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Science For A Better Life

Petition for a Determination of Nonregulated Status for Herbicide Tolerant Cotton Transformation Event GHB811

Petition 17-138-01p

OECD Unique Identifier BCS-GH811-4

Submitted By
Bayer CropScience LP
2 T.W. Alexander Drive
Research Triangle Park, NC 27709

Corresponding Author
Michael Weeks, M.Sc.
U.S. Regulatory Manager - Seeds
Telephone: 919-549-2119
E-Mail: michael.weeks@bayer.com

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Contains No Confidential Business Information

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Certification

The undersigned submits this request under 7 CFR § 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article Event GHB811 cotton should not be regulated under 7 CFR § 340.6.

The undersigned certifies, 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 petitioner which is unfavorable to the petition.

Michael Weeks, M.Sc.
Regulatory Manager, United States
Bayer CropScience LP
2 T.W. Alexander Drive
Research Triangle Park, NC 27709
Telephone: 919-549-2119
E-Mail: michael.weeks@bayer.com

Summary

Bayer CropScience LP requests a determination from USDA APHIS that herbicide tolerant cotton event GHB811 and any progeny derived from crosses of this event with traditional or transgenic cotton varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340.

Event GHB811 cotton was developed through *Agrobacterium*-mediated transformation using the vector pTSIH09 containing *hppdPfW336-1Pa* and *2mepsps* expression cassettes. The regulatory sequences used in this construct are derived from common plants or plant pathogens that are routinely used in plant biotechnology and have a history of safe use.

(i) The double mutant 5-enol pyruvylshikimate-3-phosphate synthase (*2mepsps*) gene that encodes for the 2mEPSPS protein. The *2mepsps* coding sequence was developed by introducing two point mutations to the wild-type *epsps* gene cloned from maize (*Zea mays*). Expression of the 2mEPSPS protein confers tolerance to glyphosate herbicides.

(ii) The *hppdPfW336-1Pa* gene encodes for the HPPD W336 protein. *The hppdPfW336-1Pa* coding sequence was developed by introducing a single point mutation to the wild type *hppd* gene derived from *Pseudomonas fluorescens*. Expression of the HPPD W336 protein confers tolerance to HPPD inhibitors, such as isoxaflutole herbicides.

The OECD identifier of event GHB811 cotton is BCS-GH811-4.

The incorporation and expression of the GHB811 transgenic locus in the cotton genome has been characterized according to international standards for the safety assessment of biotechnology products. This information is included with this petition to support the plant pest risk assessment of event GHB811 cotton. Cotton varieties containing the GHB811 event will be grown commercially in the cotton growing areas of the United States, Brazil, and possibly other cotton cultivation countries.

Molecular characterization determined that a single copy of the complete T-DNA of the pTSIH09 plasmid was inserted at a single locus of the event GHB811 cotton genome. The DNA sequence of the event GHB811 cotton transgenic locus and the corresponding insertion locus was determined. Molecular characterization analysis also demonstrated inheritance and stability of the insert across multiple generations. Bioinformatics analysis on the transgenic locus of the event GHB811 cotton revealed no evidence supporting any potential creation of unintended effects or any disruption of pre-existing genes resulting from the genetic modification.

Food safety evaluation of the 2mEPSPS and HPPD W336 proteins was undertaken utilizing guidance provided by Codex. No health-related adverse effects have been associated with the proteins.

The source organism for the 2mEPSPS protein, maize (*Zea mays*), is a safe crop plant widely used for food and feed with little pathogenic, toxic or allergenic effects on humans or animals. EPSPS proteins are ubiquitous in nature, being widely expressed in food and feed crops. No health-related adverse effects have been associated with the proteins. The 2mEPSPS protein has no amino acid sequence similarity to known allergens and is rapidly degraded in simulated gastric fluid and simulated intestinal fluid assays. The 2mEPSPS

protein has no amino acid sequence similarity to known toxins and exhibited no effects in an acute oral mouse toxicity test.

The HPPD protein source organism, *Pseudomonas fluorescens*, is a non-pathogenic bacterium which is ubiquitous in nature and has a good history of safe use. HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals. The HPPD W336 protein has no amino acid sequence similarity to known allergens and is rapidly degraded in simulated gastric fluid and simulated intestinal fluid assays. The HPPD W336 protein has no amino acid sequence similarity to known toxins and exhibited no effects in an acute oral mouse toxicity test.

Taken together, the safety data for both proteins support the conclusion that neither protein is a potential allergen or toxin. Both proteins are ubiquitous in nature and neither has been associated with any known adverse effects on humans or animals.

Levels of 2mEPSPS and HPPD W336 were measured in event GHB811 cotton tissues. Exposure levels to humans and animals of both proteins are exponentially lower than the doses tested in the acute oral mouse study.

Composition analysis and the comparative assessment demonstrated that nutrient and anti-nutrient levels in GHB811 cottonseed are comparable to that of the non-GM counterpart and reference varieties.

The agronomic performance of GHB811 was observed in 15 field trials conducted in the cotton growing regions of the United States. Based on the agronomic assessment, GHB811 cotton demonstrated no biologically relevant differences compared to the non-GM conventional counterpart and showed equivalent agronomic performance in the field to cotton reference varieties.

Acronyms and scientific terms

ai	active ingredient
A	acre
ADF	Acid Detergent Fiber
ANOVA	Analysis Of Variance
APHIS	Animal and Plant Health Inspection Service
BBCH	Uniform coding of phenologically similar growth stages of plants
BC1, BC2, etc.	Backcross 1, 2 etc.
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BCS	Bayer CropScience
BLASTx	BLAST search of protein databases using a translated nucleotide query
bp	base pairs
bw	body weight
d	day
Da	Daltons
DW	Dry weight
DKN	Diketonitrile
DNA	DeoxyriboNucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
EMBOSS	European Molecular Biology Open Software Suite
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
2mEPSPS	modified 5-enolpyruvylshikimate-3-phosphate synthase
2mepsps	modified 5-enolpyruvylshikimate-3-phosphate synthase gene
F1, F2, etc.	Filial generation 1, 2 etc.
FAO	Food and Agriculture Organization of the United Nations
FASTA	A DNA and protein sequence alignment software pakage
FDA	Food and Drug Administration
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
FW	Fresh weight
g	gram
gDNA	Genomic DNA
GetORF	EMBOSS database for ORFs
GM	Genetically Modified
ha	Hectare
HPPD	p-hydroxyphenylpyruvate dioxygenase
HPPD W336	modified p-hydroxyphenylpyruvate dioxygenase
hppdPfW336	modified p-hydroxyphenylpyruvate dioxygenase gene
ID	identification
IFT	isoxaflutole
kDa	kiloDalton
kg	kilogram
LB	Left Border
LLOQ	Lower Limit of Quantitation
LOQ	Limit of Quantitation
mg	milligram
mL	milliter
MOPS	3-(N-morpholino)propanesulfonic acid
µg	microgram
NA	Not Applicable
NBT	Nitro blue tetrazolium
ng	nanogram
ND	Not Determined
OECD	Organization for Economic Co operation and Development
ORF	Open Reading Frame
P. fluorescens, Pf	*Pseudomonas fluorescens*
PCR	Polymerase Chain Reaction
RB	Right Border
RBD	Refined, Bleached, and Deodorized oil of cottonseed
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
T_1, T_2, etc	generations after T_0 (transformation)
T-DNA	transfer DNA from Agrobacterium
UPLC-UV-MS	Ultra Performance Liquid Chromatography Ultraviolet Mass Spectrometry
U.S. EPA	United States Environmental Protection Agency
U.S.	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization
WSSA	Weed Science Society of America
wt	Wild type
Z. mays	*Zea mays,* corn

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1. Rationale for non-regulated status

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Services (APHIS) is responsible for protection of the U.S. agricultural infrastructure against noxious pests and weeds. Under the Plant Protection Act (7 USC § 7701-7772) APHIS considers certain organisms altered or produced by genetic engineering as regulated articles under 7 CFR §340 which cannot be released into the environment without appropriate approvals. APHIS provides that petitions may be filed under 7 CFR §340.6 to evaluate data to determine that a particular regulated article does not present a plant pest risk. Should APHIS determine that the submitted article does not present a plant pest risk, the article may be deregulated and released without further restrictions.

This petition serves an application for a determination of non-regulated status for event GHB811 cotton.

1.1. Description of the trait and intended use of the product

GHB811 cotton was developed through *Agrobacterium*-mediated transformation using the vector pTSIH09 containing *hppdPfW336-1Pa* and *2mepsps* expression cassettes.

(i) The double mutant 5-enol pyruvylshikimate-3-phosphate synthase (*2mepsps*) gene that encodes for the 2mEPSPS protein. The *2mepsps* coding sequence was developed by introducing two point mutations to the wild-type *epsps* gene cloned from maize (*Zea mays*). Expression of the 2mEPSPS protein confers tolerance to glyphosate herbicides.

(ii) The *hppdPfW336-1Pa* gene encodes for the HPPD W336 protein. The *hppdPfW336-1Pa* coding sequence was developed by introducing a single point mutation to the wild type *hppd* gene derived from *Pseudomonas fluorescens*. Expression of the HPPD W336 protein confers tolerance to HPPD inhibitors, such as isoxaflutole herbicides.

The OECD identifier is BCS-GH811-4.

Cotton is primarily used worldwide for its lint. Lint is produced on the seed coat, and is spun into fine strong threads. Only the United States and a few other countries have developed major commercial uses for the seed. Raw unprocessed cottonseed may be fed to ruminants in the form of cottonseed meal and hulls or the seed can be processed for oil, the primary component consumed by humans. Linters, the short fibers that remain on the hulls after the removal of the lint have both edible and non-edible use.

1.2. Description of the benefits and anticipated adoption of the product

Planting double-herbicide-tolerant cotton GHB811 varieties provides growers with new options for weed control using isoxaflutole (IFT) and/or glyphosate herbicide. Glyphosate is widely used in cotton and other agricultural production systems. IFT herbicide offers an alternative weed control option for the cotton grower to help manage problem weed species and as an alternative mode of action tool to help slow the spread of herbicide resistant weeds. With IFT, a new site of action is introduced in cotton that is efficacious against many weeds currently found in cotton fields.

Efficacious Weed Control

Both IFT and glyphosate have been shown to be efficacious components of a weed management system with GHB811 herbicide tolerant cotton. IFT and glyphosate offer complementary weed management options. IFT provides control of weeds before emergence, while glyphosate provides control of weeds that have already emerged.

Simplicity and Convenience

GHB811 herbicide-tolerant cotton provides an easy-to-use system that allows a highly efficient weed control in the crop. High efficiency allows for the cultivation of additional acreage and expansion of production operations with the existing level of infrastructure. Additionally, some equipment costs and labor may be eliminated in situations where cultivation equipment is no longer necessary, such as no-till practices.

Economic Benefit to Growers

Use of glyphosate-tolerant cotton has been shown to increase grower returns in the form of higher yields and reduced overhead production costs.

Environmental Benefits

The main environmental benefit of GHB811 herbicide-tolerant cotton is the continued use of reduced and no-till production systems (glyphosate resistant weeds are threatening the use of these practices). Reduced and no-tillage practices significantly contribute to reductions in soil erosion from water and wind. Reduced tillage also contributes to reduced fossil fuel use, less air pollution from dust, improved soil moisture retention, and reduced soil compaction.

1.3. Regulatory status at other U.S. agencies

A Premarket Biotechnology Notification (PBN) was submitted to the U.S. Food and Drug Administration on April 17, 2017. To date an administrative number has not been assigned to this PBN.

The U.S. EPA does not regulate GHB811 cotton as it does not produce a Plant Incorporated Protectant. Bayer CropScience LP will submit for a label expansion to allow for the use of IFT herbicide in HPPD-inhibitor tolerant cotton.

2. The biology of cotton (*Gossypium spp.*)

The OECD consensus document on cotton biology (OECD, 2008) provides information pertaining to the following aspects of cotton biology:

- Taxonomy, morphology and uses
- Centers of origin of the species and domestication
- Agronomic practices
- Reproductive biology and dispersal
- Genetics and hybridization
- Interactions with other organisms

3. Development of event GHB811 cotton

3.1. Description of the transformation technology

3.1.1. Transformation methods

Seeds of cotton variety Coker 312 were germinated on Murashige & Skoog (MS) medium. Hypocotyl segments were dissected from the cotton seedlings and were transformed with the transformation vector pTSIH09 using a cotton hypocotyl *Agrobacterium tumefaciens* (*A. tumefaciens*) transformation method.

3.1.2. Description of the transformation vector and gene construct

The vector pTSIH09 is derived from pGSC1700 and pUC19. The map of the vector pTSIH09 is presented in Figure 3.1 and the genetic elements are described in Table 3.1.

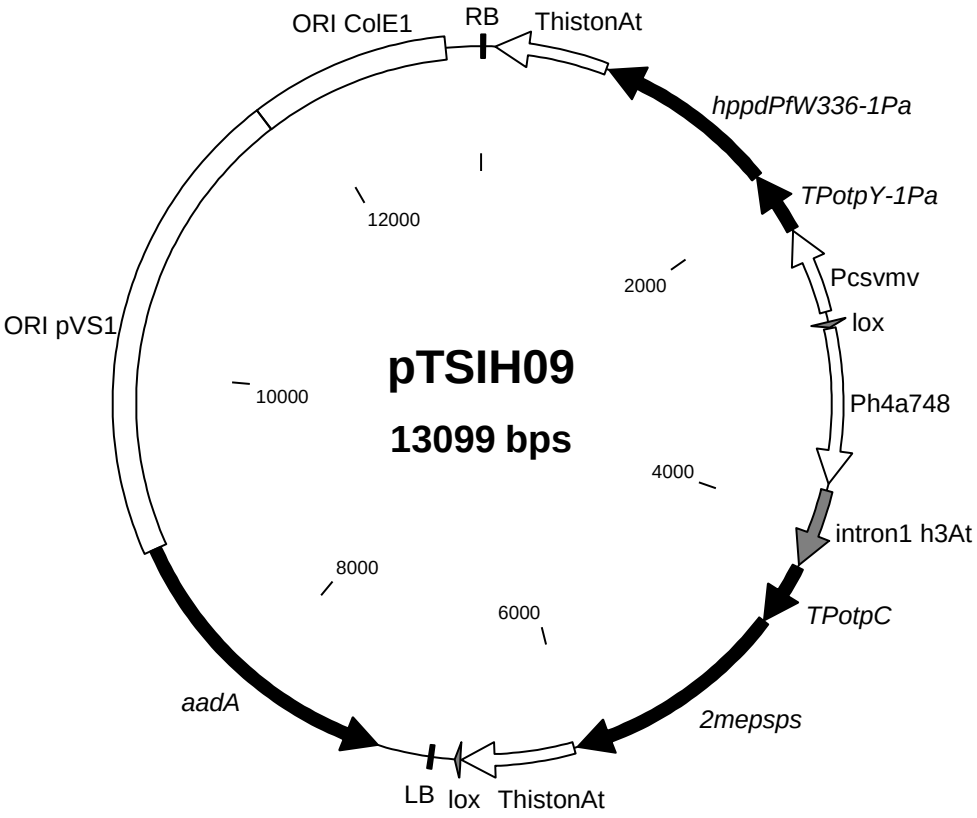


Figure 3.1. Map of vector pTSIH09

Table 3.1. Description of the genetic elements of pTSIH09

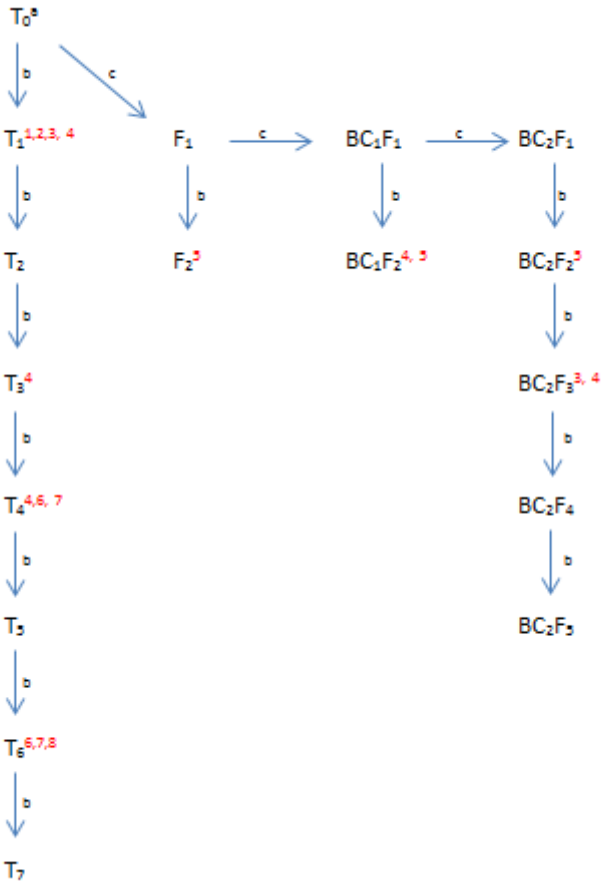
Nt Positions	Orientation	Origin
1 – 25		**RB:** right border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
26 – 82		Synthetic polylinker derived sequences
83 – 749	Counter clockwise	**ThistonAt:** sequence including the 3´ untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987)
750 – 765		Synthetic polylinker derived sequences
766 - 1842	Counter clockwise	***hppdPfW336-1Pa*:** coding sequence of the 4-hydroxyphenylpyruvate dioxygenase gene of *Pseudomonas fluorescens* strain A32 modified by the replacement of the amino acid Glycine 336 with a Tryptophane (Boudec *et al*., 2001), adapted to cotton codon usage
1843 - 2214	Counter clockwise	***TPotpY-1Pa:*** coding sequence of an optimized transit peptide derivative (position 55 changed into Tyr), containing sequence of the RuBisCO small subunit genes of *Zea mays* and *Helianthus annuus* (Lebrun *et al*., 1996), adapted for cotton codon usage
2215 - 2222		Synthetic polylinker derived sequences
2223 - 2735	Counter clockwise	**Pcsvmv:** sequence including the promoter region of the Cassava Vein Mosaic Virus (Verdaguer *et al.,* 1996)
2736 - 2795		Synthetic polylinker derived sequences
2796 - 2829	Clockwise	**lox:** sequence including the 34bp recognition sequence for the Cre recombinase of bacteriophage P1 (Hoess and Abremski, 1985)
2830 - 2833		Synthetic polylinker derived sequences
2834 - 3750	Clockwise	**Ph4a748:** sequence including the promoter region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al.,* 1987)
3751 - 3789		Synthetic polylinker derived sequences
3790 - 4255	Clockwise	**intron1 h3At:** first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et al.,* 1992)
4256 - 4268		Synthetic polylinker derived sequences
4269 - 4640	Clockwise	***TPotpC:*** coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of *Zea mays* and *Helianthus annuus* (Lebrun *et al*., 1996)
4641 - 5978	Clockwise	***2mepsps:*** coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of *Zea mays* (Lebrun *et al*., 1997)
5979 - 5998		Synthetic polylinker derived sequences
5999 - 6665	Clockwise	**ThistonAt:** sequence including the 3´ untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987)
6666 - 6669		Synthetic polylinker derived sequences
6670 - 6703	Clockwise	**lox:** sequence including the 34bp recognition sequence for the Cre recombinase of bacteriophage P1 (Hoess and Abremski, 1985)
6704 - 6831		Synthetic polylinker derived sequences
6832 - 6856		**LB:** left border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
6857 - 7161		Ti-plasmid sequences of pTiAch5 flanking the left border repeat (Zhu *et al*., 2000).
7162 - 8946	Counter Clockwise	***aadA*:** fragment including the aminoglycoside adenyltransferase gene of *Escherichia coli* (Fling *et al*., 1985).

