 by acres planted were Iowa, Illinois, Minnesota, Missouri, Indiana, Nebraska, Ohio, South Dakota, North Dakota, and Kansas (USDA 2012a).

### XII.A. Soybean Production

Successful soybean production requires the integration of diverse inputs and crop management. Proper choice of cultivar, tillage system, planting date, planting density, row spacing, fertilization, scouting for pests, and weed control are necessary for optimizing yield and return on grower investment. Most soybeans are planted in a rotation with other annual crops such as corn, cotton, or spring wheat, and they can be planted in the same year following the harvest of winter wheat (a practice known as double cropping). Very little continuous soybean is planted in the U.S.

Tillage in soybean production is used to prepare the seedbed for planting, reduce soil compaction, improve soil drainage, and incorporate fertilizers and herbicides, as wells as for mechanical weed control (Heatherly and Elmore 2004). Conventional tillage raises soil temperature, improves seedbed consistency, and facilitates more rapid soybean germination and emergence. Conventional tillage can lead to increased yield but also increases grower fuel and labor costs and increases the risk of soil erosion. Conservation tillage and no-till practices leave significant surface residue of the prior crop but can preserve soil moisture and reduce labor and fuel costs. Pre-emergent and post-emergent herbicides are used for weed control in these various tillage systems. Tillage primarily for weed control in soybean has largely been replaced by the use of glyphosate in conjunction with planting of glyphosate-tolerant cultivars. The increase in no-till soybean acres in recent years has paralleled adoption of glyphosate-tolerant soybeans; Figure XII-1 shows trends in tillage-system use from 1998 to 2011.

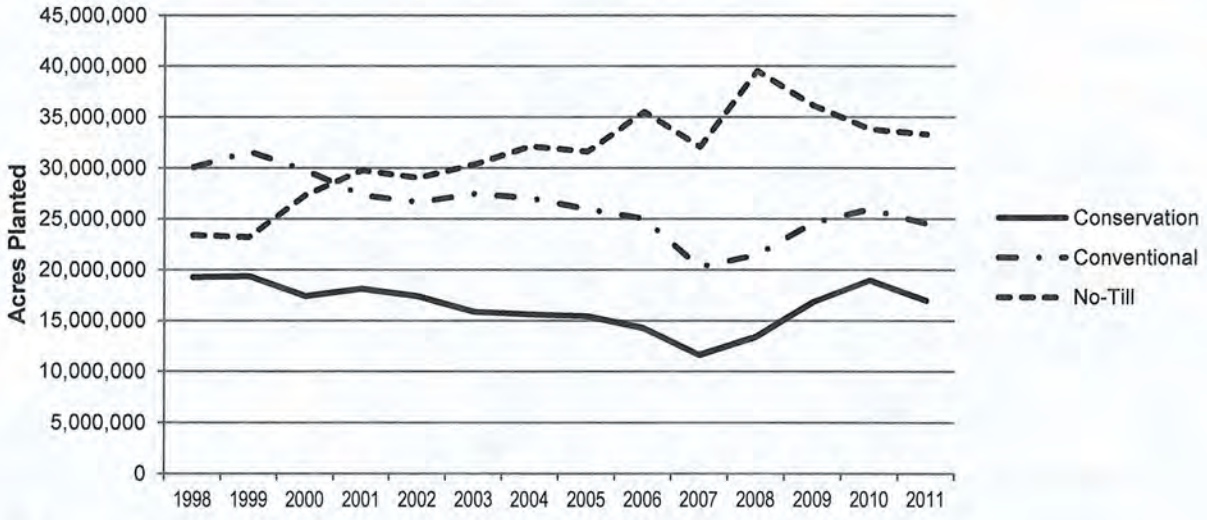


**Figure XII-1. Type of tillage system used in soybean production (Syngenta 2011)**

### XII.B. Weed Control

Profitable soybean production depends upon successful management of weeds (Buhler and Hartzler 2004). Weeds compete with soybean for water, nutrients, and light. Control of early-season weeds is one of the most critical components of a profitable soybean production. Weed control during the first two to four weeks of the growing season is essential for maximizing crop yield and allows sufficient time for early crop growth and crop canopy closure. Soybean is a poor competitor with weeds when cool soil temperatures cause slow germination and reduced early stand growth, but is more competitive in warmer soils when seed germination and seedling growth are more rapid. Adoption of an integrated weed management program is the best long-term approach for managing weeds. The goal of such a program is to integrate the cropping system with all available weed-control strategies into a comprehensive weed management program that is environmentally and economically sustainable.

The weeds of major concern to soybean producers are listed in Table XII-1, which lists those weeds reported by growers to have been treated with herbicides on more than one million acres in 2011 (Syngenta 2011). Annual weed species are a greater pest problem than perennial species in both soybean and corn production (Aref and Pike 1998).

**Table XII-1. Weeds infesting more than one million soybean acres in 2011 and requiring treatment with at least one herbicide**

| Annual broadleaf weeds | Annual grass weeds |
|---|---|
| Redroot pigweed *(Amaranthus retroflexus)* | Foxtail *(Setaria* spp.*)* |
| Common waterhemp *(Amaranthus tuberculatus)* | Giant foxtail *(Setaria faberi)* |
| Velvetleaf *(Abutilon theophrasti)* | Yellow foxtail *(Setaria glauca)* |
| Common cocklebur *(Xanthium strumarium)* | Volunteer corn *(Zea maize)* |
| Comon lambsquarters *(Chenopodium album)* | Barnyardgrass *(Echinochloa crus-galli)* |
| Marestail *(Conyza canadensis)* | Crabgrass *(Digitaria* spp.*)* |
| Giant ragweed *(Ambrosia trifida)* | Green foxtail *(Setaria viridis)* |
| Common ragweed *(Ambrosia artemisiifolia)* | Fall panicum *(Panicum dichotomiflorum)* |
| Morning glory *(Ipomoea* spp.*)* | Wild oat *(Avena fatua)* |
| Common sunflower *(Convolvulus arvensis)* | Signalgrass *(Brachiaria platyphylla)* |
| Wild mustard *(Brassica kaber)* | Shattercane *(Sorghum bicolor)* |
| Kochia *(Kochia scoparia)* | Ryegrass *(Lolium* spp.*)* |
| Smartweed *(Polygonum* spp.*)* | |
| Henbit *(Lamium amplexicaule)* | **Perennial weeds** |
| Horseweed *(Conyza canadensis)* | Johnsongrass *(Sorghum halepense)* |
| Palmer amaranth *(Amaranthus palmeri)* | Canada thistle *(Cirsium arvense)* |
| Tall waterhemp *(Amaranthus rudis)* | Dandelion *(Taraxacum officinale)* |
| Wild buckwheat *(Polygonum convolvulus)* | Quackgrass *(Elytrigia repens)* |
| Chickweed *(Stellaria media)* | |
| Prickly sida *(Sida spinosa)* | |
| Black nightshade *(Solanum nigrum)* | |
| Sicklepod *(Cassia obtusifolia)* | |

### XII.B.1. Herbicides Used in Soybean Production

Herbicides are the primary means of weed control for most growers. In addition to herbicides, much of the tillage performed in soybean production, including seedbed preparation tillage, is a component of a weed-control program. Proper weed species identification shortly after emergence is required for selection of the appropriate herbicide(s). Herbicide applications generally fit into one of four use categories:

1. Preplant residual herbicides are applied prior to planting of the crop, before tillage, or when a field is being tilled. These herbicides are typically soil-incorporated, have residual activity, and will kill weeds as they germinate or emerge.
2. Preplant burndown herbicides require foliar contact with weeds and have no soil activity. They are used to clear a field of weeds prior to crop planting.
3. Pre-emergent herbicides are applied to the soil at the time of planting or before emergence of the crop, generally within seven days of planting. They kill weeds as they germinate or emerge. To be most effective, they require rainfall for activation.
4. Post-emergent herbicides are applied to weeds after the crop has emerged. These herbicides can be part of a directed application between rows soon after crop emergence, part of an over-the-top application of herbicides that are not active against soybean, or part of an over-the-top application to cultivars that are tolerant to the herbicide.

Dozens of herbicides are approved for use in soybean cropping systems. The most widely applied herbicide active ingredients are listed in Table XII-2, which lists those herbicide active ingredients applied to at least 1% of the total 74,835,004 planted soybean acres in 2011 (Syngenta 2011). The use of glyphosate in conjunction with planting of glyphosate-resistant soybean varieties has been the mainstay of U.S. soybean weed-control programs for the past 15 years. Only 3% of the soybeans planted in the U.S. in 2011 were conventional (i.e., nontransgenic). Roundup Ready and Roundup Ready2 Yield varieties comprised 75.5% and 20%, respectively, of the soybean acres planted in 2011 (Syngenta 2011). For many years, growers were able to effectively control virtually all weeds in soybean with glyphosate alone. However, the emergence of weeds resistant to glyphosate and a shift in weed populations to biotypes more tolerant to the herbicide have led to the use of additional herbicides and more intensive weed management practices in soybean production systems in many regions of the country.

**Table XII-2. Most widely used herbicide active ingredients for weed control in soybean production**

| Herbicide | Spectrum of Activity[1] | Use Category[2] |
|---|---|---|
| Glyphosate | NS | PB, PS |
| Chlorimuron | dicots | PR, PE, PS |
| Flumioxazin | dicots | PR, PE |
| 2,4-Dichlorophenoxyacetic acid (2,4-D) | dicots | PB |
| Fomesafen | dicots | PR, PE, PS |
| Imazethapyr | NS | PR, PE, PS |
| *s*-Metolachlor | monocots | PR, PE, PS |
| Clethodim | monocots | PS |
| Cloransulam-methyl | dicots | PR, PE, PS |
| Thifensulfuron | dicots | PS |
| Sulfentrazone | dicots | PR, PE |
| Metribuzin | dicots | PR, PE |
| Saflufenacil | dicots | PB |
| Pendimethalin | monocots | PR, PE |
| Fluthiacet-methyl | dicots | PS |
| Paraquat | NS | PB |
| Trifluralin | monocots | PR |
| Lactofen | dicots | PE, PS |
| Glufosinate-ammonium | NS | PB, PS |
| Flumiclorac | dicots | PS |
| Fluazifop | monocots | PS |
| Acetochlor | monocots | PS |
| Dicamba | dicots | PB |

[1]NS = nonselective.
[2]PR = preplant residual, PB = preplant burndown, PE = pre-emergent, PS = post-emergent.

### XII.B.2. Weed Resistance

Extensive use of glyphosate-only weed-control programs in corn, soybean, and cotton in the U.S. over the past 15 years has led to selection for glyphosate-resistant weeds and a shift in weed populations to species that are more tolerant to the herbicide. Examples include the escalation of problems from marestail (*Conyza canadensis*) in soybean and cotton, Palmer amaranth (*Amaranthus palmeri*) in southeastern U.S. cotton, and giant ragweed (*Ambrosia trifida*) in midwest soybean (Owen 2008). To date, 23 weed species resistant to glyphosate have been identified globally, 13 of which are found in the U.S. (Heap 2012). Table XII-3 includes a list of species found in the U.S. that are confirmed to be resistant to glyphosate. The number of weed species resistant to glyphosate has been increasing, as has the number of acres infested with these resistant biotypes. This situation has led to the need for alternative control technologies that can be used as part of an integrated weed management program.