Nt Positions	Orientation	Origin
8947 - 11736		**ORI pVS1:** fragment including the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens* (Hajdukiewicz *et al*., 1994).
11737 - 12893		**ORI ColE1:** fragment including the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* (Bolivar *et al*., 1977).
12894 - 13099		Ti-plasmid sequences of pTiAch5 flanking the right border repeat (Zhu *et al*., 2000)

3.2. Description of the breeding process for the parent organism

Following *Agrobacterium*-mediated transformation of the conventional breeding line, Coker312 resulting in event GHB811, T_0 plants were treated with tembotrione (HPPD-inhibitor herbicides) to select for the expression of the *hppdPfw336-1Pa* genes. The surviving plants were then self-pollinated to generate T_1 seed. All subsequent T_2 to T_7 generations were produced through self-pollination. A subsample of the T_1 and T_2 plants were sprayed with glyphosate to ensure expression of the *2mepsps* gene at those generations. In the T_3 through T_7 generations which were grown in the field, each selfed generation was sprayed with glyphosate to ensure the expression of the *2mepsps* gene. In the development of GHB811 cotton varieties, T_0 plants were back-crossed into a conventional commercial cotton line.

The breeding program for the development of event GHB811 and its introgression into commercial cotton germplasm is demonstrated in Figure 3.2 below. Table 3.2 describes the GHB811 generations used for analysis and the associated reports describing these studies.



a: Coker312 was used for transformation
b: selfing
c: crossing with Stoneville 457 variety

Figure 3.2. Pedigree of event GHB811 cotton

Table 3.2. Generations used for analysis of event GHB811 cotton

No. in Tree	Experiment	Generation(s)	Comparator
1	DNA sequencing of insert and flanking region	T1	Coker312
2	Insert Characterization by Southern Analysis	T1	Coker312
3	Absence of Vector Backbone by Southern Analysis	T1, BC2F3	Coker312
4	Structural Stability by Southern Analysis	T1, T3, T4, BC1F2, BC2F3	Coker312
5	Inheritance of the Insert	F2, BC1F2, BC2F2	None
6	Agronomic and phenotypic Analysis	T4, T6	Coker312
7	Composition Analysis	T4, T6	Coker312
8	Protein Expression Analysis	T6	None

4. Genetic material used for transformation event GHB811 cotton

4.1. Description of the transferred genes and gene products

2mEPSPS

The wild-type maize 5-enol pyruvylshikimate-3-phosphate synthase (*epsps*) gene was mutated using site directed mutagenesis. The wild-type maize *epsps* gene was mutated at positions 102 (substitution of threonine by isoleucine) and position 106 (substitution of proline by serine) (Lebrun *et al*., 1997). These modifications confer to the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants bearing this gene become tolerant to glyphosate herbicides (Lebrun *et al*., 1997). The modified protein is designated as 2mEPSPS. The amino acid sequence of the 2mEPSPS protein is provided in Figure 4.1.

HPPD W336

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase of *Pseudomonas fluorescens* strain A32 was modified by the replacement of the amino acid glycine 336 with a tryptophan (Boudec *et al*., 2001). This modification confers to the protein an improved tolerance against HPPD inhibitors. The modified protein is designated as HPPD W336 (Boudec *et al*., 2001). The amino acid sequence of HPPD W336 is provided in Figure 4.2.

```
1   magaeeivlq pikeisgtvk lpgskslsnr illlaalseg ttvvdnllns edvhymlgal
61  rtlglsvead kaakravvvg cggkfpveda keevqlflgn agiamrslta avtaaggnat
121 yvldgvprmr erpigdlvvg lkqlgadvdc flgtdcppvr vngigglpgg kvklsgsiss
181 qylsallmaa plalgdveie iidklisipy vemtlrlmer fgvkaehsds wdrfyikggq
241 kykspknayv egdassasyf lagaaitggt vtvegcgtts lqgdvkfaev lemmgakvtw
301 tetsvtvtgp prepfgrkhl kaidvnmnkm pdvamtlavv alfadgptai rdvaswrvke
361 termvairte ltklgasvee gpdyciitpp eklnvtaidt yddhrmamaf slaacaevpv
421 tirdpgctrk tfpdyfdvls tfvkn
```

Figure 4.1. Amino acid sequence of the 2mEPSPS protein

```
1   madlyenpmg lmgfefiefa sptpgtlepi feimgftkva thrsknvhly rqgeinliln
61  nepnsiasyf aaehgpsvcg mafrvkdsqk aynralelga qpihidtgpm elnlpaikgi
121 ggaplylidr fgegssiydi dfvylegver npvgaglkvi dhlthnvyrg rmvywanfye
181 klfnfreary fdikgeytgl tskamsapdg miriplnees skgagqieef lmqfngegiq
241 hvafltddlv ktwdalkkig mrfmtappdt yyemlegrlp dhgepvdqlq argilldgss
301 vegdkrlllq ifsetlmgpv ffefiqrkgd dgfgewnfka lfesierdqv rrgvltad
```

Figure 4.2. Amino acid sequence of the HPPD W336 protein

5. Genetic characterization of event GHB811 cotton

5.1. Structural stability

The structural stability of GHB811 cotton was investigated by performing a Southern blot analysis on individual plants from five different generations (T1, T3, T4, BC1F2 and BC2F3 generations).

Seeds from five different seed lots, each corresponding to a different generation, were used to produce cotton GHB811 leaf material. The identity of the produced plant material was confirmed.

Non-genetically modified (non-GM) cotton variety Coker 312 (non-GM counterpart) was used as a negative control. The transforming plasmid of cotton GHB811, pTSIH09 was used as a positive control.

The non-GM counterpart-derived gDNA and a set of individual gDNA samples from GHB811 cotton were digested using the restriction enzyme combination *Psi*I/*Sap*I. Additional restriction digests of the non-GM counterpart gDNA were prepared using respectively the *Hinc*II and the *Eco*RI restriction enzymes. Plasmid DNA of pTSIH09 was digested using the *Hinc*II restriction enzyme, as recommended by the manufacturer (New England BioLabs).

Hybridization was performed with the T-DNA probe P009 (Table 5.1). A schematic overview of the GHB811 transgenic locus, with indication of the restriction enzymes, the T-DNA probe used and the expected fragments is presented in Figure 5.1. The hybridization results are presented in Figure 5.2 to Figure 5.6. A summary of the results obtained is presented in Table 5.2 to Table 5.6.

Each membrane used for the analysis contained one negative control which was never shown to hybridize with the T-DNA probe. This confirmed the absence of any background hybridization. Similarly, each reported membrane contained one positive control. For all hybridizations, the expected fragments were detected for the positive control, indicating that the conditions of the Southern blot experiments allowed specific hybridization of the used probes with the target sequences.

Genomic DNA from individual GHB811 cotton plants was digested with restriction enzyme combination *Psi*I/*Sap*I and hybridized to the T-DNA probe, with genomic DNA extracted from one plant represented in each lane. For all individual plants from the T1, T3, T4, BC1F2 and BC2F3 generation, all expected three fragments (3300 bp, 1588 bp, and 2600 bp) were obtained (Figure 5.2 to Figure 5.6 and Table 5.2 to Table 5.6).

Taken together, all obtained results demonstrate the structural stability of GHB811 cotton in the T1, T3, T4, BC1F2 and BC2F3 generations.

Table 5.1. Information on the used probe for structural stability analysis

Probe ID	Description	Primer pair	Primer sequence (5' → 3')	Primer position in pTSIH09 (bp)	Size probe (bp)
P009	T-DNA probe	GLPA467	AAGGCCCGATCAAATCTGAG	79 → 98	6700
		GLPA468	GTGCCGTAATGCCGTAATGC	6778 → 6759	

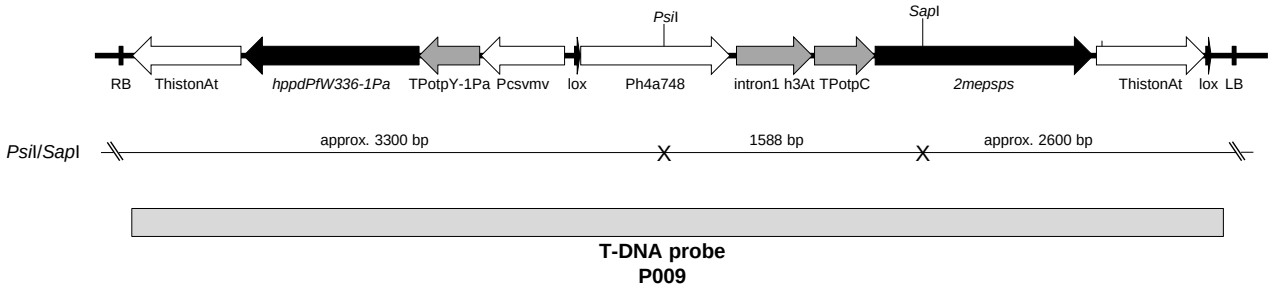


Figure 5.1. Schematic overview of the GHB811 cotton transgenic locus with indication of the restriction sites, the probe used and the expected fragment sizes in bp (based on the corresponding detailed insert characterization study)

Table 5.2. Stability of cotton GHB811 in the individual plants of the T1 generation - Expected and obtained hybridization fragments (Figure 5.2)

Sample	Reference to lane N° in Figure 5.2	Fragment size (bp)	Fragment description	Probe P009 T-DNA	
				Exp.	Obt.
18 samples Cotton GHB811, T1 generation – *Psi*I/*Sap*I	Lane 2 to 19	approx. 3300 *	5' integration fragment	Yes	Yes
		1588*	Internal fragment	Yes	Yes
		approx. 2600 *	3' integration fragment	Yes	Yes
non-GM counterpart - *Psi*I/*Sap*I	Lane 20	/	Negative control	/	/
non-GM counterpart – *Hinc*II + equimolar amount pTSIH09 – *Hinc*II	Lane 21	1113	Positive control	Yes	Yes
		2476		Yes	Yes
		3169		Yes**	No
		6341		Yes	Yes

* Results determined in the detailed insert characterization and confirmation of absence of vector backbone study of cotton GHB811

** Due to the small overlap of this fragment with the T-DNA probe, the likelihood to visualize this fragment is very low

Table 5.3. Stability of cotton GHB811 in the individual plants of the T3 generation - Expected and obtained hybridization fragments (Figure 5.3)

Sample	Reference to lane N° in Figure 5.3	Fragment size (bp)	Fragment description	Probe P009 T-DNA	
				Exp.	Obt.
16 samples Cotton GHB811, T3 generation – *Psi*I/*Sap*I	Lane 2 to 17	approx. 3300 *	5' integration fragment	Yes	Yes
		1588*	Internal fragment	Yes	Yes
		approx. 2600 *	3' integration fragment	Yes	Yes
non-GM counterpart - *Psi*I/*Sap*I	Lane 18	/	Negative control	/	/
non-GM counterpart – *Hinc*II + equimolar amount pTSIH09 – *Hinc*II	Lane 19	1113	Positive control	Yes	Yes
		2476		Yes	Yes
		3169		Yes**	No
		6341		Yes	Yes

* Results determined in the detailed insert characterization and confirmation of absence of vector backbone study of cotton GHB811

** Due to the small overlap of this fragment with the T-DNA probe, the likelihood to visualize this fragment is very low

Table 5.4. Stability of cotton GHB811 in the individual plants of the T4 generation - Expected and obtained hybridization fragments (Figure 5.4)

Sample	Reference to Lane N° in Figure 5.4	Fragment size (bp)	Fragment description	Probe P009 T-DNA	
				Exp.	Obt.
15 samples Cotton GHB811, T4 generation – *Psi*I/*Sap*I	Lane 2 to 16	approx. 3300 *	5' integration fragment	Yes	Yes
		1588*	Internal fragment	Yes	Yes
		approx. 2600 *	3' integration fragment	Yes	Yes
non-GM counterpart - *Psi*I/*Sap*I	Lane 17	/	Negative control	/	/
non-GM counterpart – *Hinc*II + equimolar amount pTSIH09 – *Hinc*II	Lane 18	1113	Positive control	Yes	Yes
		2476		Yes	Yes
		3169		Yes**	Very faint
		6341		Yes	Yes

* Results determined in the detailed insert characterization and confirmation of absence of vector backbone study of cotton GHB811

** Due to the small overlap of this fragment with the T-DNA probe, the likelihood to visualize this fragment is very low

Table 5.5. Stability of cotton GHB811 in the individual plants of the BC1F2 generation - Expected and obtained hybridization fragments (Figure 5.5)

Sample	Reference to lane N° in Figure 5.5	Fragment size (bp)	Fragment description	Probe P009 T-DNA	
				Exp.	Obt.
14 samples Cotton GHB811, BC1F2 generation – *Psi*I/*Sap*I	Lane 2 to 15	approx. 3300 *	5' integration fragment	Yes	Yes
		1588*	Internal fragment	Yes	Yes
		approx. 2600 *	3' integration fragment	Yes	Yes
non-GM counterpart - *Psi*I/*Sap*I	Lane 16	/	Negative control	/	/
non-GM counterpart – *Hinc*II + equimolar amount pTSIH09 - *Hinc*II	Lane 17	1113	Positive control	Yes	Yes
		2476		Yes	Yes
		3169		Yes**	No
		6341		Yes	Yes

* Results determined in the detailed insert characterization and confirmation of absence of vector backbone study of cotton GHB811

** Due to the small overlap of this fragment with the T-DNA probe, the likelihood to visualize this fragment is very low

Table 5.6. Stability of cotton GHB811 in the individual plants of the BC2F3 generation - Expected and obtained hybridization fragments (Figure 5.6)

Sample	Reference to lane N° in Figure 5.6	Fragment size (bp)	Fragment description	Probe P009 T-DNA	
				Exp.	Obt.
15 samples Cotton GHB811, BC2F3 generation – *Psi*I/*Sap*I	Lane 2 to 16	approx. 3300 *	5' integration fragment	Yes	Yes
		1588*	Internal fragment	Yes	Yes
		approx. 2600 *	3' integration fragment	Yes	Yes
non-GM counterpart - *Psi*I/*Sap*I	Lane 17	/	Negative control	/	/
non-GM counterpart – *Hinc*II + equimolar amount pTSIH09 – *Hinc*II	Lane 18	1113	Positive control	Yes	Yes
		2476		Yes	Yes
		3169		Yes**	No
		6341		Yes	Yes

* Results determined in the detailed insert characterization and confirmation of absence of vector backbone study of cotton GHB811

** Due to the small overlap of this fragment with the T-DNA probe, the likelihood to visualize this fragment is very low

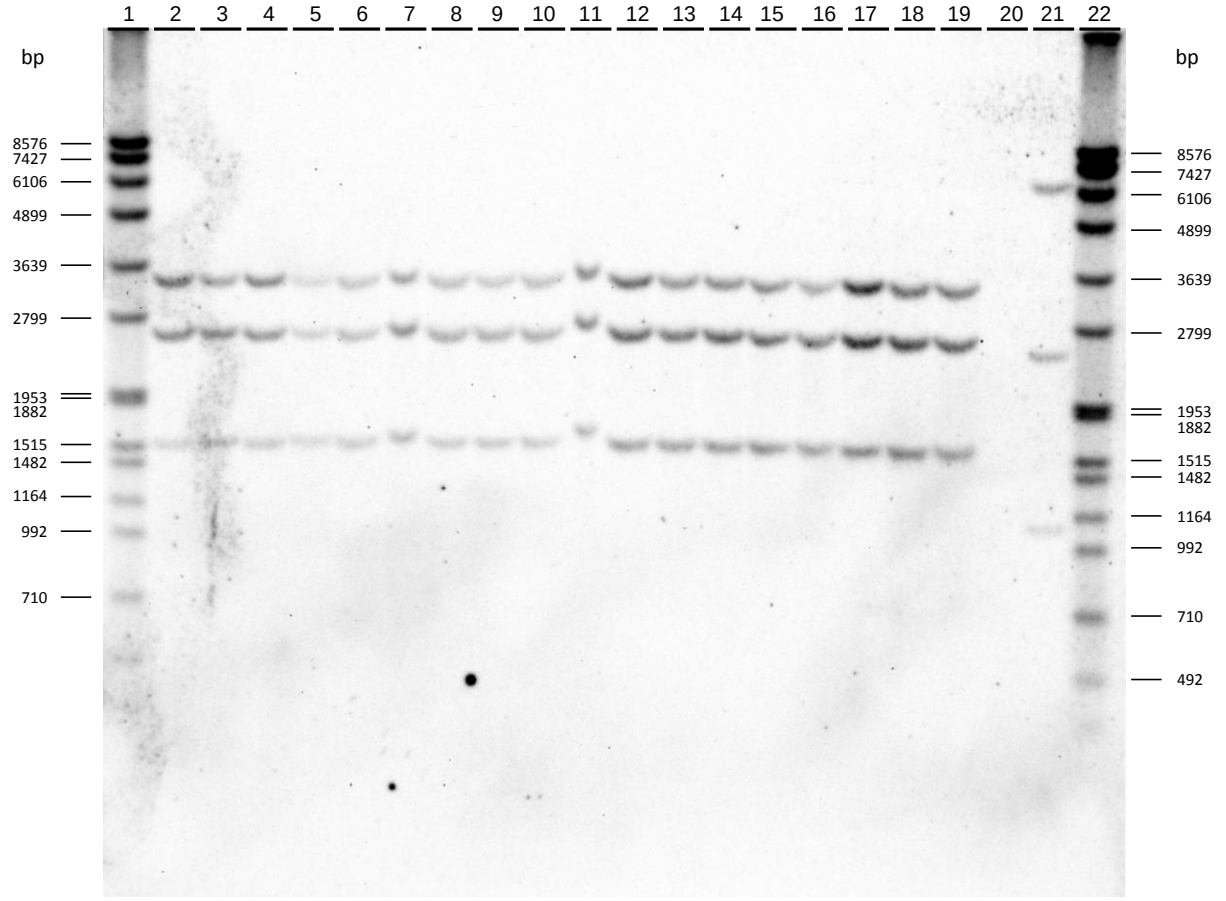


Figure 5.2. Southern blot analysis of cotton GHB811 – Hybridization performed with the T-DNA probe to assess structural stability of the individual plants of the T1 generation

Digital image ID: H1/THT068A/05-F3

Genomic DNA was isolated from individual cotton GHB811 plants of the T1 generation and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme combination *Psi*I/*Sap*I and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-5).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lanes 2 to 11: 5 µg gDNA of individual hemizygous samples of cotton GHB811 of the T1 generation (one copy of GHB811) – *Psi*I/*Sap*I digested
Lanes 12 to 19: 5 µg gDNA of individual homozygous samples of cotton GHB811 of the T1 generation (two copies of GHB811) – *Psi*I/*Sap*I digested
Lane 20: 5 µg gDNA of the non-GM counterpart – *Psi*I/*Sap*I digested (negative control)
Lane 21: 5 µg gDNA of the non-GM counterpart – *Hin*cII digested + an equimolar amount of plasmid pTSIH09 – *Hin*cII digested (positive control)
Lane 22: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested

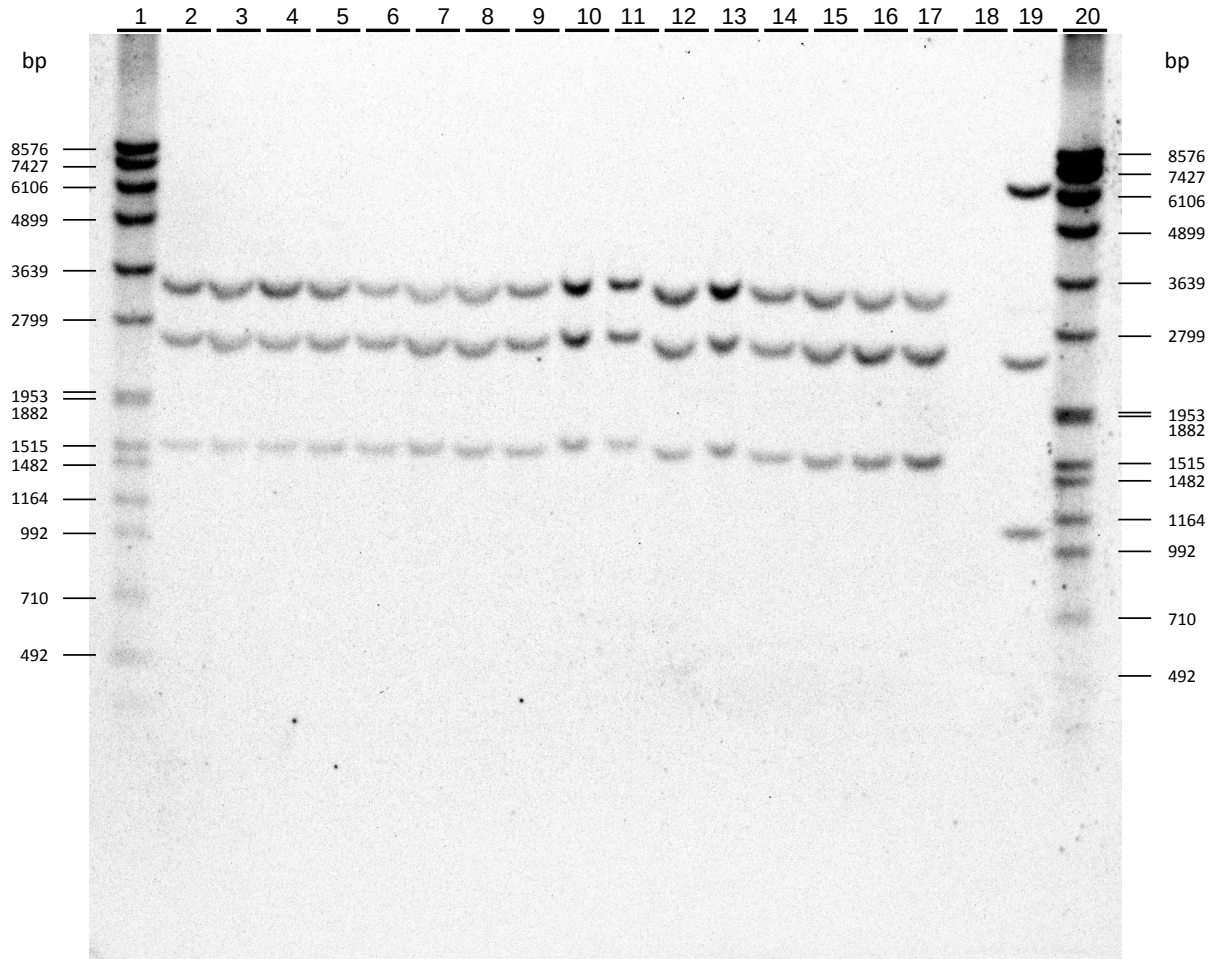


Figure 5.3. Southern blot analysis of cotton GHB811 – Hybridization performed with the T-DNA probe to assess structural stability of the individual plants of the T3 generation

Digital image ID: H1/THT068A/04-F3

Genomic DNA was isolated from individual cotton GHB811 plants of the T3 generation and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme combination *Psi*I/*Sap*I and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-5).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 2.5 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lanes 2 to 17: 2.5 µg gDNA of individual homozygous samples of cotton GHB811 of the T3 generation – *Psi*I/*Sap*I digested
Lane 18: 2.5 µg gDNA of the non-GM counterpart – *Psi*I/*Sap*I digested (negative control)
Lane 19: 2.5 µg gDNA of the non-GM counterpart – *Hin*cII digested + an equimolar amount of plasmid pTSIH09 – *Hin*cII digested (positive control)
Lane 20: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 2.5 µg of gDNA of the non-GM counterpart – *Eco*RI digested

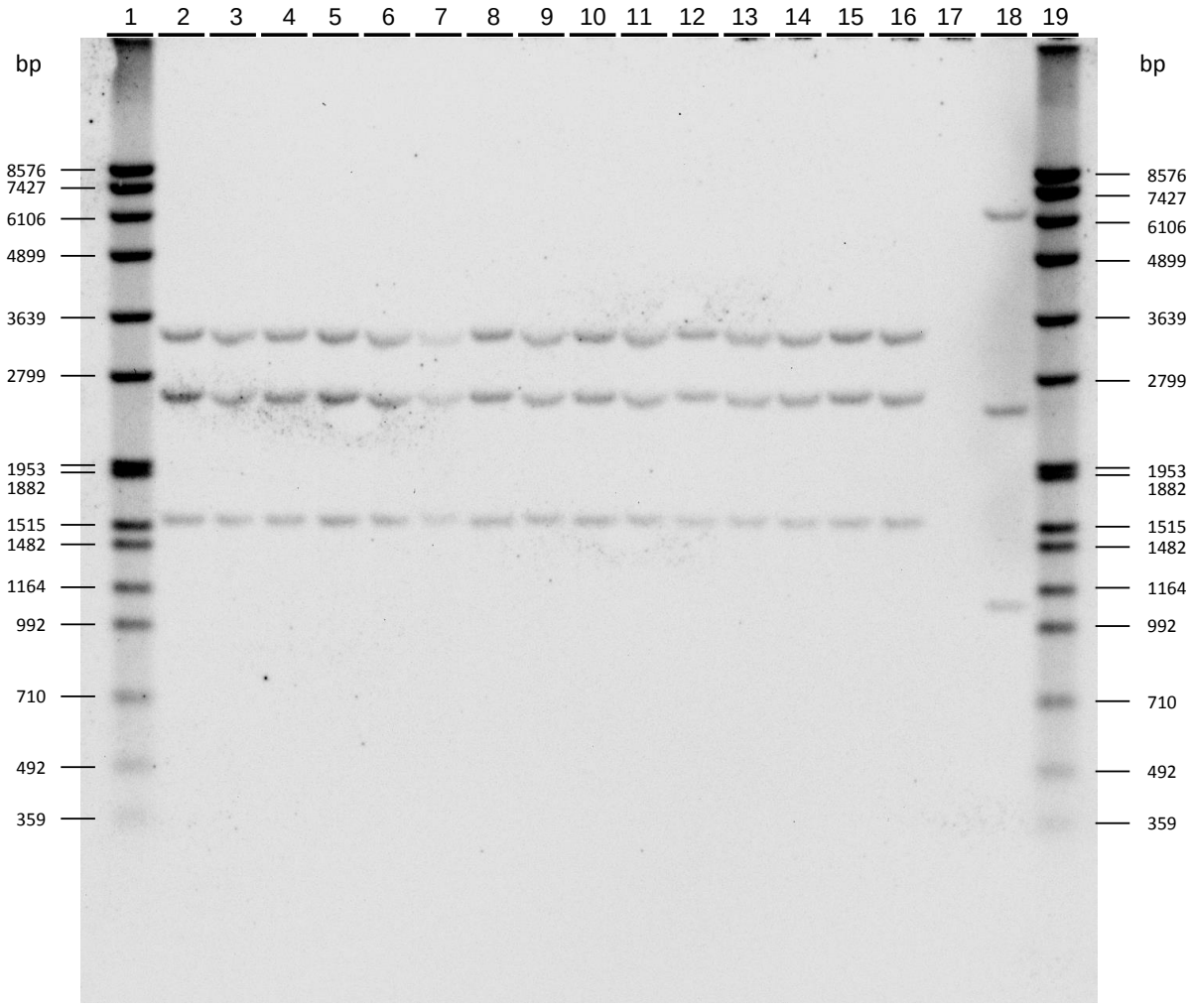


Figure 5.4. Southern blot analysis of cotton GHB811 – Hybridization performed with the T-DNA probe to assess structural stability of the individual plants of the T4 generation

Digital image ID: H1/THT068A/01-F3

Genomic DNA was isolated from individual cotton GHB811 plants of the T4 generation and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme combination *Psi*I/*Sap*I and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-5).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lanes 2 to 16: 5 µg gDNA of individual homozygous samples of cotton GHB811 of the T4 generation – *Psi*I/*Sap*I digested
Lane 17: 5 µg gDNA of the non-GM counterpart – *Psi*I/*Sap*I digested (negative control)
Lane 18: 5 µg gDNA of the non-GM counterpart – *Hin*cII digested + an equimolar amount of plasmid pTSIH09 – *Hin*cII digested (positive control)
Lane 19: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested

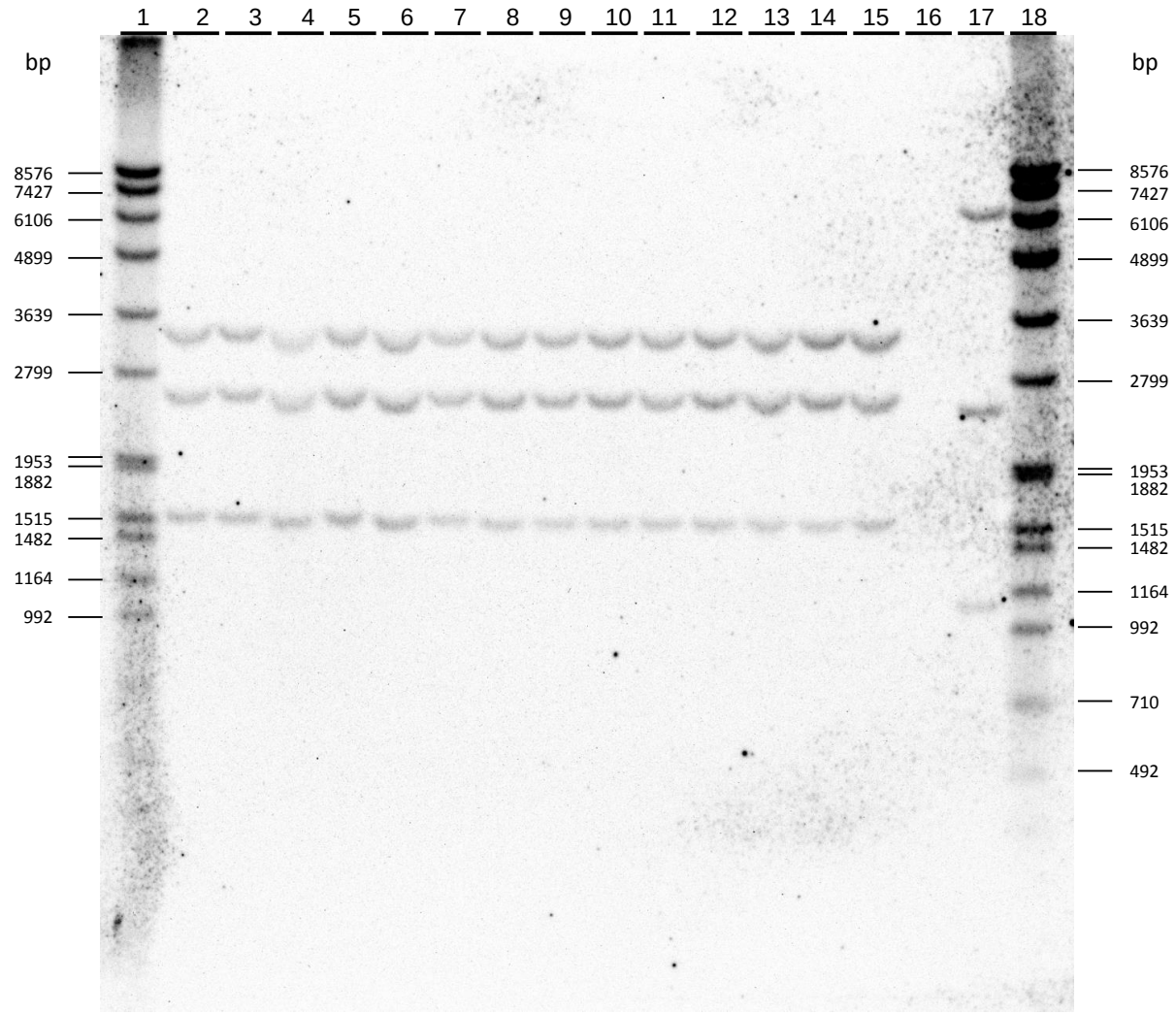


Figure 5.5. Southern blot analysis of cotton GHB811 – Hybridization performed with the T-DNA probe to assess structural stability of the individual plants of the BC1F2 generation

Digital image ID: H2/THT068A/03-F4

Genomic DNA was isolated from individual cotton GHB811 plants of the BC1F2 generation and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme combination *Psi*I/*Sap*I and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-5).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lanes 2 to 15: 5 µg gDNA of individual hemizygous samples of cotton GHB811 of the BC1F2 generation – *Psi*I/*Sap*I digested
Lane 16: 5 µg gDNA of the non-GM counterpart – *Psi*I/*Sap*I digested (negative control)
Lane 17: 5 µg gDNA of the non-GM counterpart – *Hin*cII digested + an equimolar amount of plasmid pTSIH09 – *Hin*cII digested (positive control)
Lane 18: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested

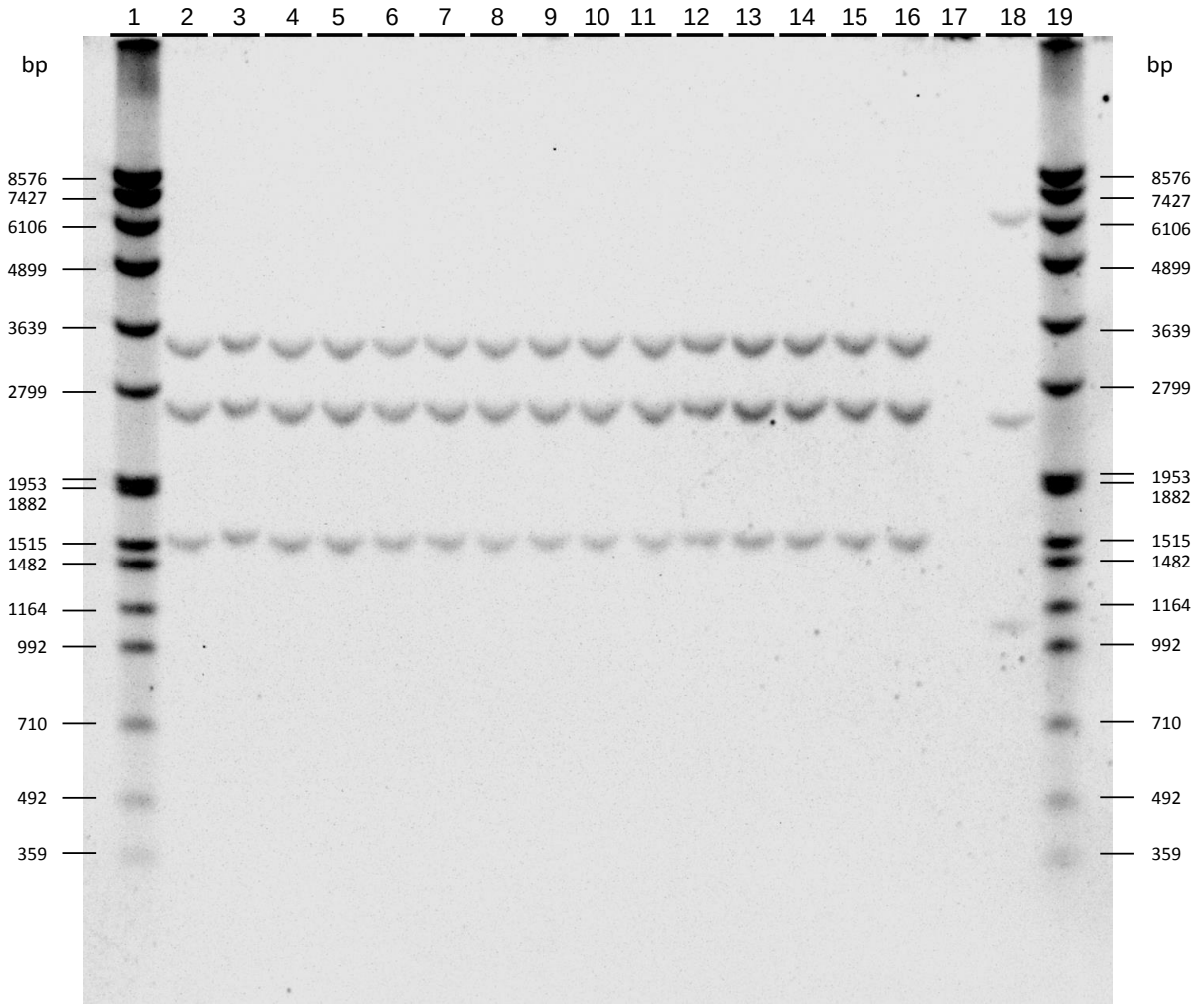


Figure 5.6. Southern blot analysis of cotton GHB811 – Hybridization performed with the T-DNA probe to assess structural stability of the individual plants of the BC2F3 generation

Digital image ID: H1/THT068A/02-F1

Genomic DNA was isolated from individual cotton GHB811 plants of the BC2F3 generation and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme combination *Psi*I/*Sap*I and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-5).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lanes 2 to 16: 5 µg gDNA of individual homozygous samples of cotton GHB811 of the BC2F3 generation – *Psi*I/*Sap*I digested
Lane 17: 5 µg gDNA of the non-GM counterpart – *Psi*I/*Sap*I digested (negative control)
Lane 18: 5 µg gDNA of the non-GM counterpart – *Hin*cII digested + an equimolar amount of plasmid pTSIH09 – *Hin*cII digested (positive control)
Lane 19: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 5 µg of gDNA of the non-GM counterpart – *Eco*RI digested

5.2. Number of insertion sites, and arrangement and copy number of transferred DNA

The transgenic locus of cotton GHB811 (T1 generation) was characterized by means of Southern blot analysis on genomic DNA (gDNA) prepared from leaf material.

A set of restriction enzymes were chosen to produce different restriction fragments containing portions of the insert and adjacent genomic DNA for each enzyme, which generated a specific banding pattern on the Southern blots. The selection and design of probes used in this study allowed the investigation of the T-DNA insert organization. Probes used in this study are summarized in Table 5.7. Probes covering the different features of the transgenic cassettes (P001 to P008) as well as the probe covering the complete T-DNA region were used (P009) (Figure 5.7).

Figure 5.8 shows the expected fragments for a complete single copy of the complete T-DNA integration in a single locus of GHB811 cotton genome. Expected and obtained hybridization fragments are listed in Table 5.8. The hybridization results to characterize the T-DNA insertion in GHB811 cotton are presented in Figure 5.9 to Figure 5.17.

Each membrane contained one negative control, which showed no hybridization with any of the probes used (Figure 5.9 to Figure 5.17, lane 13). Consequently, the absence of any background hybridization was demonstrated for all the probes used.

Similarly, each membrane contained a positive control. This positive control, consisting of pTSIH09 plasmid DNA, was digested with *Hin*cII and an equimolar amount was spiked in *Hin*cII digested gDNA from the non-GM counterpart. For each of the probes used, the expected fragments were detected (Figure 5.9 to Figure 5.17, lane 14), confirming that the applied experimental conditions allowed specific hybridization of the used probes with the target sequences.

Restriction digestion with *Afl*II

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, digestion of the insert with the *Afl*II restriction enzyme should generate two integration fragments and three internal fragments (Figure 5.8). The 5' integration fragment is expected to be greater than 1905 bp and contains genomic DNA flanking the 5' end of the insert, the right border (RB) sequence and the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene. The internal fragment with expected length of 1417 bp contains a small part of the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene and the TPotpY-1Pa sequence. The internal fragment with expected length of 2222 bp contains a small part of the TPotpY-1Pa sequence, the Pcsvmv and Ph4a748 promoter sequences, a lox recognition site, the intron1 h3At sequence and a small part of the TPotpC sequence. The 1682 bp internal fragment contains the TPotpC sequence, the *2mepsps* gene sequence and a small part of the ThistonAt terminator sequence following the *2mepsps* gene. The 3' integration fragment is expected to be greater than 2102 bp and contains the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, left border (LB) sequences and genomic DNA flanking the 3' end of the insert.

Hybridization of the *Afl*II digested cotton GHB811 gDNA with *hppdPfW336-1Pa* probe results in one band of 1417 bp (Figure 5.10, lane 3). This band corresponds to one of the internal fragments and was also observed with the TPotpY-1Pa probe and the T-DNA probe, as

expected (Figure 5.11 and Figure 5.17, lane 3). Hybridization of the *Afl*II digested cotton GHB811 gDNA with Pcsvmv, Ph4a748 + lox, intron1 h3At probe resulted in one band of 2222 bp (Figure 5.12 to Figure 5.14, lane 3). This band corresponds to one of the internal fragments and was also observed with the TPotpC probe, the TPotpY-1Pa probe and the T-DNA probe, as expected (Figure 5.11, Figure 5.15, and Figure 5.17, lane 3). Hybridization of the *Afl*II digested cotton GHB811 gDNA with *2mepsps* probe resulted in one band of 1682 bp (Figure 5.16, lane 3). This band corresponds to one of the internal fragments and was also observed with the TPotpC probe and the T-DNA probe, as expected (Figure 5.15 and Figure 5.17, lane 3). In addition, two strong bands with lengths of 2500 bp and 4400 bp were observed after hybridization with the ThistonAt probe and the T-DNA probe (Figure 5.9 and Figure 5.17, lane 3). These bands correspond to the two integration fragments. Yet, with this experimental setup, it was not possible to determine which of these two fragments represented the 5' or the 3' integration fragment.

In conclusion, the *Afl*II restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Bsp*HI

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, digestion of the insert with the *Bsp*HI restriction enzyme produces two integration fragments (Figure 5.8). The 5' integration fragment is expected to be 1365 bp and contains genomic DNA flanking the 5' end of the insert, the RB sequence, the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene and a part of the *hppdPfW336-1Pa* gene. The 3' integration fragment is expected to be greater than 7084 bp and contains part of the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence, the Pcsvmv promoter sequence, a lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence, the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, the LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization with the TPotpY-1Pa, Pcsvmv, Ph4a748 + lox, intron1 h3At, TPotpC and *2mepsps* probes (Figure 5.11 to Figure 5.16, lane 4) showed, as expected, the presence of only one band of 8300 bp which corresponds to the 3' integration fragment. Hybridization with the ThistonAt, *hppdPfW336-1Pa* and T-DNA probes (Figure 5.9, Figure 5.10 and Figure 5.17, lane 4) showed besides the band of 8300 bp, a second band of 1365 bp which corresponds to the 5' integration fragment.

In conclusion, the *Bsp*HI restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Psi*I/*Sap*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the double digestion of the insert with the *Psi*I and *Sap*I restriction enzymes produces two integration fragments and one internal fragment (Figure 5.8). The 5' integration fragment is expected to be 3423 bp and contains genomic DNA flanking the 5' end of the insert, the RB sequences, the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence, the Pcsvmv promoter

sequence, a lox recognition site and a part of the Ph4a748 promoter sequence. The internal fragment with expected length of 1588 bp contains a part of the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence and a part of the *2mepsps* gene sequence. The 3' integration fragment is expected to be 2644 bp and contains part of the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, the LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Psi*I/*Sap*I digested cotton GHB811 gDNA with the intron1 h3At and the TPotpC probe (Figure 5.14 and Figure 5.15, lane 5) showed the presence of one band of 1588 bp. This band corresponds to the internal fragment and was also observed with the Ph4a748 + lox probe, the *2mepsps* probe and the T-DNA probe, as expected (Figure 5.13, Figure 5.16 and Figure 5.17, lane 5). In addition, two strong bands with lengths of 2644 bp and 3423 bp are observed after hybridization with several probes. These bands correspond to the two integration fragments. The 3423 bp band, corresponding to the 5' integration fragment was observed with ThistonAt, *hppdPfW336-1Pa*, TPotpY-1Pa, Pcsvmv, Ph4a748 + lox, and T-DNA probes (Figure 5.9 to Figure 5.13, and Figure 5.17, lane 5), as expected. The 2644 bp band, corresponding to the 3' integration fragment, was observed with the ThistonAt, the *2mepsps* and the T-DNA probes (Figure 5.9, Figure 5.16 and Figure 5.17, lane 5), as expected.

In conclusion, the *Psi*I/*Sap*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Pvu*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Pvu*I restriction enzyme produces two internal fragments (Figure 5.8). The integration fragments are not expected to be observed because there is no or only very small overlap with the selected probes. The internal fragment with expected length of 5784 bp contains the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence, the Pcsvmv promoter sequence, a lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence and a part of the *2mepsps* gene sequence. The internal fragment with expected length of 921 bp contains a part of the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene and the lox recognition site.

Hybridization of the *Pvu*I digested cotton GHB811 gDNA with the ThistonAt, *2mepsps* and T-DNA probes, resulted in a band which has an estimated size of 1050 bp. This size differed more than 10 % from the expected size of 921 bp (Figure 5.9, Figure 5.16, and Figure 5.17, lane 6). The fact that the size of this fragment (1050 bp) corresponds to the summation of the 921 bp internal fragment and a 136 bp 3' integration fragment demonstrates that this fragment is due to an incomplete digestion of a second, nearby *Pvu*I restriction site located on the 3' flanking sequence. Hybridization of the *Pvu*I digested cotton GHB811 gDNA with all feature probes and the T-DNA showed the presence of a band corresponding with the 5784 bp internal fragment (Figure 5.9 to Figure 5.17, lane 6).

In conclusion, the *Pvu*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Sac*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Sac*I restriction enzyme produces two integration fragments and one internal fragment (Figure 5.8). The 5' integration fragment is expected to be greater than 2740 bp and contains genomic DNA flanking the 5' end of the insert, the RB sequence, the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, and a part of the *hppdPfW336-1Pa* gene. The internal fragment with expected length of 1119 bp contains a part of the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence and part of the Pcsvmv promoter sequence. The 3' integration fragment is expected to be 4494 bp and contains part of the Pcsvmv promoter sequence, the lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence, the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene, the LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Sac*I digested cotton GHB811 gDNA with the TPotpY-1Pa probe (Figure 5.11, lane 7) showed one fragment of 1119 bp. This band corresponds to the internal fragment and was also observed with the *hppdPfW336-1Pa* probe, the Pcsvmv probe and the T-DNA probe as expected (Figure 5.10, Figure 5.12 and Figure 5.17, lane 7). In addition, two bands with lengths of 4494 bp and >10 000 bp are observed after hybridization with several probes. These bands correspond to the two integration fragments. As the > 10 000 bp band is only observed with probes ThistonAt, *hppdPfW336-1Pa* and the T-DNA probe (Figure 5.9, Figure 5.10 and Figure 5.17, lane 7), this fragment corresponds to the 5' integration fragment. The 4494 bp band, corresponding to the 3' integration fragment is obtained with the ThistonAt, Pcsvmv, Ph4a748 + lox, intron1 h3At, TPotpC, the *2mepsps* and the T-DNA probes (Figure 5.9 and Figure 5.12 to Figure 5.17, lane 7), as expected.

Hybridization of the *Sac*I digested cotton GHB811 gDNA with the ThistonAt, Ph4a748 + lox, intron1 h3At, TPotpC, the *2mepsps* and the T-DNA probes, which are all probes fully overlapping the *Sac*I 3' integration fragment, resulted also in a weak fragment of 4900 bp (Figure 5.9, Figure 5.13 to Figure 5.17, lane 7). The weakness of the signal, combined with the fact that the size of this fragment (4900 bp) corresponds to the approximate summation of the 4494 bp 3' integration fragment and a 374 bp fragment in the 3' flanking sequence demonstrates that this fragment is due to incomplete digestion of the *Sac*I restriction site in the 3' flanking sequence (Figure 5.8).

In conclusion, the *Sac*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Sca*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Sca*I restriction enzymes produces three internal fragments and a 3' integration fragment (Figure 5.8). The 5' integration fragment is not expected to be observed because there is no overlap with the selected probes. The internal fragment with expected length of 1890 bp contains the ThistonAt terminator sequence

following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene and a small part of the TPotpY-1Pa sequence. The internal fragment with expected length of 342 bp contains the TPotpY-1Pa sequence and a small part of the Pcsvmv promoter sequence. The internal fragment with expected length of 2925 bp contains the Pcsvmv promoter sequence, a lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence and a part of the *2mepsps* gene sequence. The 3' integration fragment is expected to be greater than 2950 bp and contains a part of the *2mepsps* gene, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Sca*I digested cotton GHB811 gDNA with Pcsvmv probe, the Ph4a748 + lox probe, the intron1 h3At probe and the TPotpC probe results in one band of 2925 bp (Figure 5.12 to Figure 5.15, lane 8). This band corresponds to one of the internal fragments and was also observed with the *2mepsps* probe and the T-DNA probe, as expected (Figure 5.16 and Figure 5.17, lane 8). Hybridization of the *Sca*I digested cotton GHB811 gDNA with *hppdPfW336-1Pa* probe resulted in one band of 1890 bp (Figure 5.10, lane 8). This band corresponds to one of the internal fragments and was also observed with the ThistonAt probe, the TPotpY-1Pa probe and the T-DNA probe, as expected (Figure 5.9, Figure 5.11 and Figure 5.17, lane 8). Hybridization of the *Sca*I digested cotton GHB811 gDNA with TPotpY-1Pa probe resulted, besides the 1890 bp fragment, in another fragment of 342 bp (Figure 5.11, lane 8). This band corresponds to the third internal fragment. This fragment is not observed using the T-DNA probe (Figure 5.17, lane 8), as expected, because of the small overlap with the probe in proportion to the large size of the probe. In addition, hybridization with the ThistonAt, the *2mepsps* and the T-DNA probes (Figure 5.9, Figure 5.16 and Figure 5.17, lane 8) resulted in a band with length of 5900 bp. This band corresponds to the 3' integration fragment.

In conclusion, the *Sca*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Pac*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Pac*I restriction enzymes produces a 3' integration fragment (Figure 5.8). The 5' integration fragment is not expected to be observed because there is no overlap with the selected probes. This 3' integration fragment is expected to be 7399 bp and contains the whole T-DNA starting from the ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene until the LB sequence and a part of the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Pac*I digested cotton GHB811 gDNA with all feature probes and the T-DNA probe (Figure 5.9 to Figure 5.17, lane 9) showed the presence of a band with length of 7399 bp. This band corresponds to the 3' integration fragment.

In conclusion, the *Pac*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Sty*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Sty*I restriction enzyme produces two integration fragments and one internal fragment (Figure 5.8). The 5' integration fragment is expected to be greater than 3405 bp and contains the genomic DNA flanking the 5' end of the insert, RB sequences, ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene and the TPotpY-1Pa sequence. The internal fragment with expected length of 2882 bp contains the Pcsvmv promoter sequence, a lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence and a part of the *2mepsps* gene sequence. The 3' integration fragment is expected to be 2356 bp and contains a part of the *2mepsps* gene, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Sty*I digested cotton GHB811 gDNA with the Pcsvmv probe, the Ph4a748 + lox probe, the intron1 h3At probe and the TPotpC probe resulted in one band of 2882 bp (Figure 5.12 to and Figure 5.15, lane 10). This band corresponds to the internal fragment and was also observed with the *2mepsps* probe and the T-DNA probe (Figure 5.16 and Figure 5.17, lane 10), as expected. In addition, two strong bands with lengths of 3400 bp and 2356 bp were observed after hybridization with several probes. The obtained fragment size of 3400 bp was smaller than the expected fragment size (>3405 bp) due to rounding of values. These bands correspond to the two integration fragments. As the 3400 bp band was only observed with probes ThistonAt, *hppdPfW336-1Pa*, TPotpY-1Pa and the T-DNA probe, this band corresponds to the 5' integration fragment (Figure 5.9 to Figure 5.11, and Figure 5.17, lane 10). The 2356 bp band was obtained with the ThistonAt, the *2mepsps* and the T-DNA probes and not with the other probes (Figure 5.9, Figure 5.16 and Figure 5.17, lane 10). Therefore, this band corresponds to the 3' integration fragment.