**Table XII-3. Weeds found in the U.S. with confirmed resistance to glyphosate**

| Species | Common name | First identified (year) | Controlled by[1] | |
|---|---|---|---|---|
| | | | Mesotrione | Glufosinate |
| *Amaranthus palmeri* | Palmer amaranth | 2005 | x | x |
| *Amaranthus tuberculatus* | common waterhemp | 2005 | x | x |
| *Ambrosia artemisiifolia* | common ragweed | 2004 | x | x |
| *Ambrosia trifida* | giant ragweed | 2004 | x | x |
| *Conyza bonariensis* | hairy fleabane | 2007 | | |
| *Conyza canadensis* | horseweed | 2000 | x | x |
| *Echinochloa colona* | junglerice | 2008 | | x |
| *Eleusine indica* | goosegrass | 2010 | | x |
| *Kochia scoparia* | Kochia | 2007 | x | x |
| *Lolium multiflorum* | Italian ryegrass | 2004 | | |
| *Lolium rigidum* | rigid ryegrass | 1998 | | |
| *Poa annum* | annual bluegrass | 2010 | | x |
| *Sorghum halepense* | johnsongrass | 2007 | | x (seedlings) |

[1]The weed is listed on a U.S. EPA–approved label for mesotrione or glufosinate-ammonium herbicide products.

### XII.B.3. Current Weed-Control Practices

Most weed management programs in U.S. soybean production now employ more than one herbicide. The following four programs represent typical herbicide use programs currently used by U.S. soybean growers:

- Program 1 – employed in regions where glyphosate-resistant weeds are not a problem. Although they are declining in number, many growers can still rely on a glyphosate-only herbicide program. Such a program in no-till soybeans would employ a preplant burndown of weeds followed by one or two over-the-top applications of glyphosate after weeds have emerged. In conventional-tillage soybeans, one or two over-the-top applications of glyphosate would be used.

- Program 2 – employed in regions where glyphosate-resistant weeds are not prevalent and control of input costs is important. This program would include a soil-incorporation or pre-emergence application of an inexpensive residual herbicide such as trifluralin or pendamethalin. If there are significant weed escapes after planting, glyphosate can be applied when weeds reach 3 to 4 inches in height.

- Program 3 – employed in regions where glyphosate weed resistance is becoming prevalent. This program would be based on post-emergence applications of glyphosate in a tank mix with a second herbicide, such as fomesafen or lactofen, to control many weeds resistant to glyphosate. In a no-till system, this program might be preceded by a preplant burndown application of glyphosate plus 2,4-D or paraquat.

- Program 4 – employed in regions with heavy infestation of glyphosate-resistant weeds or infestations of several glyphosate-resistant weed species. This program incorporates both pre- and post-emergence applications of multiple-mode-of-action herbicide mixtures. A tank mixture of several residual herbicides (e.g., flumioxazin, fomsafen, metribuzin, or *s*-metolachlor) would be applied pre-planting or pre-emergence, followed by a post-

emergence application of either glyphosate or glufosinate-ammonium in a tank mixture with a residual herbicide. Deploying combinations of herbicides with different modes of action is the foundation of the program. In no-till systems, this program includes glyphosate plus 2,4-D or paraquat preceding or tank-mixed with preplant soil residual herbicides. This type of program might typically be used in the Southeast and mid-South, where glyphosate-resistant weeds are common.

Variations on the above scenarios are based on a grower's tillage practices, the productivity of the land involved, management practices, and the economics unique to each farm. Some growers will also employ more than one herbicide program.

### XII.C. Potential Impact of SYHT0H2 Introduction on Soybean Production

Mesotrione is a systemic, translocated herbicide with soil residual activity that is used for control of predominantly dicot weed species in a number of crops, including corn. It has been used for weed control in corn since 2001, mostly in mixtures with other herbicides to provide a broad spectrum of weed control. Current uses in corn include applications pre-planting or pre-emergence at rates of up to 225 g a.i./ha and post-emergence at up to 105 g a.i./ha through the V8 growth stage. The mesotrione EPA label allows up to two applications per season in corn, one pre-planting or pre-emergence, and one post-emergence.

Glufosinate-ammonium is a nonselective contact herbicide with no soil residual activity that is used in numerous glufosinate-tolerant crops, typically following application of a pre-emergent herbicide. The EPA label currently allows for a single preplant burndown or post-emergence application in soybeans at a rate up to 738 g a.i./ha. One additional post-emergence application may be made at up to 595 g a.i./ha. A season maximum rate is approximately 1333 g a.i./ha. Post-emergence applications may be made from crop emergence up to but not including the R1 growth stage.

Adoption of the SYHT0H2 soybean technology by growers is not expected to change their production practices in a material way. The technology will, however, bring tangible benefits to growers. Upon EPA approval, HPPD-inhibiting herbicides, such as mesotrione and isoxaflutole, will become available for use in soybean to manage glyphosate-resistant weeds. These herbicides employ a different mode of action from all other herbicides currently used in soybean production. Deploying mixtures of herbicides with different modes of action is a key component of any integrated weed resistance management program. Combining mesotrione tolerance and glufosinate tolerance traits in the same plant enables growers to apply mixtures of the two herbicides post-emergence. The results of field performance trials for these two traits can be found in Appendix E.

The use of mesotrione with SYHT0H2 soybean will allow for applications of the herbicide prior to crop planting and up through the R1 growth stage. Applications pre-planting and pre-emergence to early post-emergence can be made at a rate of up to 225 g a.i./ha and post-emergence at a rate of 105 g a.i./ha. Upon EPA approval, a pre-planting or pre-emergence application followed by a post-emergence application would be allowed, for a maximum of two applications per season.

Pre-planting or pre-emergence applications of mesotrione will provide season-long control for a multitude of annual dicot weeds, including redroot pigweed *(Amaranthus retroflexus)*, common and tall waterhemp *(A. tuberculatus and A. rudis),* Palmer amaranth *(A. palmeri),* lambsquarters *(Chenopodium album),* eastern black nightshade *(Solanum ptycanthum),* black nightshade *(S. nigrum),* common ragweed *(Ambrosia artimisiifolia),* Pennsylvania smartweed *(Polygonum pensylvanicum),* and velvetleaf *(Abutilon theophrasti).* Mesotrione can be applied in a mixture with other herbicides approved for soybeans (e.g., *s*-metolachlor, fomesafen, or metribuzin) to broaden the spectrum of weed control and provide overlapping activity and multiple modes of action against the target weed species. These soil-applied residual herbicides would be a key component of an effective weed management program, as well as part of the weed resistance management strategy. See Appendix F for further discussion of weed resistance prevention. Adding mesotrione to the pool of available soil-applied herbicides will increase the effectiveness and sustainability of weed control in soybeans.

Post-emergence applications of mesotrione would typically occur between the V2 and R1 stages of development or when weeds are 3 to 4 inches tall and would control emerged sensitive weeds, as well as provide soil residual activity. Weeds controlled post-emergence would include those listed above plus common cocklebur *(Xanthium strumarium),* giant ragweed *(Ambrosia trifida),* common sunflower *(Helianthus annuus),* and large crabgrass *(Digitaria sanguinalis),* as well as partial control or suppression of other species. For post-emergence use, mesotrione would be mixed with other herbicides such as glufosinate-ammonium, fomesafen, or *s*-metolachlor and would likely be applied following preplant or pre-emergent herbicides. Many of the weed species that mesotrione controls have previously developed resistance to other herbicides, such as acetolactase synthase inhibitors (e.g., imazethapyr) and glyphosate.

SYHT0H2 soybean will also enable the use of glufosinate-ammonium over the top of emerged soybean. Glufosinate-ammonium can be applied to SYHT0H2 soybeans from emergence up to the pre-flowering growth stage, but it is most effective for controlling small weeds (2 to 6 inches high). Rates for single applications of glufosinate-ammonium would be from 450 to 735 g a.i./ha. Two post-emergence applications would be allowed upon approval by EPA, with a seasonal maximum total of 1330 g a.i./ha. Herbicide programs that include mesotrione and/or glufosinate-ammonium on SYHT0H2 soybeans coupled with other soybean-selective herbicides would provide several modes of action with overlapping activity against the target weed species, to delay or prevent the onset of weed resistance.

Addition of a glyphosate tolerance trait to SYHT0H2 soybean through breeding would allow for mixtures of glyphosate and mesotrione and/or glufosinate-ammonium to be applied, which would facilitate broad-spectrum control of weeds, with flexibility in application timing and reduced risk of weed resistance. Growers would not need to alter their tillage practices, because mesotrione can be applied pre-planting, pre-emergence, or post-emergence, and glufosinate-ammonium can be applied as part of a preplant burndown program or post-emergence. Volunteer SYHT0H2 soybean in corn can be controlled with triazines or synthetic auxin herbicides. In cereal crops, SYHT0H2 soybean volunteers can be controlled with synthetic auxin or sulfonylurea herbicides. Appendix F describes the stewardship program that the Petitioners will put in place to preserve the viability of the SYHT0H2 soybean, mesotrione, and glufosinate-ammonium technologies.

## XII.D. References


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## XIII. Adverse Consequences of Introduction

Syngenta and BCS are not aware of any unfavorable information that would have a bearing on a decision by USDA to deregulate SYHT0H2 soybean. The development and testing of SYHT0H2 soybean has not revealed any data or observations indicating that deregulation of this new cultivar would pose a greater risk to the environment than conventional soybean.

## Appendix A. USDA Notifications for SYHT0H2 Soybean

Field trials with SYHT0H2 soybean have been conducted in the U.S. under USDA–APHIS notifications since 2008. A complete listing of these notifications and their report status is provided in Table A-1.

**Table A-1. USDA notifications for field releases of SYHT0H2 soybean**

| Notification no. | States | Effective dates | Report status |
|---|---|---|---|
| 08-105-104n | IL | 5/2/08–5/2/09 | submitted |
| 08-106-110n | no plantings | 5/2/08–5/2/09 | submitted |
| 08-238-106n | PR | 9/18/08–9/18/09 | submitted |
| 09-054-106n | IL, IN, IA, MO | 4/1/09–4/1/10 | submitted |
| 10-062-113n | AR, HI, IL, IN, IA, KS, LA, MN, MS, MO, NE, NC, OH, PR, SD, TN, WI | 4/1/10–4/1/11 | submitted |
| 10-064-116n | IL, IA, IN, MO, NE, NC, PA, WA | 3/29/10–3/29/11 | submitted |
| 10-078-107n | IL, IN, IA, KS, MN | 4/12/10–4/12/11 | submitted |
| 10-258-103n | HI | 10/11/10–10/11/11 | submitted |
| 11-041-125n | AR, GA, HI, IA, IL, IN, KS, LA, MD, MN, MO, MS, NC, NE, OH, PR, SD, TN, WI | 4/1/11–4/1/12 | pending |
| 11-060-108n | NC | 3/23/11–3/23/12 | submitted |
| 11-061-103n | MN | 3/24/11–3/24/12 | submitted |
| 11-082-106n | IL, IN, IA, KS, MD, MN, MS, OH | 4/21/11–4/21/12 | pending |
| 11-194-102n | PR | 8/1/11–8/1/12 | pending |
| 11-355-101r | HI, IL, PR | 01/10/12–01/10/13 | pending |
| 12-041-101n | IL, IN, IA, MS, MO, TN | 4/1/12–4/1/13 | pending |
| 12-046-101n | AR, CO, GA, HI, IL, IN, IA, KS, LA, MD, MI, MN, MO, MS, NE, NC, ND, OH, PR, SD, TN, WI | 4/1/12–4/1/13 | pending |
| 12-052-106n | IA | 4/1/12–4/1/13 | pending |
| 12-060-106n | IL, IN, IA, KS, MN, MO, NE, NC, PA, WI | 4/1/12–4/1/13 | pending |
| 12-058-105n | AR, GA, KS, MS, MO, TN | 03/19/12–03/19/13 | pending |
| 12-083-104n | IL, IN, IA, MN, MS | 04/20/12–04/20/13 | pending |
| 12-128-102n | IL, IA, MI, MO, NE, PA, PR, WI | 05/15/12–05/15/2013 | pending |

## Appendix B. Methods Used for Genetic Characterization of SYHT0H2 Soybean

This appendix provides details of the materials and methods used in the studies performed to genetically characterize Event SYHT0H2 soybean. The study designs, results, and conclusions are described in Section V of this petition.

### B.1. Plant Material

SYHT0H2 and nontransgenic control soybean seeds were planted and grown in a glasshouse under standard conditions and then processed to extract genomic DNA. The generations of SYHT0H2 plants used and the corresponding control plants used for each study are specified in Section III of this petition. Appropriate quality-control methods were used to verify the purity and identity of the plant material used in each study.

### B.2. Real-Time PCR Analysis

Genomic DNA was isolated from leaf tissue of each individual plant by a method adapted from the Promega Wizard Magnetic 96 DNA Plant System. Each plant was individually analyzed for the presence the SYHT0H2 insert by real-time PCR analysis (Ingham *et al.* 2001). A control assay targeting a soybean native alcohol dehydrogenase 1 (*adh1*) gene was included to monitor soybean DNA quality and the performance of PCR components (such as buffers, reagents, and equipment). The forward primer binding site was located in the soybean genomic sequence, the reverse primer binding site in the SYHT0H2 insert, and the probe binding site in the SYHT0H2 insert.

### B.3. Genomic DNA Extraction

The genomic DNA used for Southern blot analyses was isolated from the pooled leaf tissue by a method modified from that described by Saghai-Maroof *et al.* (1984). The pooled leaf tissue was ground into a fine powder with a pre-chilled mortar and pestle under liquid nitrogen and placed in a bottle for storage.

For each DNA extraction, approximately 5 g of leaf tissue and 25 ml of pre-warmed cetyltrimethyl ammonium bromide (CTAB) buffer (100 mM 2-amino-2[hydroxymethyl]-1,3-propanediol [tris] pH 8.0, 20 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 1.4 M sodium chloride, 2% CTAB [w/v], and 0.2% β-mercaptoethanol [v/v]) were combined in a bottle; the sample was then mixed gently and incubated for approximately 60 to 120 minutes at 65°C ± 5°C. An equal volume of chloroform:isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature.

The resulting aqueous phase was transferred to a clean container, and 10 µg of ribonuclease A per milliliter of aqueous phase was added. The sample was mixed and incubated for 30 to 60 minutes at 37°C ± 2°C. An equal volume of chloroform:isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature. The aqueous phase was collected in a clean bottle, and the DNA was precipitated with a 0.7 volume of isopropanol. The DNA was then pelleted by centrifugation at 291 × *g* for 10 minutes or collected with a sterile loop, washed once with 70% (v/v) ethanol, and centrifuged at 7277 × *g*

for 10 minutes. The DNA pellet was air-dried and dissolved in pre-warmed 0.1X tris-EDTA buffer.

Genomic DNA extractions with lyophilized tissue were performed by the method above, with the following exceptions. Plant samples were ground individually into a fine powder in the presence of dry ice or liquid nitrogen. A subsample from each homogenous powdered sample was lyophilized for extraction. Approximately 1 g of lyophilized tissue and 25 ml of pre-warmed CTAB buffer (50 mM tris pH 8.0, 10 mM EDTA pH 8.0, 0.7 M sodium chloride, 1% CTAB [w/v], and 0.1% β-mercaptoethanol [v/v]) were combined in a bottle.

The DNA was quantified using an Invitrogen Quant-iT PicoGreen dsDNA assay kit.

### B.4. Sequencing of the T-DNA Insert

Prior to PCR amplification, genomic DNA from SYHT0H2 soybean was digested with the restriction enzymes *Bgl*II, *Ahd*I, *Bsp*EI, and *Bsr*DI in separate reactions. The digested genomic DNA fragments were used as a template for the Sigma-Aldrich JumpStart REDAccuTaq LA DNA Polymerase PCR system to amplify nine overlapping DNA fragments that covered the entire SYHT0H2 insert.

The PCR products were cloned into the Invitrogen pCR4-TOPO TA vector, and four to six colonies from each cloning reaction were randomly selected and grown. The plasmid DNA from each colony was extracted and digested with the restriction enzyme *Eco*RI to confirm the presence of an insert of the expected size. Three DNA clones were then randomly selected, and the presence of the expected insert in each clone was confirmed by single sequencing runs on the ends of the clones, using primers located in the TOPO cloning vector.

Three clones for each of the nine overlapping PCR products were sequenced individually and aligned with Applied BioSystems Sequence Analysis software v. 5.3 and DNASTAR Lasergene software v. 8 to generate a consensus sequence for each clone. The three clone sequences were then aligned to obtain a consensus sequence for each PCR product. Gene CodesSequencher v. 4.9 was used to generate the final sequence of the SYHT0H2 insert from the nine PCR products' consensus sequences, and Vector NTI v. 10 was used to compare the SYHT0H2 insert sequence with the sequence of the transformation plasmid pSYN15954 T-DNA.

### B.5. Southern Blot Analyses

Southern blot analyses were performed according to standard molecular biology techniques (Chomczynski 1992). Each lane contained 3 µg of genomic DNA that was digested with the appropriate restriction enzyme(s) for six to seven hours.

To demonstrate the sensitivity of the analyses, each Southern blot analysis included a positive control, which consisted of digested DNA from plasmid pSYN15954 representing one copy of a DNA fragment of known size in the soybean genome. The positive control was loaded in a well together with 3 µg of digested DNA from nontransgenic 'Jack' soybean, 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.

The molecular-weight marker (serving as the reference substance), the digested genomic DNA, and the corresponding positive control were loaded onto 1% Lonza SeaKem Gold agarose gels, and the DNA fragments were separated by electrophoresis in 1X tris-acetate-EDTA buffer.

Following a 10- to 15-minute depurination in 0.25 N hydrochloric acid, the DNA in the gel was denatured in 0.5 M sodium hydroxide, 1.5 M sodium chloride, and 2 mM EDTA for 30 to 35 minutes. A Bio-Rad Appligene vacuum blotter was then used to transfer the DNA to a Bio-Rad Zeta-Probe GT membrane by downward alkaline transfer for 90 to 180 minutes. The membrane was rinsed briefly in 2X sodium chloride–sodium citrate (SSC) buffer, and ultraviolet light was used to crosslink the DNA to the membrane.

All PCR-generated probes (each element-specific probe, the T-DNA–specific probes, and the pSYN15954 plasmid-backbone-specific probe) and the molecular-weight-marker-specific probe were labeled with alpha-phosphorus-32-deoxycytidine triphosphate by random priming with the Invitrogen RadPrime or GE Healthcare Megaprime DNA labeling system. The NOS terminator–specific probe was also labeled with alpha-phosphorus-32-deoxyadenosine triphosphate. The unincorporated label was removed through the use of Bio-Rad Micro Bio-Spin chromatography columns or GE Healthcare ProbeQuant G-50 Micro Columns.

The membranes were incubated in 30 ml of Sigma-Aldrich PerfectHyb Plus hybridization buffer with or without denatured calf thymus DNA at 100 µg/ml for at least 15 minutes at 65°C ± 5°C. Both the molecular-weight-marker-specific probe and either the corresponding element-specific probe or the pSYN15954 plasmid-backbone-specific probe were added to the hybridization solution. The membranes were incubated for 3 to 22 hours at 65°C ± 5°C. Incubation was followed by a combination of washes at 65°C ± 5°C in 2X SSC buffer with 0.1% sodium dodecyl sulfate (SDS) buffer and washes at 65°C ± 5°C in 0.1X SSC buffer with 0.1% SDS buffer. Finally, the membranes were subjected to imaging with a Molecular Dynamics Storm 860 phosphorimager or X-ray film.

### B.6. Sequencing of the Soybean Genomic Regions Flanking the T-DNA

SYHT0H2 soybean genomic DNA was used as a template for the JumpStart REDAccuTaq LA DNA Polymerase PCR system to amplify the soybean genomic sequences flanking the 5′ and 3′ regions of the SYHT0H2 insert. The sequences of the PCR primers are shown in Table B-6. Table B-7 lists the thermal cycling parameters for PCR amplification. Three separate PCR reactions were performed to amplify each flanking sequence.

The PCR products were cloned into the Invitrogen pCR4-TOPO TA vector. From each cloning reaction, three colonies were randomly selected and grown. The plasmid DNA from each colony was extracted and digested with the restriction enzyme *Eco*RI to confirm the presence of an insert of the expected size. One clone was then randomly selected from each cloning reaction, and the presence of the expected insert in each clone was confirmed by single sequencing runs on the ends of the clone, using primers located in the TOPO cloning vector.

For each flanking sequence, the three clones, obtained from separate PCR reactions and confirmed to contain the expected insert, were sequenced individually. Applied BioSystems DNA Sequence Analysis software v. 5.3 and DNASTAR Lasergene v. 8 were used to generate a consensus sequence for each flanking sequence.

### B.7. Sequencing of the Genomic Insertion Site in Nontransgenic 'Jack' Soybean

The SYHT0H2 insertion site was amplified from genomic DNA extracted from nontransgenic 'Jack' soybean via PCR analysis. Three separate PCR reactions were performed. The design of the PCR primers was based on the genomic sequences flanking the 5′ and 3′ ends of the SYHT0H2 insert. PCR amplification was carried out with the JumpStart REDAccuTaq LA DNA Polymerase PCR system.

The PCR fragments were cloned into the Invitrogen pCR4-TOPO TA vector. From each cloning reaction, three colonies were randomly selected and grown. The plasmid DNA from each colony was extracted and digested with the restriction enzyme *Eco*RI to confirm the presence of the expected size insert. Following this confirmation, one clone was then randomly selected for each cloning reaction. The presence of the expected insert in each clone was confirmed by single sequencing runs on the ends of the clone using primers located in the TOPO cloning vector.

The three clones confirmed to contain the expected insert were sequenced individually. The sequences of the clones were analyzed and aligned using DNA Sequence Analysis software v. 5.3 from Applied BioSystems and Lasergene v. 8 from DNASTAR to generate a consensus sequence for each clone. Finally, the sequences of the three clones were aligned to obtain the final consensus sequence for the SYHT0H2 genomic insertion site.

### B.8. Flanking Sequence Analysis to Determine whether T-DNA Was Inserted into a Known Soybean Gene (BLASTN Analyses)

BLASTN analyses were used to screen the soybean genomic sequences flanking the SYHT0H2 insert for similarity to sequences in the NCBI nr/nt database (NCBI 2012) and the PlantGBD Viridiplantae EST database (PlantGDB 2012). The following parameters were used for the BLASTN analyses:

- Expect = 10. The *E*-value is a measure of the probability that matches between sequences occurred by chance. Search results involving comparisons between nucleotides with highly similar sequences yield *E*-values approaching zero; the probability that sequence similarities occurred by chance increases with higher *E*-values (Ponting 2001). The search identified all sequences in the database with search results yielding an *E*-value of 10 or lower.
- Match/mismatch scoring = default for nucleotide searches: +1 for a match and –3 for a mismatch.
- Gap costs: existence = 5; extension = 2. A gap is a space introduced into an alignment to compensate for insertions or deletions in one sequence relative to another. The introduction of a gap causes the deduction of a fixed value from the alignment score to prevent the accumulation of excessive gaps in an alignment. Extension of the gap to encompass additional nucleotides is also penalized in determining the score of an alignment. The resultant score is derived from the number of identical matches between the query sequence and the database entry, with higher scores indicating greater similarity between the two sequences.