In conclusion, the *Sty*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Hin*dIII

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Hin*dIII restriction enzyme produces two integration fragments and one internal fragment (Figure 5.8). The 5' integration fragment is expected to be greater than 3978 bp and contains the genomic DNA flanking the 5' end of the insert, RB sequences, ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence and the Pcsvmv promoter sequence. The internal fragment with expected length of 979 bp contains a lox recognition site and the Ph4a748 promoter sequence. The 3' integration fragment is expected to be 4013 bp and contains the intron1 h3At sequence, the TPotpC sequence, the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Hin*dIII restriction digestion of cotton GHB811 with the Ph4a748 + lox probe and the T-DNA probe confirmed the presence of the 979 bp internal fragment (Figure

5.13 and Figure 5.17, lane 11). Hybridization of the *Hin*dIII restriction digestion of cotton GHB811 with probes intron1 h3At, TPotpC and *2mepsps* resulted in one band of 4013 bp (Figure 5.14 to Figure 5.16, lane 11). This band corresponds to the 3' integration fragment and was also observed with the ThistonAt probe and the T-DNA probe (Figure 5.9 and Figure 5.17, lane 11), as expected. Hybridization of the *Hin*dIII restriction digestion of cotton GHB811 gDNA with probes ThistonAt, *hppdPfW336-1Pa*, TPotpY-1Pa, Pcsvmv and the T-DNA probe resulted in another band of 4300 bp (Figure 5.9 to Figure 5.12, and Figure 5.17, lane 11) which corresponds to the 5' integration fragment.

Hybridization of the *Hin*dIII restriction digestion of cotton GHB811 with the *hppdPfW336-1Pa* probe also resulted in a weak 5200 bp fragment (Figure 5.10, lane 11). The weakness of the signal, combined with the fact that the size of this fragment (5200 bp) corresponds to the approximate summation of the 4300 bp 5' integration fragment and a 979 bp internal fragment demonstrates that this fragment is due to incomplete digestion of the *Hin*dIII restriction site between the Pcsvmv and lox recognition site (Figure 5.8).

In conclusion, the *Hin*dIII restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Restriction digestion with *Ase*I

Based on the presented single copy integration model for the T-DNA region of the pTSIH09 plasmid, the digestion of the insert with the *Ase*I restriction enzyme produces two integration fragments and one internal fragment (Figure 5.8). The 5' integration fragment is expected to be 3508 bp and contains the genomic DNA flanking the 5' end of the insert, RB sequences, ThistonAt terminator sequence following the *hppdPfW336-1Pa* gene, the *hppdPfW336-1Pa* gene, the TPotpY-1Pa sequence and the Pcsvmv promoter sequence. The internal fragment with expected length of 2462 bp contains a lox recognition site, the Ph4a748 promoter sequence, the intron1 h3At sequence, the TPotpC sequence and a part of the *2mepsps* gene sequence. The 3' integration fragment is expected to be 2081 bp and contains a part of the *2mepsps* gene sequence, the ThistonAt terminator sequence following the *2mepsps* gene, the lox recognition site, LB sequences and the genomic DNA flanking the 3' end of the insert.

Hybridization of the *Ase*I restriction digestion of cotton GHB811 with the Ph4a748 + lox probe, the intron1 h3At probe and the TPotpC probe resulted in one band of 2462 bp (Figure 5.13 to Figure 5.15, lane 12). This band corresponds to the internal fragment and was also observed with the *2mepsps* probe and the T-DNA probe (Figure 5.16 and Figure 5.17, lane 12), as expected. Hybridization of the *Ase*I restriction digestion of cotton GHB811 gDNA with probes *hppdPfW336-1Pa*, TPotpY-1Pa and Pcsvmv resulted in one band of 3508 bp (Figure 5.10 to Figure 5.12, lane 12). This band corresponds to the 5' integration fragment and was also observed with the ThistonAt and the T-DNA probes (Figure 5.9 and Figure 5.17, lane 12), as expected. Hybridization of the *Ase*I restriction digestion of cotton GHB811 with probes ThistonAt, *2mepsps* and the T-DNA probe resulted in another band of 2081 bp (Figure 5.9, Figure 5.16 and Figure 5.17, lane 12) which corresponds to the 3' integration fragment.

In conclusion, the *Ase*I restriction digestion of the cotton GHB811 gDNA and subsequent hybridization with all used probes confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09.

Conclusion

Digestions of gDNA from cotton GHB811 by a set of restriction enzymes and subsequent hybridizations with the different probes that spanned the complete T-DNA of pTSIH09 confirmed the integration model based on a single copy of the complete T-DNA region of pTSIH09. A few weak additional bands were observed in hybridization of the *Sac*I or *Hin*dIII digested cotton GHB811 gDNA. The weakness of the signal, combined with the fact that the size of those bands corresponds to the approximate summation of two fragments produced by complete digestion, demonstrates that those additional weak bands are due to incomplete digestion of the *Sac*I *or Hin*dIII restriction site. Therefore, it was demonstrated that a single copy of the complete T-DNA of the pTSIH09 plasmid was inserted at a single locus of the cotton GHB811 genome.

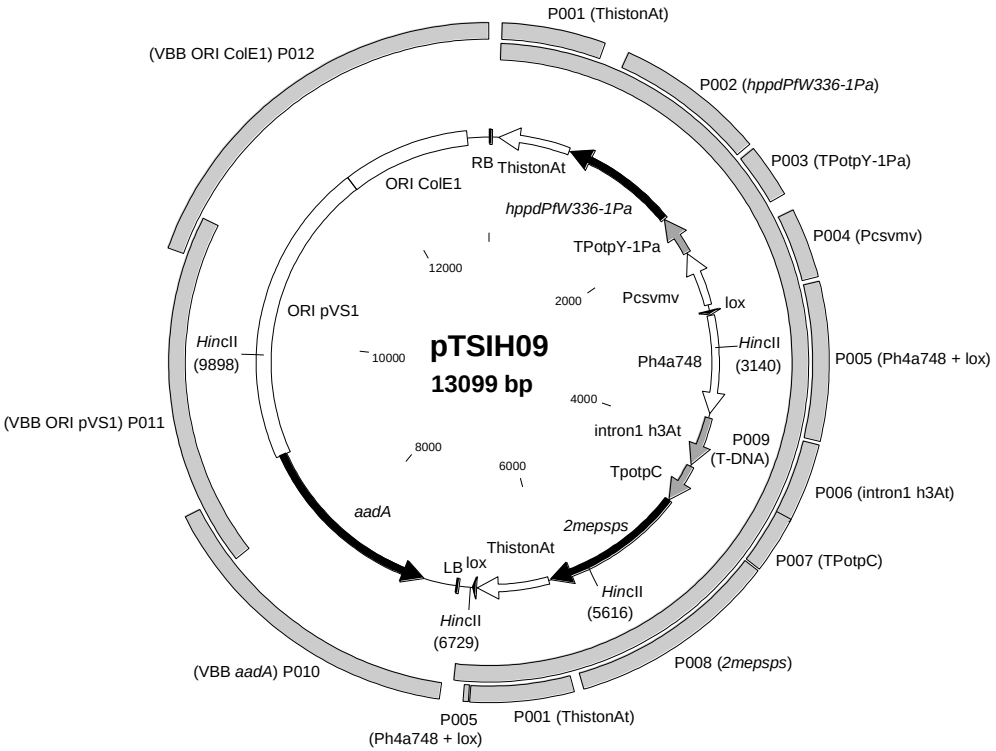


Figure 5.7. Map of transformation vector pTSIH09 with indication of restriction enzymes and probes used for Southern blot analysis

The indicated restriction enzyme positions between brackets refer to the first base after the cleavage site of the restriction enzyme.

Table 5.7. Information on the probes used for insert characterization

Probe ID	Description	Primer pair/ Restriction digest	Primer sequence (5' □ 3')	Primer position on pTSIH09 (bp)	Size probe (bp)	Overlap between probe
P001	ThistonAt	GLPA449°	CCCGATCAAATCTGAGGGAC	83 → 102 and 6665 → 6646	646 6583* 7808*	NA
		GLPA456°	CTGGGTTTCTCACTTAAGCG	728 → 709 and 6020 → 6039		
P002	*hppdPfW336-1Pa*	GLPA457	AAACGGGTCCCATGAGAGTC	882 → 901	939	NA
		GLPA312	CTATGGGACTCATGGGTTTC	1820 → 1801		
P003	TPotpY-1Pa	GLPA459	ACCTCCGTTGCTAACATTCC	1855 → 1874	338	NA
		GLPA460	TTGCCACTGTTTCACGTACC	2192 → 2173		
P004	Pcsvmv	GLPA473	CAAATGCCGAACTTGGTTCC	2303 → 2322	439	NA
		GLPA474	GGCCGCGAAGGTAATTATCC	2741 → 2722		
P005	Ph4a748 + lox	GLPA472	CCCTGTTATCCCTAAAGCTTATTAA TATAAC	2770 → 2800	997	NA
		GLPA462	CGTGGGATCCTCTAGAGTCG	3762 → 3747**		
P006	intron1 h3At	GLPA073	TCAGGCGAAGAACAGGTATG	3785 → 3804	507	NA
		GLPA463	ACTGAGGAGGAGATCGAAGC	4291 → 4272		
P007	TPotpC	GLPA464	GCTTCGATCTCCTCCTCAGT	4272 → 4291	363	NA
		GLPA465	GATCCTTCCGCCGTTGCTGA	4634 → 4615		
P008	*2mepsps*	GLPA075	GCGCCGAGGAGATCGTGCTGC	4648 → 4668	1312	NA
		GLPA076	CTCAGCACATCGAAGTAGTC	5959 → 5940		
P009	T-DNA	GLPA467	AAGGCCCGATCAAATCTGAG	79 → 98	6700	NA
		GLPA468	GTGCCGTAATGCCGTAATGC	6778 → 6759		
P010	Vector backbone - *aadA*	GLPA032	GCCGCCGCTGCCGCTTTGC	6853 → 6871	1990	395 bp
		GLPA352	AGATCCTTGACCCGCAGTTG	8842 → 8823		
P011	Vector backbone - ORI pVS1	GLPA180	GAACCGAACAGGCTTATGTC	8448 → 8467	2354	
		GLPA469	GCGTGGTGTTTAACCGAATG	10801 → 10782		261 bp
P012	Vector backbone - ORI ColE1	GLPA470	TCCGCTACGAGCTTCCAGAC	10541 → 10560	2559	
		GLPA161	TGTCGCGTGTGAATAAGTCGC	13099 → 13079		

NA means not applicable

* Two additional PCR products of 6583 bp and 7808 bp can be generated with these primers. Only the fragment of interest (646 bp) was produced.

** Part of the GLPA462 does not bind on pTSIH09

° These primers amplify two identical regions

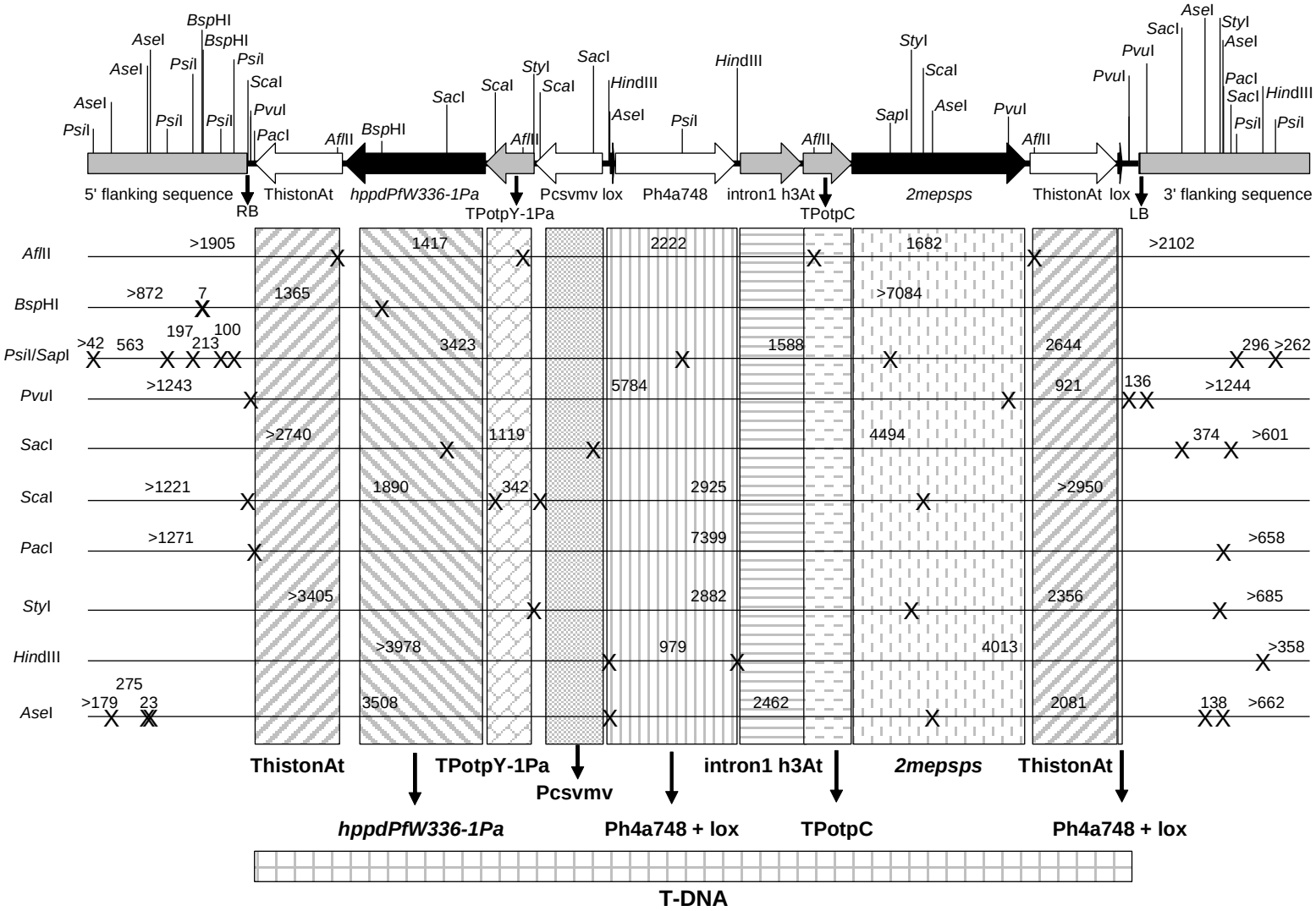


Figure 5.8. Schematic overview of the cotton GHB811 transgenic locus sequence with indication of the different restriction enzymes and probes used for the cotton GHB811 insert characterization and expected fragment sizes (bp).

Table 5.8. Expected and obtained hybridization fragments determined for the insert characterization of cotton GHB811

Sample	Expected fragment sizes (bp)[a]	Obtained fragment sizes (bp)	Fragment description	H1/THT063B/28-F3		H1/THT063B/29-F4		H1/THT063B/30-F3		H1/THT063B/31-F2		H2/THT063B/30-F3		H2/THT063B/31-F2		H3/THT063B/31-F2		H2/THT063B/29-F2		H2/THT063B/28-F2	
				P001-3 ThistonAt		P002-1 *hppdPfW336-1Pa*		P003-3 TPotpY-1Pa		P004-3 Pcsvmv		P005-2 Ph4a748 + lox		P006-2 intron1 h3At		P007-3 TPotpC		P008-2 *2mepsps*		P009-7 T-DNA	
				Figure 5.9		Figure 5.10		Figure 5.11		Figure 5.12		Figure 5.13		Figure 5.14		Figure 5.15		Figure 5.16		Figure 5.17	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
GHB811 - *Afl*II	> 1905	2500[$]	5' integration fragment	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	1417	1417	internal fragment	Yes**(17)	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	2222	2222	internal fragment	No	No	No	No	Yes**(64)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes**(79)	Yes	No	No	Yes	Yes
	1682	1682	internal fragment	Yes**(13)	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	> 2102	4400[$]	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	No	No	Yes	Yes
GHB811 - *Bsp*HI	1365	1365	5' integration fragment	Yes	Yes	Yes**(169)	Yes	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	> 7084	8300	3' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GHB811 – *Psi*I/ *Sap*I	3423	3423	5' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes
	1588	1588	internal fragment	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2644	2644	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes	Yes	Yes	Yes
GHB811 – *Pvu*I	> 1243	/	5' integration fragment	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	5784	5784	Internal fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	921	1050 [c]	Internal fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes**(126)	Yes	Yes	Yes
	136		3' integration fragment	No		No		No		No		No		No		No		No		Yes**(24)	
GHB811 – *Sac*I	> 2740	> 10 000	5' integration fragment	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	1119	1119	Internal fragment	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes

Sample	Expected fragment sizes (bp)[a]	Obtained fragment sizes (bp)	Fragment description	H1/THT063B/28-F3		H1/THT063B/29-F4		H1/THT063B/30-F3		H1/THT063B/31-F2		H2/THT063B/30-F3		H2/THT063B/31-F2		H3/THT063B/31-F2		H2/THT063B/29-F2		H2/THT063B/28-F2	
				P001-3 ThistonAt		P002-1 *hppdPfW336-1Pa*		P003-3 TPotpY-1Pa		P004-3 Pcsvmv		P005-2 Ph4a748 + lox		P006-2 intron1 h3At		P007-3 TPotpC		P008-2 *2mepsps*		P009-7 T-DNA	
				Figure 5.9		Figure 5.10		Figure 5.11		Figure 5.12		Figure 5.13		Figure 5.14		Figure 5.15		Figure 5.16		Figure 5.17	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
	4494	4494	3' integration fragment	Yes	Yes	No	No	No	No	Yes** (76)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	/	4900	additional fragment (partial digestion)	No	Yes	No	No	No	No	No	No	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
GHB811 – *Sca*I	> 1221	/	5' integration fragment	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	1890	1890	Internal fragment	Yes	Yes	Yes	Yes	Yes**(63)	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	342*	342*	Internal fragment	No	No	No	No	Yes*	Yes	No	No	No	No	No	No	No	No	No	No	Yes*	No
	2925	2925	Internal fragment	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	> 2950	5900	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes	Yes	Yes	Yes
GHB811 – *Pac*I	> 1271	/	5' integration fragment	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	7399	7399	3' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GHB811 – *Sty*I	> 3405[b]	3400[b]	5' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	2882	2882	Internal fragment	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2356	2356	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes	Yes	Yes	Yes
GHB811 – *Hin*dIII	> 3978	4300	5' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes**(15)	No	No	No	No	No	No	No	Yes	Yes
	979	979	Internal fragment	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	Yes	Yes
	4013	4013	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	/	5200	additional fragment (partial digestion)	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No

Sample	Expected fragment sizes (bp)[a]	Obtained fragment sizes (bp)	Fragment description	H1/THT063B/28-F3		H1/THT063B/29-F4		H1/THT063B/30-F3		H1/THT063B/31-F2		H2/THT063B/30-F3		H2/THT063B/31-F2		H3/THT063B/31-F2		H2/THT063B/29-F2		H2/THT063B/28-F2	
				P001-3 ThistonAt		P002-1 *hppdPfW336-1Pa*		P003-3 TPotpY-1Pa		P004-3 Pcsvmv		P005-2 Ph4a748 + lox		P006-2 intron1 h3At		P007-3 TPotpC		P008-2 *2mepsps*		P009-7 T-DNA	
				Figure 5.9		Figure 5.10		Figure 5.11		Figure 5.12		Figure 5.13		Figure 5.14		Figure 5.15		Figure 5.16		Figure 5.17	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
GHB811 – *Ase*I	3508	3508	5' integration fragment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes**(22)	No	No	No	No	No	No	No	Yes	Yes
	2462	2462	Internal fragment	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2081	2081	3' integration fragment	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes	Yes	Yes	Yes
non-GM counterpart (Coker 312) - *Bsp*HI	/	/	negative control	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
non-GM counterpart - *Hinc*II + one equimolar amount pTSIH09 - *Hinc*II	6341	6341	positive control	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes
	2476	2476	positive control	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	1113	1113	positive control	Yes	Yes	No	No	No	No	No	No	Yes**(34)	No	No	No	No	No	Yes	Yes	Yes	Yes
	3169	3169	positive control	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes**(50)	Yes

Exp. : expected; Obt.: obtained;

[a] Expected fragment sizes are based on the full sequence of GHB811

[b] The obtained fragment size is smaller than the expected fragment size because of rounding of the values

[c] Instead of the expected fragment of 921 bp in the *Pvu*I digest, a 1050 bp fragment was obtained, which was the result of incomplete digestion at the *Pvu*I restriction site neighboring the LB region. The size of this fragment (1050 bp) corresponds to the summation of the 921 bp internal fragment and a 136 bp 3' integration fragment.