- Filter = low complexity. The low complexity filter masks regions of low compositional complexity that could cause spurious or misleading results.

### B.9. References


Chomczynski P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Analytical Biochemistry* 201:134–139.

Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* 31:132–140.

NCBI. 2012. Entrez Nucleotide Database. Bethesda, MD: National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide.

PlantGDB. 2012. PlantGDB EST Assemblies. http://www.plantgdb.org/prj/ESTCluster (updated January 26, 2012).

Ponting C. 2001. Issues in predicting protein function from sequence. *Briefings in Bioinformatics* 2:19–29.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Science of the United States of America* 81:8014–8018.

## Appendix C. Methods Used to Quantify AvHPPD-03 and PAT Proteins in Soybean Tissues

### C.1. Test, Control, and Reference Substances

The test substance for this study was SYHT0H2 soybean seed in the genetic background 'Jack' (Nickell *et al.* 1990). The control substance was nontransgenic near-isogenic soybean seed of the same genetic background as the test substance. Field-grown seed lots of the test and control substances were characterized by real-time polymerase chain reaction testing (Ingham *et al.* 2001) to confirm identity and purity.

### C.2. Plant Tissue Production and Collection

During the 2011–2012 growing season, soybean plants were grown according to local agronomic practices at four separate field-trial locations in Argentina that are representative of agricultural regions where soybean is commercially cultivated and that are suitable for the cultivation of variety 'Jack.' These locations included Gahan, Provincia de Buenos Aires; Los Angeles, Provincia de Buenos Aires; Inés Indart, Provincia de Buenos Aires; and Salto, Provincia de Buenos Aires. Table 1 shows the plant samples collected for analysis. Two samples were collected from the control entry and five samples from the test entry. All samples were placed on dry ice after collection and transported to Investigaciones Biotecnológicas en el Campo Argentino (IBCA), Salto, Provincia de Buenos Aires, Argentina, and stored at –80°C ± 10°C until they were prepared for protein extraction and analysis.

**Table C-1. Tissue samples collected for analysis**

| Growth stage | Tissues collected | Sample description |
|---|---|---|
| V4 | leaves | all healthy trifoliate leaves from one plant |
| V8 | leaves | all healthy trifoliate leaves from one plant |
| | roots | all root tissue from one plant |
| V10 | leaves | all healthy trifoliate leaves from one plant |
| R6 | leaves | all healthy trifoliate leaves from one plant. |
| | roots | all root tissue from one plant |
| | forage | the entire above-ground portion of one plant |
| R8 | seed | all seed from the pods of one plant |

### C.3. Plant Tissue Sample Preparation

The plant tissue samples were ground individually into a fine powder in the presence of dry ice at IBCA and stored at –80°C ± 10°C until shipment. A subsample from each homogeneous powdered sample was lyophilized for protein extraction and analysis and stored at –80°C ± 10°C. The percent dry weight of each sample was determined from the fresh weight of the sample before lyophilization and the dry weight after lyophilizat\ion by the following formula:

$$\%\,\mathrm{DW} = \left(\frac{\mathrm{DW\ (g)}}{\mathrm{FW\ (g)}}\right) \times 100$$

### C.4. Protein Extraction and ELISA Analysis

Protein extractions were performed on representative aliquots of the lyophilized samples. ELISA methodology was used to quantify AvHPPD-03 and PAT in each extract. Nontransgenic plant tissue extracts were analyzed concurrently to confirm the absence of plant-matrix effects in each ELISA. For each ELISA, a standard curve was generated with known amounts of the corresponding reference protein. The mean absorbance for each sample extract was plotted against the appropriate standard curve to obtain the amount of protein as nanograms per milliliter of extract.

Phosphate-buffered saline with 0.05% Tween 20 surfactant (PBST) buffer was added to the lyophilized plant tissue at a ratio of 3 ml of buffer to 30 mg of sample (Table C.2). The samples were mixed, placed on wet ice, homogenized in an Omni-Prep Multi-Sample Homogenizer, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at –20°C ± 5°C until analysis.

**Table C-2. Reagents and buffers used for extraction and ELISA of AvHPPD-03 and PAT**

| Item | Constituents |
|---|---|
| Phosphate-buffered saline with 0.05% Tween 20 surfactant | 138 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4, and 0.05% Tween 20 surfactant |
| EnviroLogix QualiPlate Kit for HPPD in Soy or QualiPlate Kit for LibertyLink PAT/*pat* | 96-well plate precoated with anti-AvHPPD-03 antibody, AvHPPD-03 enzyme conjugate solution, and substrate solution; or 96-well plate precoated with anti-PAT antibody, PAT enzyme conjugate solution, and substrate solution |

### C.5. AvHPPD-03 Quantification

The appropriate number of 96-well plates pre-coated with the capture antibody and the appropriate amounts of antibody/enzyme conjugate solution and substrate solution (all provided in the QualiPlate Kit for HPPD in Soy) were removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature. The tube containing the substrate solution was covered to prevent exposure to light. Dilutions of each sample extract and the ELISA standard (prepared with protein reference substance AvHPPD-03-0209), prepared in PBST, were applied to the plates at a volume of 50 µl/well. The plates were incubated at room temperature for 30 minutes while shaking. The plates were then washed five times prior to addition of the AvHPPD-03 enzyme conjugate (50 µl/well) and incubated at room temperature for 30 minutes while shaking. The plates were washed five times prior to addition of the substrate solution (100 µl/well) and covered to prevent exposure to light during incubation at room temperature for five minutes while shaking. The colorimetric reaction was stopped by the addition of 1 N hydrochloric acid (100 µl/well), and absorbance was measured with a dual-wavelength spectrophotometer at 450 and 650 nm. The results were analyzed with Molecular Devices SoftMax Pro GxP microplate data compliance software, v. 5.4.1. The 650-nm reference measurement was subtracted from the 450-nm measurement prior to further analysis. Concentrations were interpolated from a standard curve generated with a four-parameter algorithm.

### C.6. PAT Quantification

The appropriate number of 96-well plates pre-coated with the capture antibody and the appropriate amounts of antibody/enzyme conjugate solution and substrate solution (all provided in the Qualiplate Kit for LibertyLink PAT/*pat*) were removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature. The tube containing the substrate solution was covered to prevent exposure to light. The PAT enzyme conjugate solution was applied to each well at a volume of 50 µl/well. Dilutions of each tissue extract and the appropriate serial dilutions of the ELISA standard (prepared with the protein reference substance), prepared in PBST, were applied to the plates at a volume of 50 µl/well. The plates were mixed in a rapid circular motion on the benchtop for 10 seconds and incubated at room temperature for one hour. The plates were washed five times with PBST in a microplate washer, and the substrate solution was added at a volume of 100 µl/well. The plates were covered to prevent exposure to light during incubation at room temperature for 15 minutes. The colorimetric reaction was stopped by the addition of 1 N hydrochloric acid (100 µl/well), and absorbance was measured with a dual-wavelength spectrophotometer at 450 and 650 nm. The results were analyzed with Molecular Devices SoftMax Pro GxP microplate data compliance software, v. 5.4.1. The 650-nm reference measurement was subtracted from the 450-nm measurement prior to further analysis. Concentrations were interpolated from a standard curve generated with a quadratic curve-fitting algorithm.

### C.7. Adjustments for Extraction Efficiency

Predetermined extraction efficiencies were used to adjust the AvHPPD-03 and PAT concentrations to the estimated total AvHPPD-03 and PAT concentration in the corresponding tissue sample. Extraction efficiency and method sensitivity data, determined during validation of the AvHPPD-03 quantitation method (prior to this study), are summarized in Tables C-3 and C-4.

**Table C-3. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the AvHPPD-03 ELISA**

| Sample Type | Minimum Dilution Factor | Extraction Efficiency | LOD (µg/g DW) | LOQ (µg/g DW) |
|---|---|---|---|---|
| Soybean leaf | 1 | 81% | 0.0313 | 0.0625 |
| Soybean root | 1 | 81% | 0.0313 | 0.250 |
| Soybean seed | 1 | 94% | 0.0313 | 0.125 |

**Table C-4. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the PAT ELISA**

| Sample Type | Minimum Dilution Factor | Extraction Efficiency | LOD (µg/g DW) | LOQ (µg/g DW) |
|---|---|---|---|---|
| Soybean leaf | 1 | 77% | 0.025 | 0.025 |
| Soybean root | 1 | 75% | 0.060 | 0.060 |
| Soybean seed | 1 | 94% | 0.025 | 0.060 |

The AvHPPD-03 concentrations in forage samples were adjusted with the extraction efficiency determined for leaves, which was lower than that for root or seed matrices and therefore provided the most conservative adjustment. The PAT concentrations in forage samples were adjusted with the extraction efficiency determined for roots, which was lower than that for leaves or seed matrices and therefore provided the most conservative adjustment.

### C.8. References

Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* 31:132–140.

Nickell CD, Noel GR, Thomas DJ. 1990. Registration of 'Jack' soybean. *Crop Science* 30:1365.

## Appendix D. Methods Used for Characterization of the AvHPPD-03 and PAT Proteins

This appendix presents materials and methods used in analyses of the biochemical properties and biological activity of the AvHPPD-03 and PAT proteins in SYHT0H2 soybean and in the corresponding microbially produced proteins used in safety studies.

### D.1. Source of Microbially Produced AvHPPD-03

Microbially produced test substance containing AvHPPD-03 protein was prepared from an *E. coli* overexpression system and was the source of AvHPPD-03 in the biochemical characterization studies. The test substance was characterized in detail and was determined to contain 72.2% AvHPPD-03 by weight; the molecular weight of AvHPPD-03 was consistent with the predicted molecular weight of 47.0 kDa. The AvHPPD-03 test substance was resolubilized in purified water and included in Western blot, glycosylation status, peptide mass mapping, and N-terminal sequencing analyses. The microbially produced AvHPPD-03 was resolubilized in 25 mM ascorbic acid containing 4 µg/ml bovine catalase for use within the specific enzymatic activity analysis.

### D.2. Source of Microbially Produced PAT

Microbially produced PAT was prepared by BCS in 1995 from a recombinant *E. coli* expression system and further purified and lyophilized into a powder. The resulting dry formulation was used in a number of studies supporting the safety of the PAT protein. The genes expressed in the microbial system and in SYHT0H2 soybean encode proteins identical in amino acid sequence. The PAT test substance was determined to contain 78.3% PAT by weight; the molecular weight of PAT was consistent with the predicted molecular weight of 20.6 kDa. The microbially produced PAT was resolubilized in 10 mM tris(hydroxymethyl)aminomethane (Tris), 0.1% (v/v) Tween 20, 0.4 mM ethylenediaminetetraacetate (EDTA), pH 8.9, and used in Western blot, specific enzyme activity, and glycosylation status analyses.

### D.3. Seed Test and Control Substances

The seed test substance for this study was SYHT0H2 soybean seed. The control substance was nontransgenic, near-isogenic soybean seed variety 'Jack.'

The seed test and control substances were characterized by real-time PCR analysis (Ingham *et al.* 2001) to confirm identity and purity. Prior to this study, the seed test and control substances were grown under standard greenhouse conditions. Seed from SYHT0H2 soybean and 'Jack' soybean were collected, ground into a fine powder, and stored at 2°C to 8°C.

### D.4. Protein Extraction from Soybean Seed for Western Blot and PAT enzymatic activity analysis

Protein for AvHPPD-03 and PAT Western blot analysis, and PAT enzymatic activity analysis, was extracted from the SYHT0H2 soybean seed powder by resuspension in 100 mM borate buffer (pH 7.5) containing 0.2% (v/v) polyvinylpyrrolidone, 7.7 mM sodium azide, and 0.5% (v/v) Tween 20 surfactant, 1.2% (v/v) hydrochloric acid, and supplemented with Roche Complete Protease Inhibitor Cocktail (1 tablet/50 ml). The mixture was homogenized and

incubated on ice. After incubation on ice, the extract was centrifuged and filtered through a 0.22-µm Millipore filter unit; the resulting extract was designated the "SYHT0H2 extract". The concentrations of AvHPPD-03 or PAT and total protein were determined by ELISA and the bicinchoninic acid (BCA) protein assay, respectively.

'Jack' soybean powder was extracted in parallel with preparation of the SYHT0H2 extract, by the same method. The resulting preparation was designated the nontransgenic extract. The nontransgenic extract was analyzed by BCA to determine the concentration of total protein. The nontransgenic extract was used as a negative control in the Western blot analyses.

### D.5. Purified Protein Preparation from SYHT0H2 Extract

SYHT0H2 extract was prepared as described above, and the AvHPPD-03 and PAT proteins were immunopurified from this extract. Immunoaffinity columns prepared with monoclonal antibodies specific to each protein were used to purify AvHPPD-03 and PAT from the SYHT0H2 extract. The SYHT0H2 extract was applied to the equilibrated immunoaffinity column, the column was washed to remove unbound proteins, and AvHPPD-03 or PAT was eluted in 100 mM glycine buffer (pH 2.5) and neutralized. Folliowing immunoaffinity purification, fractions containing AvHPPD-03 were further purified via hydrophobic interaction chromatography. The purified AvHPPD-03 protein was desalted in PD-10 columns and concentrated by ultrafiltration. Following immunoaffinity purification, the purified PAT protein was dialyzed into 0.1 M sodium bicarbonate, pH 8.1, and concentrated by ultrafiltration. The resulting samples were analyzed by ELISA to determine the concentration of AvHPPD-03 or PAT and stored at –20°C ± 8°C until further use. The purified protein preparations from SYHT0H2 extract were used in Western blot, glycosylation status, *N*-terminal sequencing, and peptide mass mapping analyses.

### D.6. Extracts of SYHT0H2 Soybean Seed and Negative Control Soybean Seed for Specific Enzyme Activity Analysis for HPPD

For use in the specific enzyme activity assays, protein was extracted from the SYHT0H2 soybean powder by resuspension in 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) (pH 7.0), 4 mM dithiothreitol, 5 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, and 1% polyvinylpolypyrrolidone. The mixture was mixed thoroughly, incubated on ice, and centrifuged. The soluble portion of the extract was eluted into 50 mM BTP (pH 7.0) and 5 mM potassium chloride through a PD-10 column. The concentration of total active HPPD protein (AvHPPD-03 and endogenous HPPD) in the resulting extract was immediately determined by an active site titration (AST) assay.

Seed from the nontransgenic control soybean was extracted in parallel with preparation of the SYHT0H2 extract, employing the same method. The resulting preparation was designated as the nontransgenic extract. The nontransgenic extract was used in the AST assay and as a control in the enzyme activity assay to account for endogenous HPPD activity.

### D.7. Microbially Produced Protein Spiked into Nontransgenic Extract

Nontransgenic soybean seed extract was prepared as described in D.4 and fortified with microbially produced PAT. This sample was analyzed by Western blot and enzymatic activity

assays in order to investigate if the plant matrix had an effect on PAT enzymatic activity, mobility or immunoreactivity. Inclusion of this sample allowed for the comparison of the microbially produced and plant-produced PAT in the same matrix.

Nontransgenic soybean seed extract was prepared as described in D.4 and fortified with microbially produced AvHPPD-03. This sample was analyzed by an AST assay prior to enzymatic activity analysis, in order to investigate if the plant matrix affects specific enzymatic activity. Inclusion of this sample allowed for the comparison of the microbially produced and plant-produced AvHPPD-03 in the same matrix.

### D.8. Protein Quantification

AvHPPD-03 and PAT quantification was performed with the EnviroLogix Qualiplate ELISA kits, as described in Appendix C.

Total protein was quantified via the BCA method (Hill and Straka 1988), with bovine serum albumin as the reference protein standard. The results were analyzed with Molecular Devices SoftMax Pro GxP software, v. 5.4.1, using a four-parameter algorithm. For each sample, the mean concentration of all dilutions within the quantitative range of the BCA assay was calculated.

### D.9. Immunoreactivity and Molecular-Weight Determination

Western blot analysis was used to investigate the identity and integrity of the microbially produced and plant-produced AvHPPD-03 and PAT proteins, as well as protein purified preparations from SYHT0H2 soybean seed extract. Based on quantification by ELISA, aliquots containing 10 ng of either AvHPPD-03 or PAT prepared in lithium dodecylsulfate sample buffer were subjected to SDS-PAGE under reducing conditions. A 4-12% bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris) gel and 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer were used for AvHPPD-03, and a 12% Bis-Tris gel and 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer were used for PAT. The same running buffers were used for all other procedures described herein that required a running buffer. Based on BCA analysis, an aliquot of the nontransgenic extract with total protein equivalent to that in the samples of plant-produced AvHPPD-03 or PAT prepared for Western blotting was included in the analysis as a negative control. The molecular-weight standard was Invitrogen SeeBlue Plus2 pre-stained standard.

The protein was transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. For detection of AvHPPD-03 and PAT, the membrane was probed with a polyclonal rabbit antibody or goat antibody, respectively, and the protein was detected by binding of a polyclonal donkey anti-rabbit antibody or anti-goat immunoglobulin G, respectively, conjugated with alkaline phosphatase, which catalyzes the conversion of the colorimetric substrate 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro-blue tetrazolium chloride. The Western blot was visually examined for the presence of intact immunoreactive AvHPPD-03 or PAT or immunoreactive fragments derived from AvHPPD-03 or PAT.

### D.10. Enzyme Activity Assay for AvHPPD-03

A validated radioactive $^{14}CO_2$ trapping assay was used to determine the specific enzyme activity of HPPD from (1) microbially produced AvHPPD-03, (2) SYHT0H2 soybean seed extract, (3) nontransgenic soybean seed extract, and (4) microbially produced AvHPPD-03 spiked into the nontransgenic extract. The nontransgenic extract was used as a negative control to account for endogenous HPPD activity. HPPD catalyzes the formation of HGA and carbon dioxide from HPP and molecular oxygen (Figure D-1). The HPPD enzyme activity assay determines the amount of radiolabeled $^{14}CO_2$ generated from a $^{14}C$-labeled HPP substrate during the enzymatic reaction (Barta and Böger 1996).


**Figure D-1. Reaction catalyzed by HPPD**

AvHPPD-03 enzyme activity is reported as units per microgram of HPPD, where 1 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 under the described reaction conditions. Specific enzyme activity values differing by 30% or less are considered comparable and support a conclusion of functional equivalence.

The enzyme activity assays were performed at 25°C ± 0.2°C in duplicate at four time points (0, 1, 3, and 6 minutes). A substrate mixture consisting of 63 µM unlabeled HPP in 50 mM BTP buffer (pH 7) containing 25 mM sodium ascorbate, 4 µg/ml bovine catalase, and approximately 15 µM $^{14}C$-HPP was prepared. Prior to use, the purity of $^{14}C$-HPP was determined via high-pressure liquid chromatography (HPLC) with a β-RAM radioactivity detector.

The substrate mixture was transferred into individual reaction chambers capped with tightly fitted rubber stoppers. The specific radioactivity of the freshly prepared substrate mixture was measured, and the enzyme activity assay was initiated by addition of HPPD to the substrate mixture. A suspended filter soaked with 1 N sodium hydroxide was used to trap the $CO_2$ generated during the reaction. The reactions were stopped after 0, 1, 3, and 6 minutes by addition of 0.6 N trichloroacetic acid.

After each reaction was stopped, $CO_2$ trapping was allowed to continue for 90 minutes at 25°C ± 0.2°C. Radioactivity trapped in the filter was measured by liquid scintillation counting. The total disintegrations per minute for each time point was corrected for background by subtraction of the measured radioactivity at time zero.

### D.11. Enzyme Activity Assay for PAT

A continuous spectrophotometric assay based on the method described by Thompson *et al.* (1987) and D'Halluin *et al.* (1992) and validated for quantification of PAT activity was used to measure the specific enzyme activity of PAT from (1) microbially produced PAT, (2) SYHT0H2 soybean seed extract, (3) nontransgenic soybean seed extract (negative control), and (4) nontransgenic extract fortified with microbially produced PAT in triplicate analyses.

As shown in Figure D-2, PAT catalyzes the transfer of the acetyl group from acetyl CoA to phosphinothricin. The released free thiol (CoASH) reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoate anion ($TNB^{2-}$) under mild alkaline conditions (pH 7 to 8) (Habeeb 1972). The molar formation of $TNB^{2-}$ can be monitored by measuring the absorbance increase at 412 nm and can be directly converted to the molar acetylation of phosphinothricin by PAT. PAT enzyme activity is reported as units per microgram of PAT, where 1 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 DTNB reduced or 1 µmol of $TNB^{2-}$ produced per minute) under the described reaction conditions.

phosphinothiricin + acetyl CoA $\xrightarrow{\text{PAT}}$ *N*-acetyl-phosphinothricin + CoASH

CoASH + DTNB $\rightarrow$ mixed-disulfide CoA-TNB + $TNB^{2-}$

**Figure D-2. Reaction catalyzed by PAT**

The enzyme reactions were conducted in 96-well plates. Following a 2-minute preincubation at 25°C ± 2°C, the reaction was initiated by addition of PAT to an assay mixture containing 1 mM phosphinothricin, 1 mM acetyl CoA and 1 mM DTNB in 50 mM Tris, 2 mM EDTA, and 0.5 mg/ml BSA, pH 7.5. The total volume of the reaction mixture was 100 µl.

The formation of $TNB^{2-}$ was monitored spectrophotometrically at 412 nm with Molecular Devices SoftMax Pro GxP software, v. 5.4.1, at 25°C ± 2°C over 5 minutes, with readings taken every 12 seconds. The extinction coefficient of $TNB^{2-}$ is 14,150 $M^{-1}$ $cm^{-1}$ (Riddles *et al.* 1979, 1983), which was used to calculate the amount of $TNB^{2-}$ formed in accordance with the Beer-Lambert law (Aitken and Learmonth 1996).

### D.12. Glycosylation Analysis

TFor AvHPPD-03 and PAT, the microbially produced proteins and proteins purified preparation from SYHT0H2 soybean seed extract were analyzed with the ECL Glycoprotein Detection Module Kit to confirm the absence of glycosyl residues. Samples were subjected to SDS-PAGE under reducing conditions with a running buffer. The protein was applied to the gel at 500 and 1000 ng. Transferrin, a glycosylated protein, was applied to the gel at 10, 25, 50, and 100 ng as a positive control. Soybean trypsin inhibitor, a nonglycosylated protein, was applied to the gel at 1000 ng as a negative control. The molecular weight standard was Invitrogen SeeBlue Plus2 pre-stained standard.

The protein was electroblotted onto a PVDF membrane. While on the membrane, glycan moieties were oxidized with sodium metaperiodate, labeled with biotin, and detected with alkaline-phosphatase-linked streptavidin.