* Based on technical limitations of the Southern blotting technique, these fragments might be too small for visualization

** Due to a small overlap with the probe, these fragments are not always be visible. The size of the overlap is indicated between brackets.

° The obtained fragment size exceeded the 10 % range of the expected fragment size.

[$] With this experimental setup, it is not possible to assign this fragment to either the 5' or 3' integration fragment

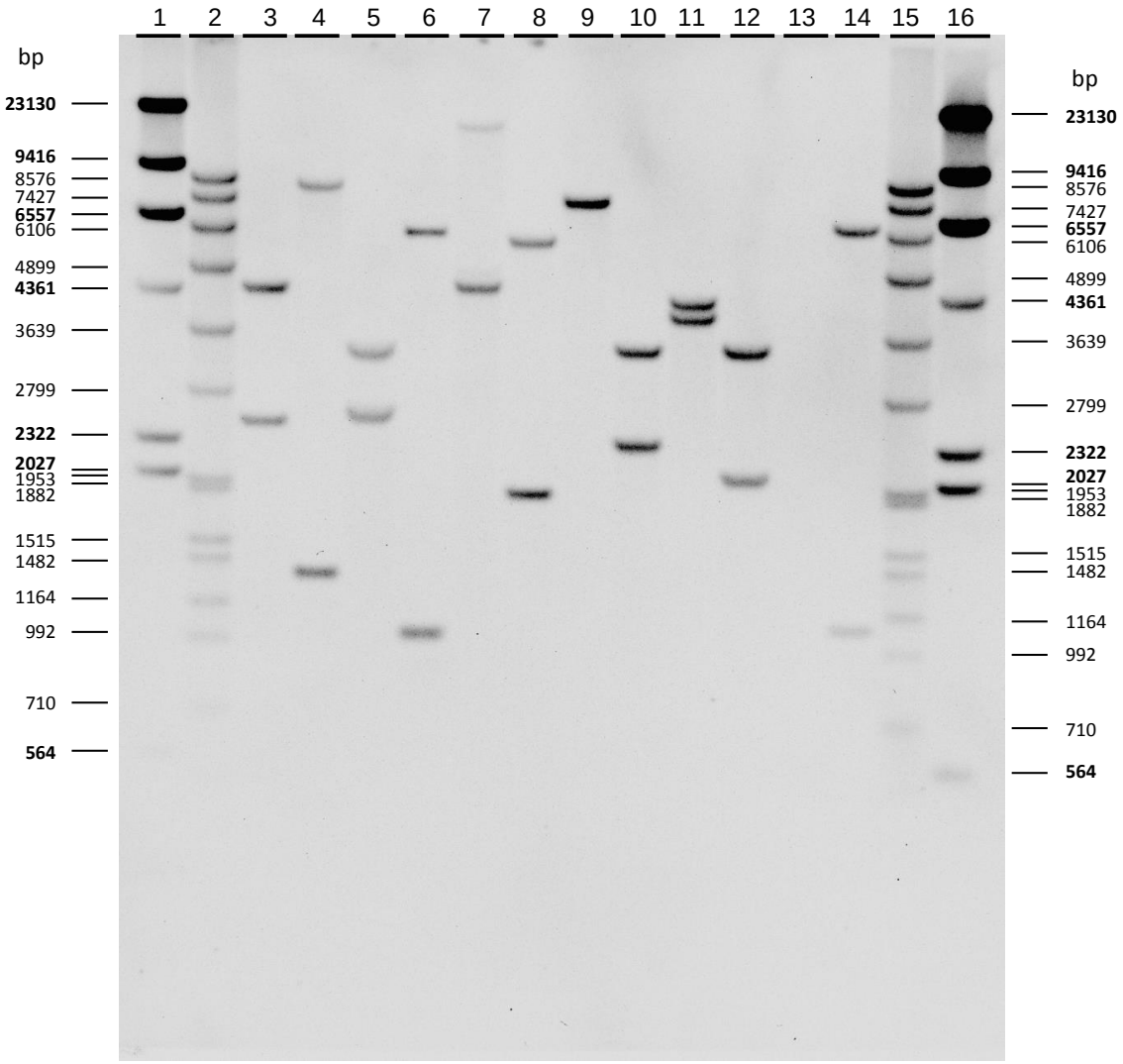


Figure 5.9. Hybridization performed with a ThistonAt probe (P001) to determine the insert organization of cotton GHB811

Digital image: H1/THT063B/28-F3

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 ThistonAt sequence (P001-3).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

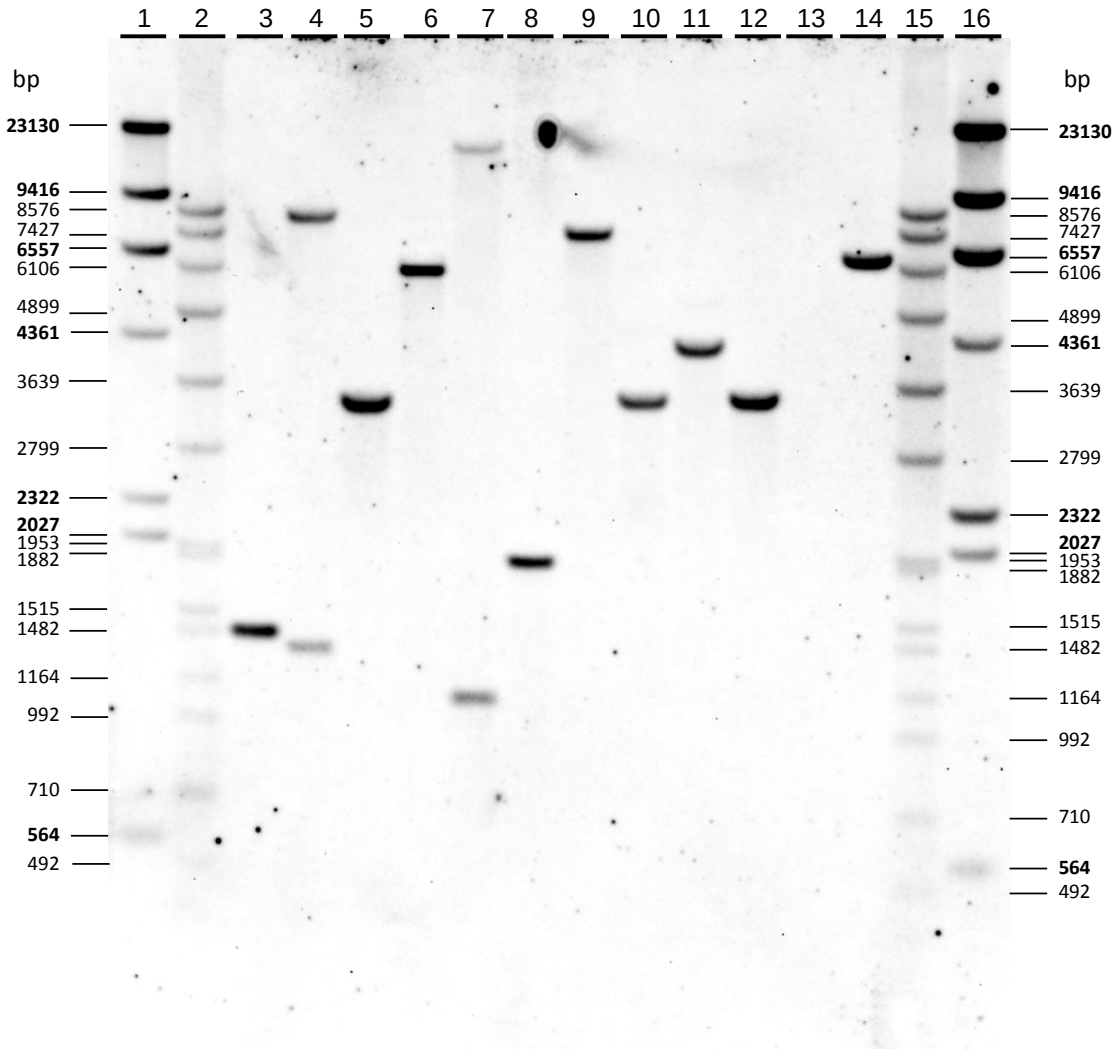


Figure 5.10. Hybridization performed with a *hppdPfW336-1Pa* probe (P002) to determine the insert organization of cotton GHB811

Digital image ID: H1/THT063B/29-F4

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 *hppdPfW336-1Pa* sequence (P002-1).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

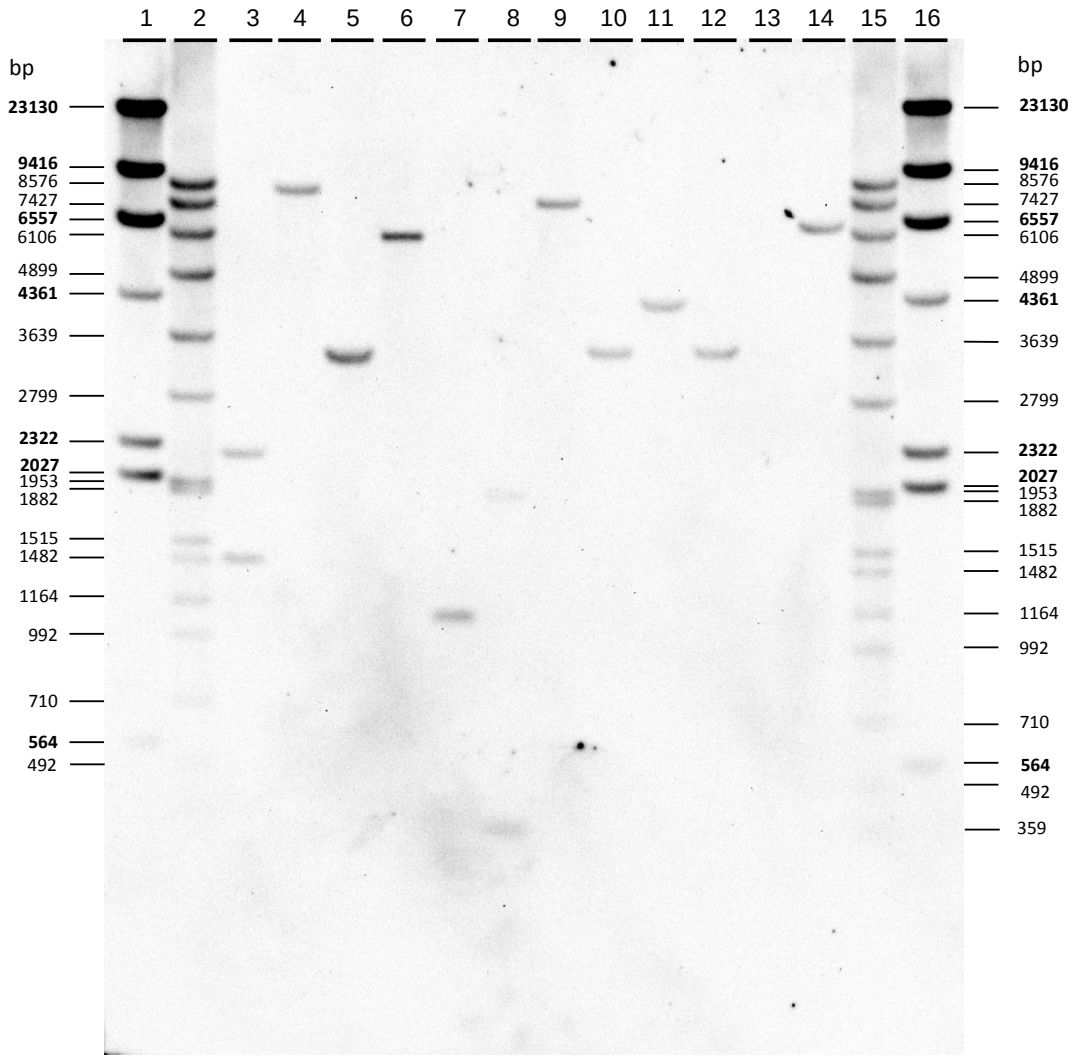


Figure 5.11. Hybridization performed with a TPotpY-1Pa probe (P003) to determine the insert organization of cotton GHB811

Digital image ID: H1/THT063B/30-F3

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 TPotpY-1Pa sequence (P003-3).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hind*III digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hind*III digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hinc*II digested + an equimolar amount of pTSIH09 - *Hinc*II digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hind*III digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

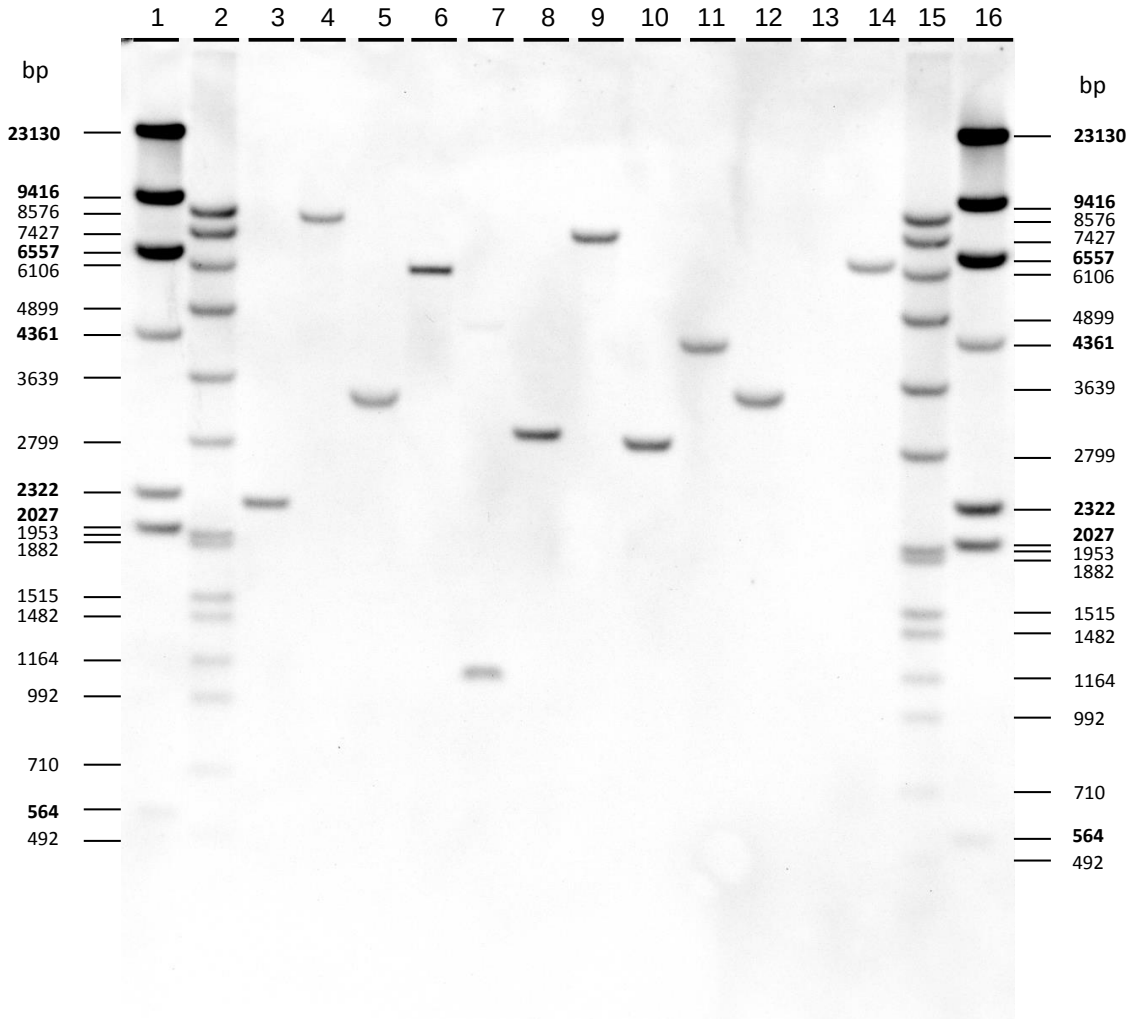


Figure 5.12. Hybridization performed with a Pcsvmv probe (P004) to determine the insert organization of cotton GHB811