### D.13. Peptide Mass Mapping Analysis

For AvHPPD-03 and PAT, aliquots containing 0.2 to 1 µg of the microbially produced protein and the purified protein preparations from SYHT0H2 soybean seed extract were subjected to SDS-PAGE under reducing conditions. The gel was stained with Coomassie blue, and the protein band consistent with the predicted molecular weight of AvHPPD-03 or PAT was excised from the gel. The protein was reduced, alkylated with iodoacetamide, and enzymatically digested with trypsin and chymotrypsin for AvHPPD-03 and trypsin, chymotrypsin, and flavastacin (endoproteinase AspN) for PAT. A separate digestion was conducted with each enzyme. The digested samples were analyzed by liquid chromatography–tandem mass spectrometry with a quadrupole time-of-flight mass spectrometer (Waters Micromass Q-Tof Premier for AvHPPD-03 or Waters Xevo QTof for PAT) coupled to a capillary liquid chromatography instrument (Waters CapLC for AvHPPD-03 or Dionex UltiMate 3000 Nano HPLC for PAT). The detected peptide masses were searched with Mascot software against a protein database containing the expected amino acid sequence of both proteins. Peptide mass mapping analysis was conducted by Syngenta Jealott's Hill International Research Centre (AvHPPD-03) (Bracknell, England, UK) and SGS M-Scan Ltd. (PAT) (Wokingham,UK).

### D.14. N-Terminal Amino Acid Sequence Analysis

For AvHPPD-03 and PAT, the *N*-terminal amino acid sequences of the microbially produced protein and purified protein preparation from SYHT0H2 soybean seed extract were determined and compared with the predicted amino acid sequence. The protein from both sources was subjected to SDS-PAGE under reducing conditions, followed by electroblotting to a PVDF membrane. The blot was stained with amido black, and the protein bands corresponding to the predicted molecular weight of AvHPPD-03 or PAT were excised and sent to SGS M-Scan, Ltd. (Wokingham, England, UK).

The samples were applied to an automated pulsed-liquid sequencer for N-terminal amino acid sequence analysis. The methodology used was developed for proteins immobilized on a PVDF membrane and optimized for automated Edman degradation analysis. The N-terminal amino acid sequencing was conducted by SGS M-Scan Ltd.

### D.15. References

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## Appendix E. SYHT0H2 Soybean: Field Evaluation of Trait Efficacy

Performance of the herbicide-tolerance traits expressed by SYHT0H2 soybean has been evaluated in several field trials. In a representative 2010 U.S. field trial, mesotrione and glufosinate-ammonium herbicides were applied in a tank mixture at twice their normal use rates to SYHT0H2 and nontransgenic control soybean at the V2 to V3 growth stage. The test substance was the seed of SYHT0H2 soybean in the genetic background 'Jack', and the control substance was seed of the nontransgenic, near-isogenic soybean 'Jack.' The test and control substances and herbicide treatments are listed in Table E-1.

**Table E-1. Test and control substances and treatments**

| | | Herbicide application rate (g a.i./ha) | |
|---|---|---|---|
| **Entry description** | **Entry identification** | **Mesotrione** | **Glufosinate** |
| Test substance | generation $T_5$ SYHT0H2/'Jack' | 210 | 900 |
| Nontransgenic, near-isogenic control | 'Jack' | 210 | 900 |
| Test substance — no herbicide | generation $T_5$ SYHT0H2/'Jack' | none | none |
| Nontransgenic control — no herbicide | 'Jack' | none | none |

Eight locations in the midwest were selected as representative of where maturity zones 2 to 4 soybean varieties would be grown commercially: Clinton, IL; Cumming, IA: Fowler, IN; Bement, IL; Sadorus, IL; Seward, NE; Slater, IA; and Westboro, MO. These trials were planted under USDA notification 10-062-113n. The trials were planted in a randomized split-plot design, with herbicide treatments as the whole plots and soybean entries as subplots and with three replicate plots per treatment. Each subplot consisted of four rows 15 feet long. Only the center two rows of each plot were sprayed with the herbicides.

The herbicides mesotrione, formulated as Callisto® herbicide, and glufosinate-ammonium, formulated as Ignite® 280 SL herbicide, were applied at the V2 to V3 growth stage, at 210 and 900 g a.i./ha, respectively. Post-emergence application at V2 to V3 is anticipated to be a likely recommendation to growers when SYHT0H2 soybeans are commercialized. The herbicides were applied at approximately twice their normal use rates to evaluate plant tolerance under conditions where an individual row might receive two applications as a result of overlapping sprays. The plots were visually rated for general phytotoxicity responses at 5 to 11, 14 to 21, and 25 to 36 days after treatment. Phytotoxicity was rated on a plot basis, not an individual plant basis, on a scale of 0% to 100%, where 0% represented no visible evidence of phytotoxicity and 100% represented maximum phytotoxicity (*i.e.* plant death). The trials were managed according to local agronomic practices.

A mean phytotoxicity rating was computed for each entry at each location, and an across-location mean rating with standard deviation was computed for each entry. Because all phytotoxicity ratings for the untreated SYHT0H2 and control soybean entries were 0%, no formal statistical analysis of differences between the untreated SYHT0H2 and control means was warranted. Likewise, a formal statistical comparison of herbicide-treated entries was not performed, because most of the control responses were 100% phytotoxicity.

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No phytotoxicity was observed in the untreated plots. Across-location mean phytotoxicity ratings for the herbicide-treated entries are shown in Figure E-1. The control plots treated with mesotrione and glufosinate-ammonium had very high phytotoxicity ratings at all observation intervals. Transient phytotoxicity, primarily chlorosis or bleaching, was observed in the SYHT0H2 plots. At 5 to 11 days after herbicide application, the mean phytotoxicity for the SYHT0H2 soybean plots across locations was 18.3%, and phytotoxicity ranged from 1% to 40% in individual plots. At 14 to 21 days, visible phytotoxicity had declined to an across-location mean rating of 9.8%, and by days 25 to 36 (4 to 5 weeks), the SYHT0H2 soybean plants had nearly fully recovered, with an across-location mean phytoxicity rating of 4%.

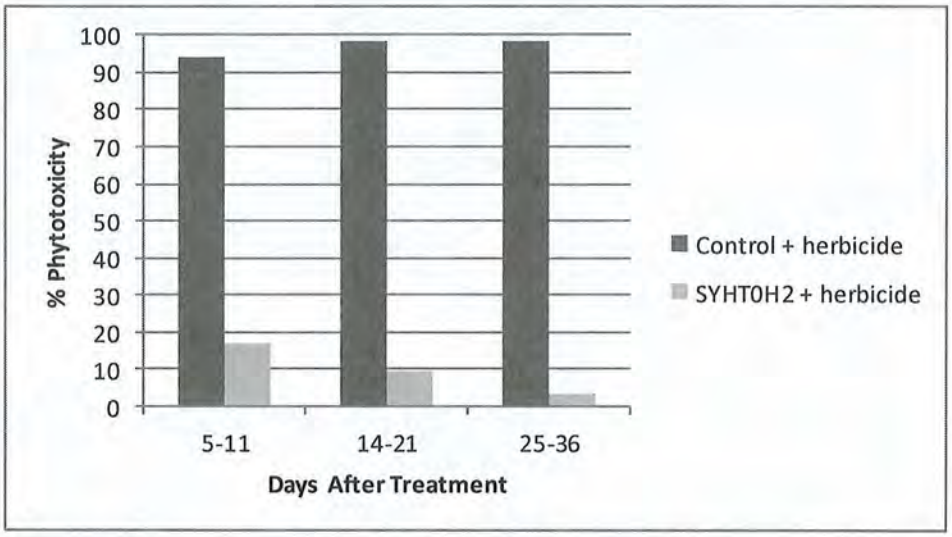


**Figure E-1. Mean phytotoxicity ratings for herbicide-treated control and SYHT0H2 soybeans**

The results of these trials demonstrate that SYHT0H2 soybean plants tolerate applications of mesotrione and glufosinate-ammonium herbicides at twice their recommended use rates. Transient phytotoxicity, evidenced primarily by chlorosis or bleaching, was observed in a multi-location replicated field trial. The plants outgrew the injury and were nearly fully recovered four to five weeks after application.

## Appendix F. Resistance Management and Sustainable Herbicide Use with SYHT0H2 Soybean

### F.1. Introduction

SYHT0H2 soybean exhibits tolerance to mesotrione and isoxaflutole herbicides, inhibitors of HPPD. Additionally, SYHT0H2 soybean exhibits tolerance to glufosinate-ammonium herbicide, an inhibitor of glutamine synthetase. The herbicide-tolerance traits in SYHT0H2 soybean will provide growers with more tools and greater flexibility and diversity in weed management options, and contribute to more sustainable weed management when used in combination with other herbicides and weed management tactics.

Syngenta and BCS are committed to actively promoting the responsible use and sustainability of this new herbicide tolerance technology in soybeans, as well as preserving the viability of HPPD-inhibiting and glufosinate-ammonium herbicides in corn and glufosinate-ammonium in other crops. Accordingly, Syngenta and BCS will coordinate and implement a comprehensive resistance management and stewardship plan for SYHT0H2 soybeans and for their HPPD-inhibiting herbicides and glufosinate-ammonium herbicide, focusing on educating and training growers, applicators, dealers, retailers, and other end users on the appropriate use of the technology.

Herbicides are the most predominant, effective, and economical weed-control tools for broad acre crops such as soybean, corn, wheat, and cotton in North America. They are critical to food, feed, fiber, and energy security and the sustainability of North American agriculture.

Over time, consolidation in agriculture and improvements in crop production technology have resulted in increased farm size. Economics and the need for simplicity and convenience in managing larger farms have caused growers to adopt monocultures, or less crop diversity, along with less herbicide diversity in their management practices. Concurrently, conservation tillage practices such as minimum-till or no-till cropping systems, which prevent soil erosion and have other environmental benefits, have reduced the use of mechanical weed control.

The overreliance upon or repeated use of a single herbicide or herbicide group with the same mode of action (MOA) can result in weed shifts (Webster and Nichols 2012) and the evolution of herbicide-resistant weeds. Herbicide resistance is defined by the Weed Science Society of America (WSSA) and the Herbicide Resistance Action Committee (HRAC) as the naturally occurring, heritable ability of some weed biotypes within a given population to survive a herbicide treatment (and reproduce) that should, under normal use conditions, effectively control that weed population. Herbicide resistance development is a function of many factors, including a weed species' genetic diversity, reproductive capability, and biology, and the selection pressure and inherent effectiveness of the herbicide on that species.

### F.2. Herbicide Resistance Evolution

Herbicide resistance is not a new phenomenon; resistance to synthetic auxins was first documented in the 1950s and 1960s (Heap 2012). At least 383 herbicide-resistant biotypes have been reported to date in 208 weed species worldwide. Over time, weeds have evolved resistance

to most major herbicide classes. The number of resistant weed biotypes identified over time is illustrated in Figure F-1.