Digital image ID: H1/THT063B/31-F2

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 Pcsvmv sequence (P004-3).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

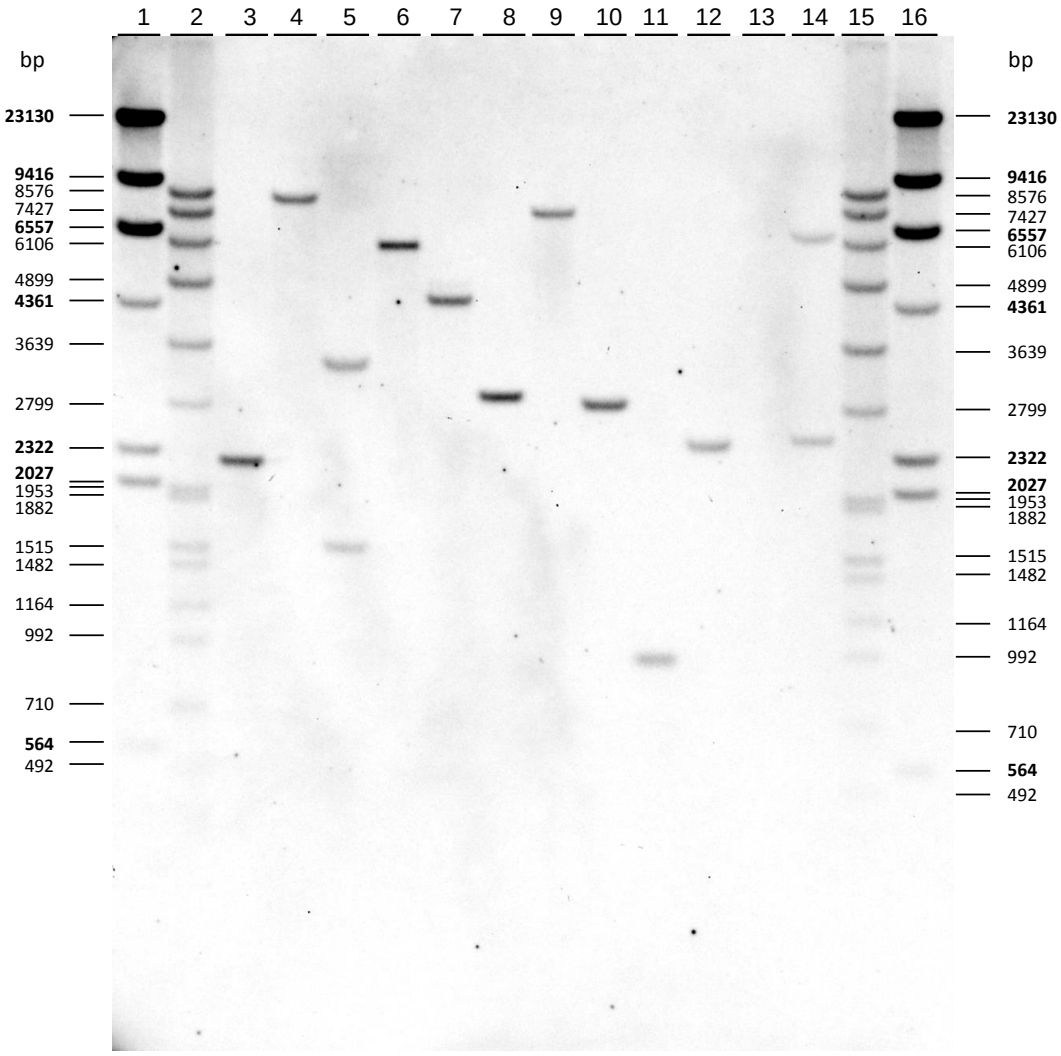


Figure 5.13. Hybridization performed with a Ph4a748 + lox (P005) to determine the insert organization of cotton GHB811

Digital image ID: H2/THT063B/30-F3

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 Ph4a748 + lox recognition site (P005-2).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

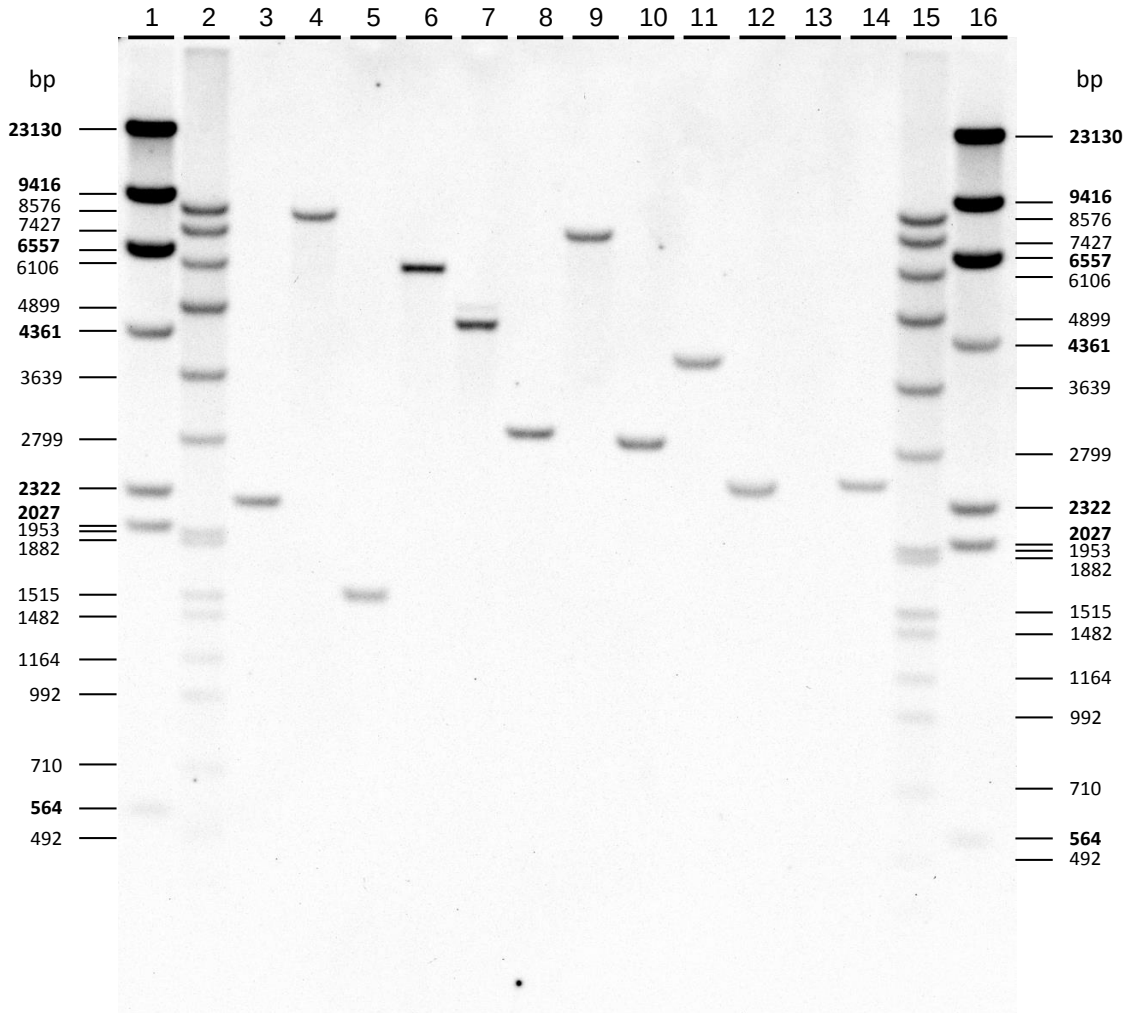


Figure 5.14. Hybridization performed with a intron1 h3At probe (P006) to determine the insert organization of cotton GHB811

Digital image ID: H2/THT063B/31-F2

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 intron1 h3At sequence (P006-2).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hind*III digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hinc*II digested + an equimolar amount of pTSIH09 - *Hinc*II digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hind*III digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

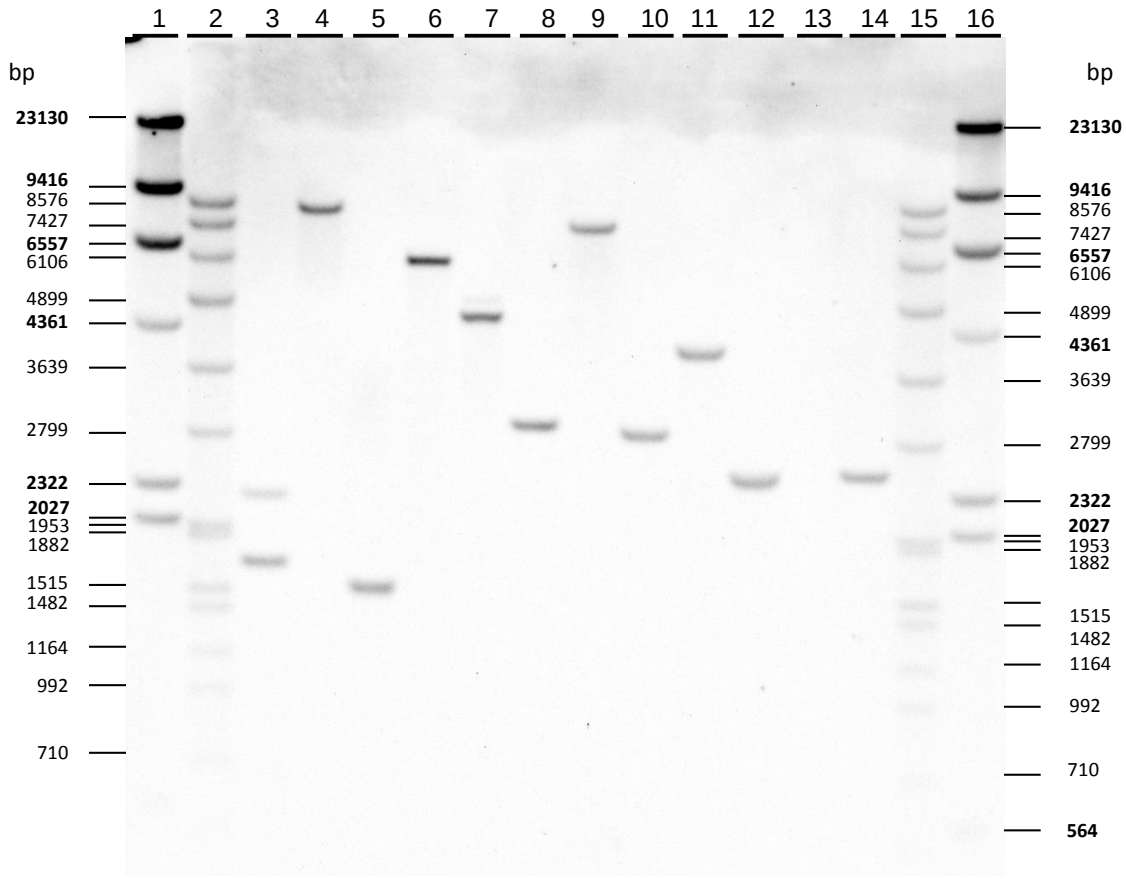


Figure 5.15. Hybridization performed with a TPotpC probe (P007) to determine the insert organization of cotton GHB811

Digital image ID: H3/THT063B/31-F2

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 TPotpC sequence (P007-3).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

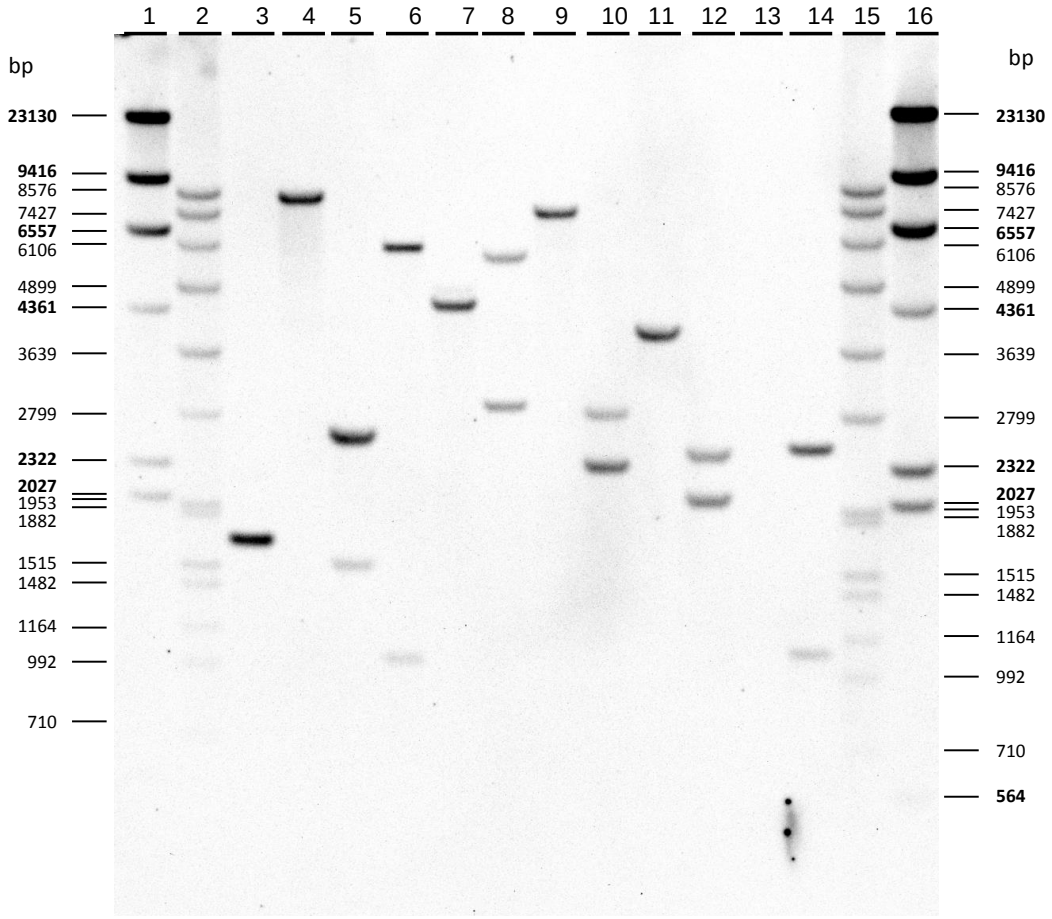


Figure 5.16. Hybridization performed with a *2mepsps* probe (P008) to determine the insert organization of cotton GHB811

Digital image ID: H2/THT063B/29-F2

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 *2mepsps* sequence (P008-2).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

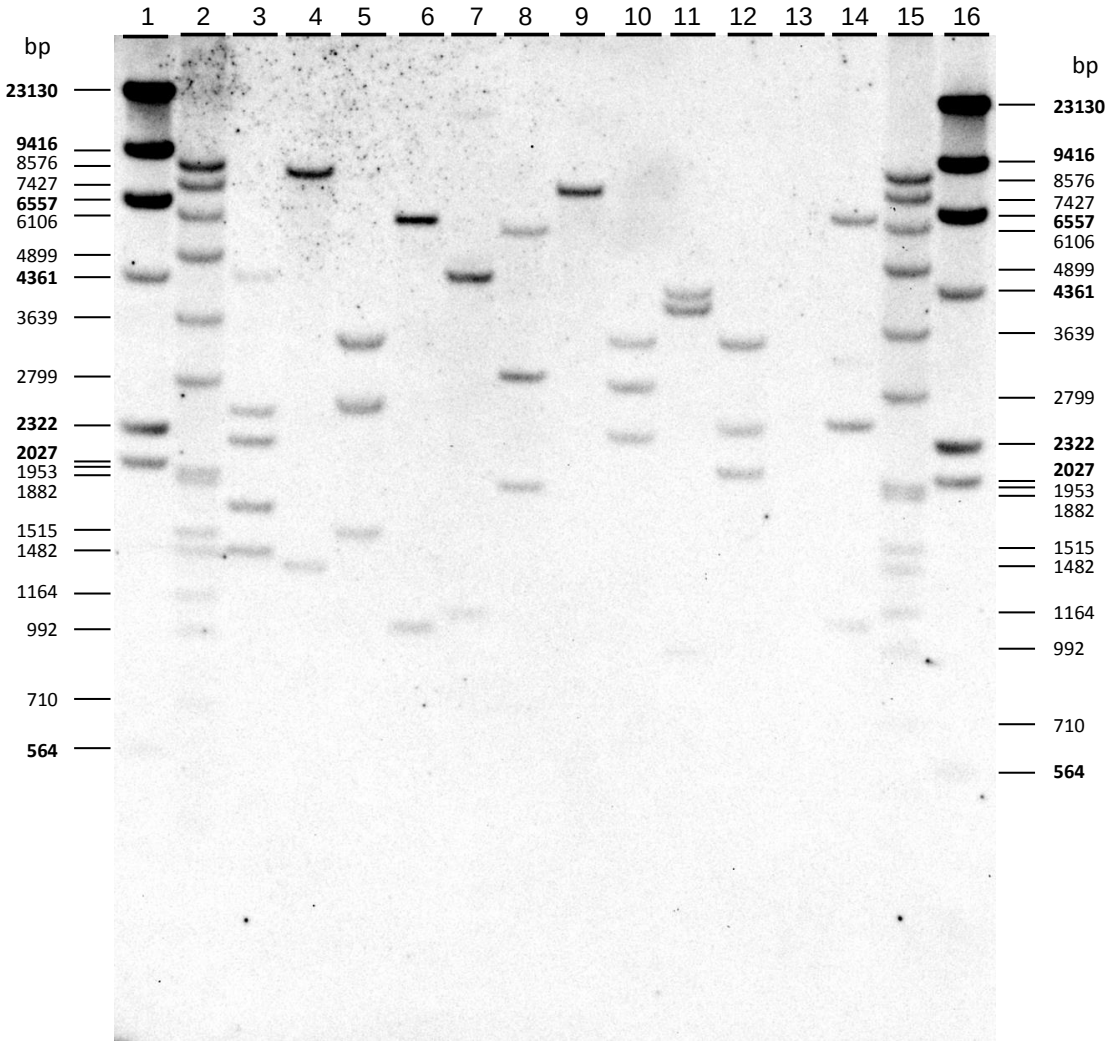


Figure 5.17. Hybridization performed with a T-DNA probe (P009) to determine the insert organization of cotton GHB811

Digital image ID: H2/THT063B/28-F2

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the cotton GHB811 T-DNA region (P009-7).