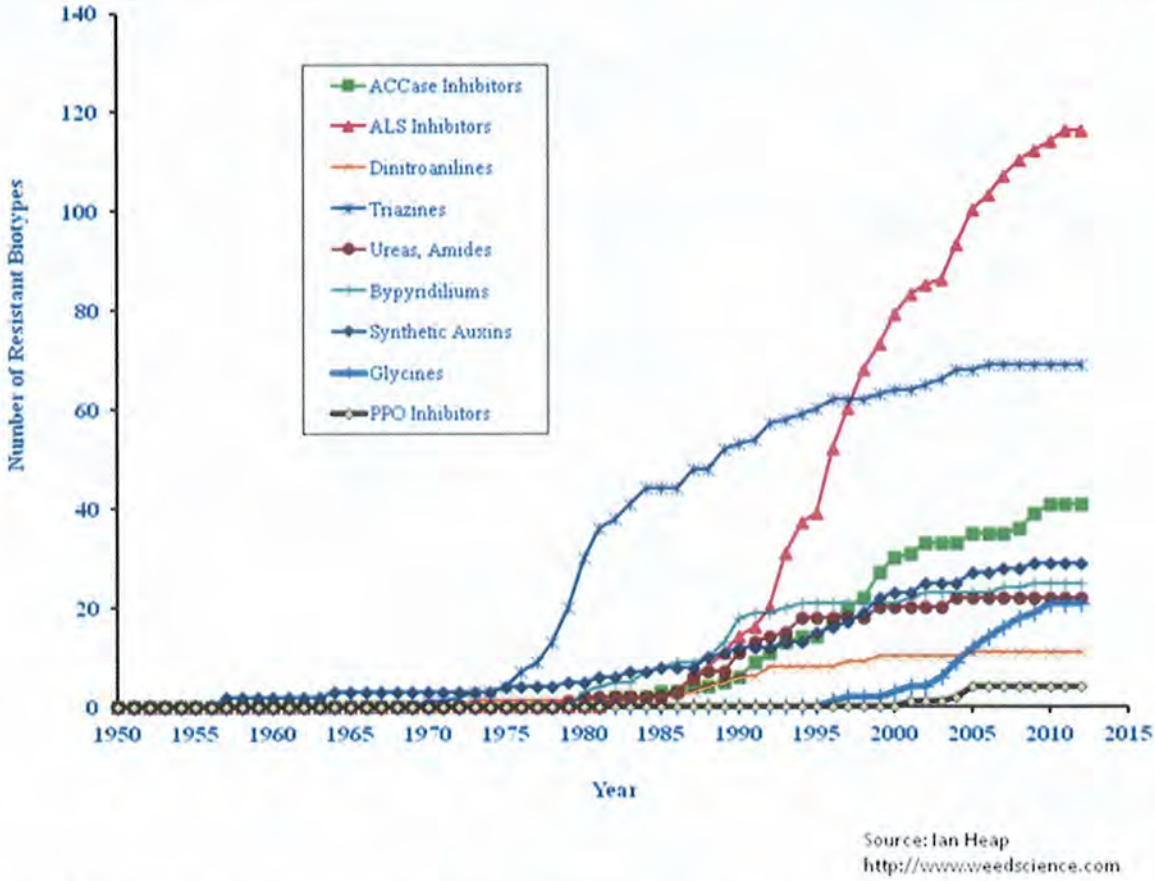


**Figure F-1. Worldwide resistance to various herbicide modes of action**

Since 1990, the number of weed biotypes resistant to acetolactate synthase (ALS) inhibitors has increased dramatically. These biotypes are now widespread and limit the utility of ALS inhibition as an MOA; to date, 43 resistant biotypes have been identified in the U.S. and 123 have been identified worldwide (Heap 2012). The effectiveness of ALS inhibitors for weed control in many crops, due to their crop safety and convenience, led to high adoption, lack of diversity in weed management strategies in these crops and, ultimately, the evolution of resistant weeds.

Glyphosate, a glycine herbicide, acts by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase. In the U.S., the convenience and effectiveness of glyphosate-tolerant crops led to rapid adoption of the technology after its introduction in soybeans in 1996, in cotton in 1997, and in corn in 1998. In turn, massive selection pressure and lack of diversity in weed management strategies in these crops have driven the evolution of glyphosate-resistant weeds. In 2011, 80% of U.S. corn acres were treated an average of 1.3 times with glyphosate, and over 95% of soybean acres were treated an average of 1.5 times (Syngenta 2011). By 2012, 13 weed species

had been confirmed resistant to glyphosate in the U.S., and 23 resistant species had been identified worldwide (Heap 2012).

As weeds evolve resistance to herbicides, greater selection pressure is exerted by an alternative herbicide with a different MOA that is applied to control the resistant biotype. Hence, weed populations can evolve resistance to more than one herbicide MOA.

### F.3. Weed Management and Sustainability Program

Syngenta and BCS will promote a multi-pronged approach to the responsible use and sustainability of their HPPD-inhibiting herbicides and glufosinate-ammonium on SYHT0H2 soybean. This integrated weed management and stewardship plan has the following specific goals:

- Promoting the most responsible use of SYHT0H2 soybean, HPPD-inhibiting herbicides, and glufosinate-ammonium for long-term effectiveness and viability for growers.
- Providing growers, dealers, retailers, agronomists, and applicators with education and training on guidelines for responsible technology use.

To meet the above goals, Syngenta and BCS are developing a comprehensive resistance management program based on input from weed scientists and agronomists and on previous experience in the following areas, which are explained in greater detail below:

- technology use grower agreements
- integrated weed management and herbicide resistance recommendations
- customer education and training
- product use information and support
- monitoring of grower use of herbicides

The elements and goals of this herbicide resistance management program are consistent with those of the WSSA, which has developed guidance at the request of the USDA Agricultural Research Service (Norsworthy *et al.* 2012).

#### F.3.a. Technology Use Grower Agreements

A Technology Use Agreement will be signed by growers at the time of SYHT0H2 soybean seed purchase. By purchasing the seed and signing the agreement, the grower will agree to practice the integrated weed management strategies described in the Technology Use Agreement. The agreement will contain information on accessing further product stewardship and weed management information from each company's web site. Telephone numbers for customer service hotlines of each company will also be included, whereby growers can obtain live technical support and answers to questions related to SYHT0H2 soybeans, product stewardship, and integrated weed management and herbicide resistance.

Seed company partners that sell SYHT0H2 soybeans through seed dealer or retail networks will also be trained on integrated weed management and stewardship of the technology. Growers will be able to contact these seed companies for product support via contact information that will

be provided on each seed bag label and bag tag. Growers who purchase SYHT0H2 soybean seed will be recorded by the seed company. These grower data will be collected by Syngenta and BCS and maintained in databases that can be used to distribute stewardship information to growers using the technology.

#### F.3.b. Herbicide Resistance Management

Proactive, integrated, and diversified weed management and cropping strategies minimize the risk of selecting for herbicide-resistant plants within a weed population. It is important not to rely on a single tactic to manage weeds. To help prevent the development of weed populations resistant to HPPD inhibitors, glufosinate-ammonium, or any other herbicide technology, Syngenta and BCS recommend the following:

- Start with clean fields.

Plant into weed-free fields and keep fields as weed-free as possible. Use tillage or effective burndown herbicides to control all emerged weeds prior to planting. Starting with clean fields reduces early-season weed competition for moisture, nutrients, and light and, therefore, increases crop yields. Use a residual herbicide pre-emergence or early post-emergence at the full labeled rate as part of every weed management program. Residual herbicides can be applied with the burndown herbicide on no-till acres. Soil-residual herbicides reduce the weed population, widen the post-emergence herbicide application window, and introduce more mechanisms of herbicide action into the management plans. Mesotrione, isoxaflutole, and glufosinate-ammonium, combined with other selective residual soybean herbicides, are effective weed management tools.

- Use several herbicide modes of action.

Use an herbicide program with several MOAs that have overlapping efficacy on the toughest-to-control or most problematic weed species in the field in rotation, sequences, or mixtures. Herbicide mixtures should include at least two MOAs that are fully effective against the target weed species. The best practice is to use a two-pass herbicide program (pre-emergence followed by post-emergence) that includes more than one effective MOA in each application. If two applications of an HPPD-inhibiting herbicide (where permitted by the product label) or glufosinate-ammonium are made during a single growing season, each application should be combined in a mixture with at least one other herbicide having a different MOA and overlapping efficacy on the target weeds.

- Apply herbicides properly.

Apply post-emergence herbicides at the proper weed size or stage using the full labeled rate with the recommended adjuvants to control the toughest or most problematic weed species in the field. Herbicides applied at rates lower than those listed on the label can result in poor weed control during the current season and can lead to increased tolerance or resistance in the weed population by allowing partially controlled weeds to reproduce. Post-emergence herbicides are most effective when applied at full rates to small, actively growing weeds. Growers and applicators must also follow labeled use directions to ensure they are making applications using the proper spray nozzles, carrier volume, and adjuvants for each herbicide, as these all can affect herbicide efficacy.

### F.3.c. Cultural Tactics for Herbicide Resistance Management

Integrating the following cultural practices whenever possible will help manage weeds and mitigate herbicide resistance, introducing more diversity and less reliance strictly on chemical weed control.

- Rotate crops.

Economics drive many planting decisions a grower makes, but crop rotation can be an important tool to introduce diversity into an integrated weed management program. Crops differ in their competitiveness with weeds and have different planting dates and cultural practices, which add diversity in the system. Crop rotation also allows herbicide diversity on a given field. Corn and soybean grown in rotation allow for the use of herbicides with different MOAs in a field.

- Use good agronomic practices.

Practicing good agronomy can facilitate weed management by increasing crop competitiveness with weeds. In soybean, incorporating the use of seed-applied fungicides and insecticides, proper fertility, and narrow row spacing can all promote soybean growth and canopy closure.

- Incorporate mechanical weed control.

Where appropriate, growers should incorporate tillage and row cultivation into their cropping practices.

- Clean equipment between fields.

Equipment should be cleaned to remove soil and plant residues, to prevent transporting weeds or weed seeds from one field or farm to another.

- Scout fields for weeds.

Growers and their agronomists or crop consultants should recognize and know the weed species and populations on their farms and fields in order to design the most effective management program for those species present. Scouting fields routinely before and after herbicide applications is essential for proper weed management and identification of any problematic weeds or escapes and to ensure that weed control is achieved. Growers, agronomists, and crop consultants should report any suspected resistant weed populations or repeated non-performance of mesotrione, isoxaflutole, or glufosinate-ammonium to their local dealer, retailer, university extension agent, or Syngenta or BCS representative.

- Reduce weed seed bank.

Weeds should not be allowed to survive and reproduce in growers' fields. Escapes should be eliminated with cultivation, hand removal, or spot application of another herbicide with a different MOA before they can reproduce or set seed.

### F.3.d. Customer Education and Training

Effective communication and education on integrated weed management is the best and most practical method for success. The education process will start internally with Syngenta and BCS

field development, product evaluation, technical service, agronomy services, and crop protection and seed sales personnel. Syngenta and BCS will also work with key influencers such as university extension weed scientists and agronomists, consultants, farm managers, and seed and crop protection retailers. These groups influence grower decisions and can help growers understand the economic impacts and reinforce the need for integrated weed management and stewardship of SYHT0H2 soybean and other technologies.

Syngenta and BCS will educate growers and end users of SYHT0H2 soybeans through grower meetings, trade shows, direct mail, and electronic communications and training. Grower education and training for SYHT0H2 soybean stewardship can be incorporated into online herbicide resistance management initiatives such as Syngenta's Resistance Fighter and BCS's Respect the Rotation web sites (www.resistancefighter.com and www.bayercropscience.us/our-commitment/respect-the-rotation, respectively).

#### F.3.e. Product Use Information and Support

Several sources of information will be available to growers to support and guide the use of appropriate crop management practices for SYHT0H2 soybean and the relevant herbicide products.

- Herbicide product labels.

Herbicide product labels are the formal and legal method of communicating use directions for the herbicides and herbicide programs that will be developed and registered for use on SYHT0H2 soybeans. In addition to directions for proper product use, the labels will and do include specific recommendations for integrated weed management and herbicide-resistant weed management.

- Seed bag labels and tags.

SYHT0H2 soybean seed bag labels and bag tags will provide product and stewardship information and customer service contact information.

- Weed management guides.

Marketing materials and product use guides or fact sheets will indicate which herbicides can be used on the crop, as well as practices growers should follow when using the technology. Syngenta and BCS support research conducted by agricultural research universities, which helps to generate regional and local weed management recommendations for growers. Both companies also support the university extension weed science community with the development of their respective weed-control or pest-management guides and handbooks, which contain use directions for herbicides and herbicide-tolerant crop traits and are valuable additional information sources available to growers.

#### F.3.f. Performance and Screening for Herbicide Resistance

Syngenta and BCS provide, and will continue to provide, in-field support to growers when seed or crop protection products do not perform as expected. When weed species survive a herbicide application and herbicide resistance is suspected, each instance is fully investigated. The

investigation considers all the factors that could account for lack of weed control, including use of the proper herbicide application rate, spray volume, and spray pressure; weed size and stage at application; or adverse environmental conditions during the time of application.

If none of these factors can explain the lack of performance, and herbicide resistance is suspected, follow-up tests can be conducted. In-field trials or seedling bioassays can be used to indicate whether a weed biotype has evolved resistance to an herbicide. Seed from suspect weeds that survived the herbicide application(s) can also be collected, grown out, and evaluated for herbicide resistance under greenhouse conditions. The first priority is to provide the grower with advice and weed management options for the current season.

If follow-up testing on the suspected resistant weed population indicates that the lack of control at labeled product rates may be attributable to herbicide resistance, and if the weed biotype has not been reported as resistant to that herbicide previously, then more detailed studies can be conducted to confirm whether the weed is resistant. As needed, the scientific community and university weed scientists can be engaged for follow-up testing for herbicide resistance.

#### F.3.g. Response to Confirmed Cases of Herbicide Resistance

Resistance will be confirmed only if two criteria outlined by the WSSA (1998) are met: (1) the suspect weed biotype survives labeled rates of the herbicide that previously controlled it, and (2) the resistance is heritable.

If herbicide resistance is verified, the weed science and grower communities will be notified, and development of specific resistance mitigation strategies for that species will be initiated in collaboration with university weed scientists. Once these resistance mitigation strategies are developed and validated, they will be communicated to growers, as appropriate, by various means, including grower and retailer meetings and training programs, fact sheets, agricultural news media, or supplemental labeling. In addition, confirmed reports of resistant weed biotypes will be logged on the International Survey of Herbicide Resistant Weeds web site (Heap 2012).

#### F.3.h. Monitoring Grower Use of Herbicides

As described above, growers who purchase SYHT0H2 soybeans will be required to sign the Technology Use Grower Agreement and will be recorded by the seed companies making the sale; grower databases will be maintained. Observance by growers of the terms in the technology use grower agreement, product labels, and integrated weed management strategy will be monitored and tracked through surveys, direct communications with growers, or on-farm visits. Growers will be required to provide (upon request) locations of fields planted with SYHT0H2 soybean and to indicate which herbicides were applied to those fields. Failure to follow the technology use agreements or product labels could result in a grower's loss of access to SYHT0H2 soybean seed.

### F.4. Weed Control with Glufosinate-ammonium

Glufosinate-ammonium provides broad-spectrum weed control, including control of weeds resistant to other herbicide MOAs. To date, there are no documented cases of glufosinate-ammonium herbicide resistance in the broad acre crops (corn, cotton, soybean, and cereals) in

North America. Regardless, the use of glufosinate-ammonium should be part of a well-rounded herbicide resistance management plan to maintain its usefulness for years to come. Glufosinate-ammonium should be used in conjunction with an HPPD inhibitor and/or other effective conventional herbicide chemistry as one approach to weed control in SYHT0H2 soybeans.

### F.5. Weed Control with and Herbicide Resistance to HPPD Inhibitors

HPPD-inhibiting herbicides provide control of a wide spectrum of broadleaf weeds, including weeds resistant to other herbicide MOAs. To date, weed resistance to HPPD inhibitors has been reported only in U.S. seed corn production fields.

Resistance to post-emergence applications of HPPD inhibitors was confirmed in a common waterhemp (*Amaranthus tuberculatus* [syn. *rudis*]) biotype in a McLean County, Illinois, seed corn production field in July 2010 (Ma *et al.* 2011, Heap 2012). The field had been in continuous seed corn production and over a 7-year period had received 11 applications of HPPD inhibitors. Greenhouse studies indicated that the McLean County waterhemp population was more tolerant to the HPPD inhibitor mesotrione applied pre-emergence than was a known mesotrione-sensitive waterhemp population. However, herbicide mixtures with *s*-metolachlor and atrazine still provided excellent control.

In 2010, post-emergence HPPD-inhibitor-resistant waterhemp was confirmed in a southeastern Iowa seed corn production field (Franssen *et al.* 2011, Heap 2012), which had received four applications of HPPD inhibitors every other year from 2001, until lack of control was observed in 2009. In 2011, a waterhemp population in a seed corn production field in eastern Nebraska was confirmed resistant to post-emergence applications of HPPD inhibitors (Heap 2012), and a waterhemp population in a seed corn production field in southeastern Iowa was confirmed resistant to both glyphosate and HPPD-inhibiting herbicides (Heap 2012). Both of these waterhemp populations had received four applications of HPPD inhibitors every other year from 2001, until a lack of control was observed in 2009–2010.

Weed management is often poor in seed corn production fields. In comparison with commercial hybrid corn, seed corn is generally less competitive with weeds, because the canopy is less dense; the male plants are removed following pollination, and the remaining rows of female plants lose significant foliage as a result of detasseling operations. Furthermore, the inbred lines used in seed corn production are often more sensitive than hybrid corn varieties to injury from some classes of herbicide chemistry, thus limiting weed-control options. Seed corn tolerates HPPD inhibitors well, and these herbicides have therefore been relied upon heavily for weed management, often with insufficient herbicide diversity to delay the evolution of resistance. A deliberate and integrated herbicide resistance management strategy is therefore required for sustainable use of HPPD inhibitors in corn and soybean to delay further resistance development.

### F.6. HPPD Inhibitor Use in Corn and Soybean

Corn is naturally tolerant to HPPD-inhibiting herbicides, which have been used by corn growers for more than a decade. Once SYHT0H2 soybean is commercialized and HPPD-inhibiting herbicides are used in soybeans, education in herbicide stewardship and resistance management practices will be important for growers, agronomists, dealers, and retailers using these products.

Conditions after soybean harvest are sometimes conducive to germination and growth of unharvested soybeans in the subsequent rotational crop. SYHT0H2 soybean volunteers can be controlled in the following crop through the use of an herbicide with a MOA that is not selective in soybeans, such as a synthetic auxin (e.g., dicamba or 2,4-D) or atrazine.

To mitigate herbicide resistance to HPPD inhibitors and glufosinate-ammonium, specific recommendations for use of these herbicides in SYHT0H2 soybean, field corn and seed corn for management of weeds, including waterhemp, are provided below.

#### F.6.a. Management Recommendations for Weed Control in Soybean and Hybrid Corn

- Always use a full rate of a residual pre-emergence herbicide as specified on the product label.
- Do not rely solely on post-emergence applications of HPPD-inhibiting herbicides for control of target weeds; integrate other herbicides that are active on the target weeds such as glufosinate-ammonium, metribuzin and fomesafen for soybeans or glufosinate-ammonium, glyphosate, atrazine, and/or dicamba for corn.
- For post-emergence herbicide applications, use full labeled rates with recommended adjuvants on small weeds.
- Use herbicide combinations with several MOAs with overlapping efficacy on target weeds (i.e., chose herbicide combinations in which each active ingredient is effective against the most difficult-to-control target weeds).
- Strive for full and effective season-long weed control. Control weeds that have escaped herbicide treatment, and do not allow weeds to go to seed.

#### F.6.b. Management Recommendations for Weed Control in Seed Corn Production

- Do not plant continuous seed corn. Rotate to another crop, and use full herbicide rates to control weeds in that crop.
- Use a pre-emergence herbicide program that includes several MOAs with overlapping efficacy against target weeds, either as a commercial premix or as a tank-mix at the maximum rate allowed for that soil type.
- Use an effective early post-emergence herbicide if no activating rainfall or irrigation is received within five to seven days following the pre-emergence herbicide application.
- Cultivate for weed control one to two weeks after the early post-emergence application; cultivation must be planned and timely.
- Use post-emergence applications as needed to control weed escapes. Use effective herbicides with residual activity, with drop nozzles or post-directed, if needed.
- After male rows are mowed, apply glyphosate plus a residual herbicide to control late-emerging weeds where crop canopy no longer provides weed suppression.
- Use post-harvest non-selective herbicides if weeds emerge after harvest.
- Do not allow weeds to go to seed.

### F.7. Modeling the Evolution of Herbicide Resistance

Using a model of herbicide resistance in cotton, Neve *et al.* (2011a, 2011b) concluded that glyphosate-resistant weeds can evolve after only four or five years of continuous glyphosate use. This model prediction is consistent with observations based on actual glyphosate use. Neve *et al.* demonstrated that management strategies, such as diversity of herbicide MOAs providing full, effective season-long weed control, could delay or mitigate glyphosate resistance in *P. amaranth* for at least 20 years.

Syngenta and BCS will explore the development of similar predictive models of herbicide resistance to support the ongoing refinement of product use recommendations for SYHT0H2 soybean and associated herbicides.

### F.8. Stewardship of SYHT0H2 Soybeans and Industry Commitment

Syngenta and BCS place high importance on the sustainability of all of their technologies. Stewardship principles guide all stages of development, from research and development through marketing and product discontinuation. Both Syngenta and BCS are founding members of Excellence Through Stewardship (www.excellencethroughstewardship.org), whose mission is the responsible management of biotechnology products through development and implementation of effective stewardship practices and providing public education on those practices.

Syngenta and BCS participate in numerous industry and professional initiatives in support of product stewardship, including the following:

- **Herbicide Resistance Action Committee** is an international industry initiative that supports cooperation between crop protection manufacturers, government and university researchers, advisors, and farmers. The objective of the Committee is to facilitate a cooperative approach to the management of herbicide resistance. Weed scientists from both Syngenta and BCS participate as members of HRAC, and both companies support its work. In addition, both companies are active in its newly formed HPPD Inhibitor Working Group.

- **CropLife America** (CLA) promotes the development of industry-wide science-based approaches to regulatory and technology management issues to maximize the benefits of plant technologies. CLA strives to enable farmers to produce the best-quality and highest-yielding crops possible, while providing consumers with a safe, affordable, and dependable food supply.

- **American Seed Trade Association** (ASTA) is a seed industry group that sets industry standards for seed quality and purity as well as product stewardship. ASTA promotes the development of better seeds to produce bettercrops for a better quality of life.

- **Weed Science Society of America** is a nonprofit professional society established to promote research, education, and extension outreach activities related to weeds and weed science. WSSA provides science-based information to the public and to policy makers and also foster awareness of weeds and their impact on managed and natural ecosystems. Both Syngenta and BCS are sustaining members of the WSSA and its Western, Southern,

North Central and Northeastern branches. Syngenta and BCS scientists are active in these weed science societies, holding offices and participating in committees.

### F.9. Conclusions

Syngenta and BCS are committed to sustainable use of SYHT0H2 soybeans through integrated weed management strategies that will maintain the viability of HPPD-inhibiting herbicides and glufosinate in soybean and other crops. Both companies are working to develop comprehensive stewardship programs and integrated weed management strategies, and will work to successfully implement those strategies in soybeans and corn. The strategies will focus on educating and training dealers, retailers, growers, agronomists, and herbicide applicators on the appropriate use of SYHT0H2 soybean and the herbicides to which it provides tolerance. A comprehensive approach will be used, employing a variety of effective tools and delivery methods, working directly with customers, university weed scientists, extension specialists, and industry organizations. The ultimate aim is the preservation of these important tools for effective, long-term use by growers.

### F.10. References


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