Lane 1: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from cotton GHB811 - *Afl*II digested
Lane 4: 3 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 5: 3 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 6: 3 µg gDNA from cotton GHB811 - *Pvu*I digested
Lane 7: 3 µg gDNA from cotton GHB811 - *Sac*I digested
Lane 8: 3 µg gDNA from cotton GHB811 - *Sca*I digested
Lane 9: 3 µg gDNA from cotton GHB811 - *Pac*I digested
Lane 10: 3 µg gDNA from cotton GHB811 - *Sty*I digested
Lane 11: 3 µg gDNA from cotton GHB811 - *Hin*dIII digested
Lane 12: 3 µg gDNA from cotton GHB811 - *Ase*I digested
Lane 13: 3 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 14: 3 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 15: 3 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 3 µg gDNA from non-GM counterpart - *Hin*dIII digested + 7.5 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

5.3. The absence of vector backbone sequences

The presence or absence of vector backbone sequences in cotton GHB811 was assessed by means of Southern blot analysis.

Genomic DNA (gDNA) from the cotton GHB811 T1 and BC2F3 generations was digested with restriction enzymes and subjected to Southern blot analysis using probes (P010 –P012) that collectively spanned the complete plasmid vector backbone (Figure 5.7). The selection and design of probes used in this study allowed for the assessment of the presence or absence of vector backbone sequences in the cotton GHB811 genome. Probes used in this study are summarized in Table 5.7. Expected and obtained hybridization fragments are listed in Table 5.9 and Table 5.10. The hybridization results in the T1 and BC2F3 generations of cotton GHB811 are presented in Figure 5.18 to Figure 5.23.

Each membrane contained one negative control, which showed no hybridization with any of the probes used (Figure 5.18 to Figure 5.23, lane 5). Consequently, the absence of any background hybridization was demonstrated for all the probes used.

Similarly, each membrane contained a positive control. This positive control, consisting of pTSIH09 plasmid DNA, was digested with *Hin*cII and an equimolar amount was spiked in *Hin*cII digested gDNA from the non-GM counterpart. For each of the probes used, the expected fragments were detected (Figure 5.18 to Figure 5.23, lane 7), confirming that the applied experimental conditions allowed specific hybridization of the used probes with the target sequences.

Additionally, a supplementary positive control was used. This additional positive control, consisting of pTSIH09 plasmid DNA, was digested with *Hin*cII and a $1/10^{th}$ equimolar amount was spiked in *Hin*cII digested gDNA from the non-GM counterpart. Both positive controls, supplemented with one or $1/10^{th}$ equimolar amount of the *Hin*cII digested gDNA from the non-GM counterpart, showed the expected hybridization fragments after hybridization with the vector backbone probes (Figure 5.18 to Figure 5.23, lanes 6 and 7). This demonstrated that the hybridizations were performed in conditions allowing detection of the possible presence of vector backbone in cotton GHB811 genome. After hybridization with the T-DNA probe, the expected fragments were obtained in the positive control spiked with one equimolar amount of pTSIH09 digested plasmid DNA (data not shown). This demonstrated that the hybridizations were performed in conditions allowing detection of the presence of T-DNA sequences.

Hybridization of the digested cotton GHB811 gDNA samples with the vector backbone probes resulted in no hybridization fragments, as expected (Figure 5.18 to Figure 5.23, lanes 3 and 4). This demonstrated the absence of vector backbone sequences in cotton GHB811 gDNA. When hybridizing the same membranes with the T-DNA probe, all expected fragments were obtained (data not shown). This demonstrated that an ample amount of a sufficient quality of digested cotton GHB811 gDNA was loaded on the gels to enable detection of vector backbone sequences in cotton GHB811.

Table 5.9. Expected and obtained hybridization fragments determined for the vector backbone assessment in T1 generation of cotton GHB811

Sample	T-DNA or plasmid fragment sizes (bp)	Fragment description	Membrane M/THT063B/12				Membrane M/THT063B/13				Membrane M/THT063B/14			
			P010-1 Vector backbone probe		P009-1 T-DNA probe		P011-1 Vector backbone probe		P009-1 T-DNA probe		P012-1 Vector backbone probe		P009-1 T-DNA probe	
			Figure 5.18				Figure 5.19				Figure 5.20			
			Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
GHB811-*Bsp*HI	1365	5' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	8300*	3' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
GHB811-*Psi*I/*Sap*I	3423	5' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	1588	Internal fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	2644	3' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
non-GM counterpart (Coker 312) - *Bsp*HI	/	/	/	/	/	/	/	/	/	/	/	/	/	/
non-GM counterpart - *Hinc*II + 0.1 equimolar amount pTSIH09 - *Hinc*II	6341	positive control	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2476	positive control	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	No°
	1113	positive control	No	No	Yes	No°	No	No	Yes	No°	No	No	Yes	No°
	3169	positive control	Yes	Yes	Yes**(50)	No	Yes	Yes	Yes**(50)	No	No	No	Yes**(50)	No
non-GM counterpart - *Hinc*II + 1 equimolar amount pTSIH09 - *Hinc*II	6341	positive control	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2476	positive control	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	1113	positive control	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	3169	positive control	Yes	Yes	Yes**(50)	No	Yes	Yes	Yes**(50)	No	No	No	Yes**(50)	No

* Fragment sizes as determined in the detailed insert characterization and vector backbone assessment in this study

** Due to a small overlap with the probe, these fragments may not be visible. The size of the overlap is indicated between brackets.

° Although not all expected fragments of positive control containing 1/10th equimolar amount of pTSIH09 were obtained after hybridization with the T-DNA probe, all expected fragments were obtained after hybridization with the vector backbone probes. Therefore, hybridization conditions were appropriate to detect vector backbone sequence.

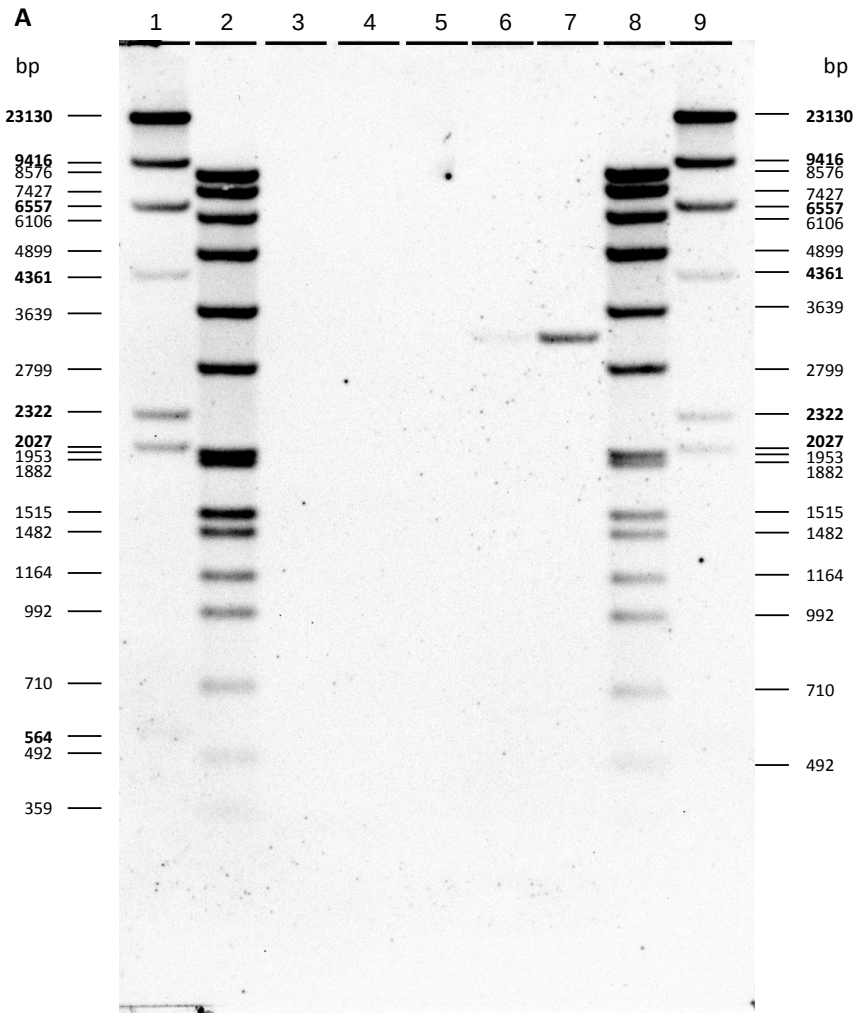
Table 5.10. Expected and obtained hybridization fragments determined for the vector backbone assessment in BC2F3 generation of cotton GHB811

Sample	T-DNA or plasmid fragment sizes (bp)	Fragment description	Membrane M/THT063B/23				Membrane M/THT063B/24				Membrane M/THT063B/25			
			P010-1 Vector backbone probe		P009-5 T-DNA probe		P011-2 Vector backbone probe		P009-2 T-DNA probe		P012-3 Vector backbone probe		P009-5 T-DNA probe	
			Figure 5.21				Figure 5.22				Figure 5.23			
			Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
GHB811-*Bsp*HI	1365	5' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	8300*	3' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
GHB811-*Psi*I/*Sap*I	3423	5' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	1588	Internal fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	2644	3' integration fragment	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
non-GM counterpart (Coker 312) - *Bsp*HI	/	/	/	/	/	/	/	/	/	/	/	/	/	/
non-GM counterpart - *Hin*cII + 0.1 equimolar amount pTSIH09 - *Hin*cII	6341	positive control	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2476	positive control	No	No	Yes	Yes	No	No	Yes	No°	No	No	Yes	Yes
	1113	positive control	No	No	Yes	Yes	No	No	Yes	No°	No	No	Yes	Yes
	3169	positive control	Yes	Yes	Yes**(50)	No	Yes	Yes	Yes**(50)	No	No	No	Yes**(50)	No
non-GM counterpart - *Hin*cII + 1 equimolar amount pTSIH09 - *Hin*cII	6341	positive control	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	2476	positive control	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	1113	positive control	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
	3169	positive control	Yes	Yes	Yes**(50)	No	Yes	Yes	Yes**(50)	No	No	No	Yes**(50)	No

* Fragment sizes as determined in the detailed insert characterization and vector backbone assessment in this study

** Due to a small overlap with the probe, these fragments may not be visible. The size of the overlap is indicated between brackets.

° Although not all expected fragments of positive control containing 1/10th equimolar amount of pTSIH09 were obtained after hybridization with the T-DNA probe, all expected fragments were obtained after hybridization with the vector backbone probes. Therefore, hybridization conditions were appropriate to detect vector backbone sequence.

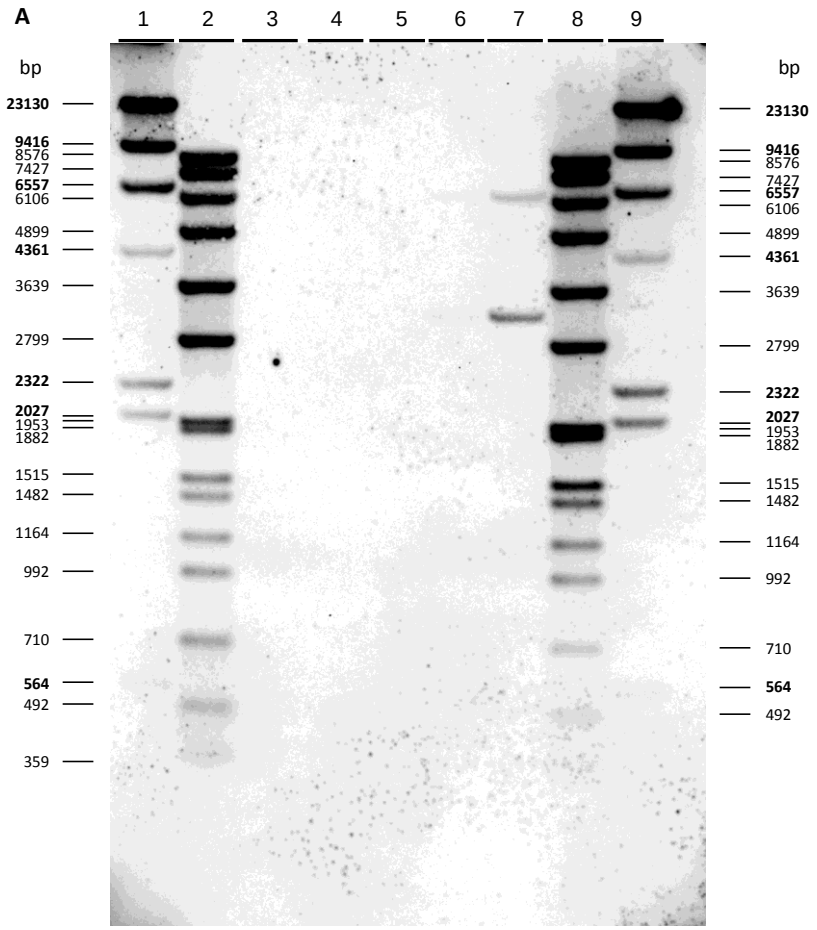


H1/THT063B/12-F6: Vector backbone probe P010-1

Figure 5.18. Hybridization performed with a vector backbone probe covering the aadA sequence (P010) to assess the vector backbone presence in the T1 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *Psi*I/*Sap*I and hybridized with a vector backbone probe (P010-1) and with the T-DNA probe (P009-1) (data not shown).

Lane 1: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3.5 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 3.5 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 3.5 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + 1/10th of an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 7: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 8: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

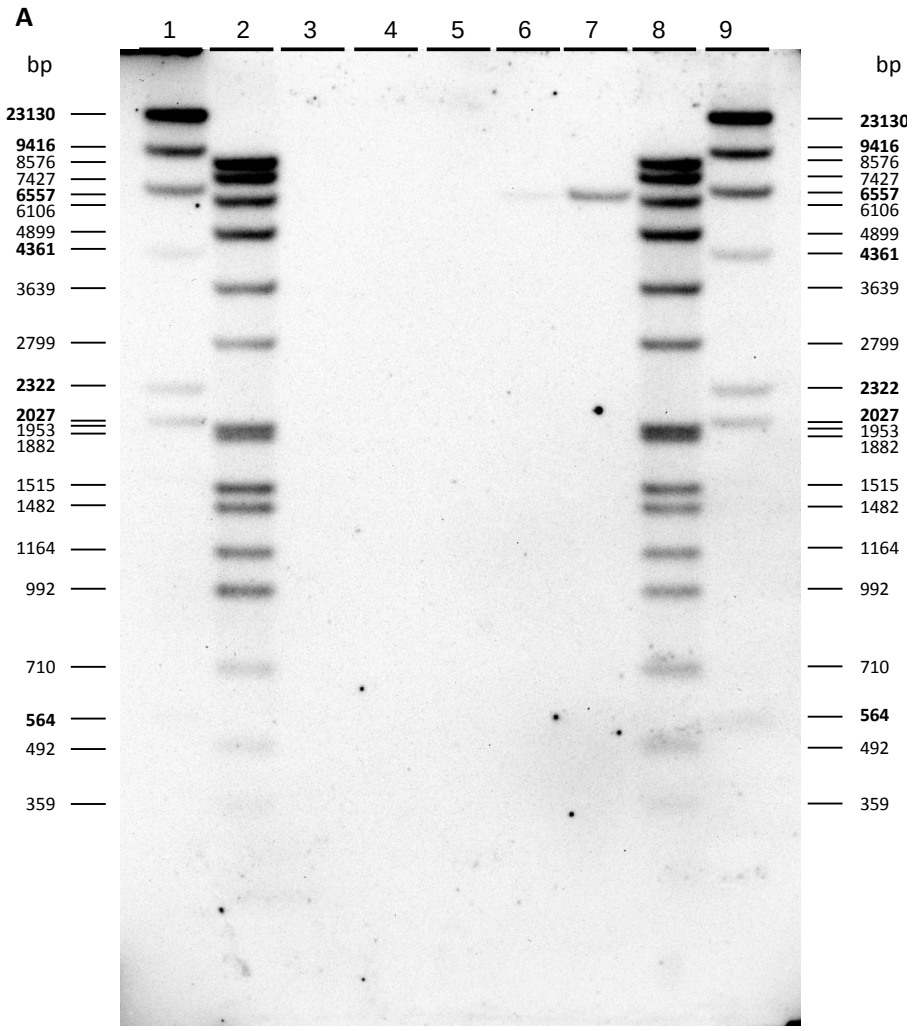


H1/THT063B/13-F4: Vector backbone probe P011-1

Figure 5.19. Hybridization performed with a vector backbone probe covering the ORI pVS1 region (P011) to assess the vector backbone presence in the T1 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *Psi*I/*Sap*I and hybridized with a vector backbone probe (P011-1) and with the T-DNA probe (P009-1) (data not shown).

Lane 1: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3.5 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 3.5 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 3.5 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + 1/10th of an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 7: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 8: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

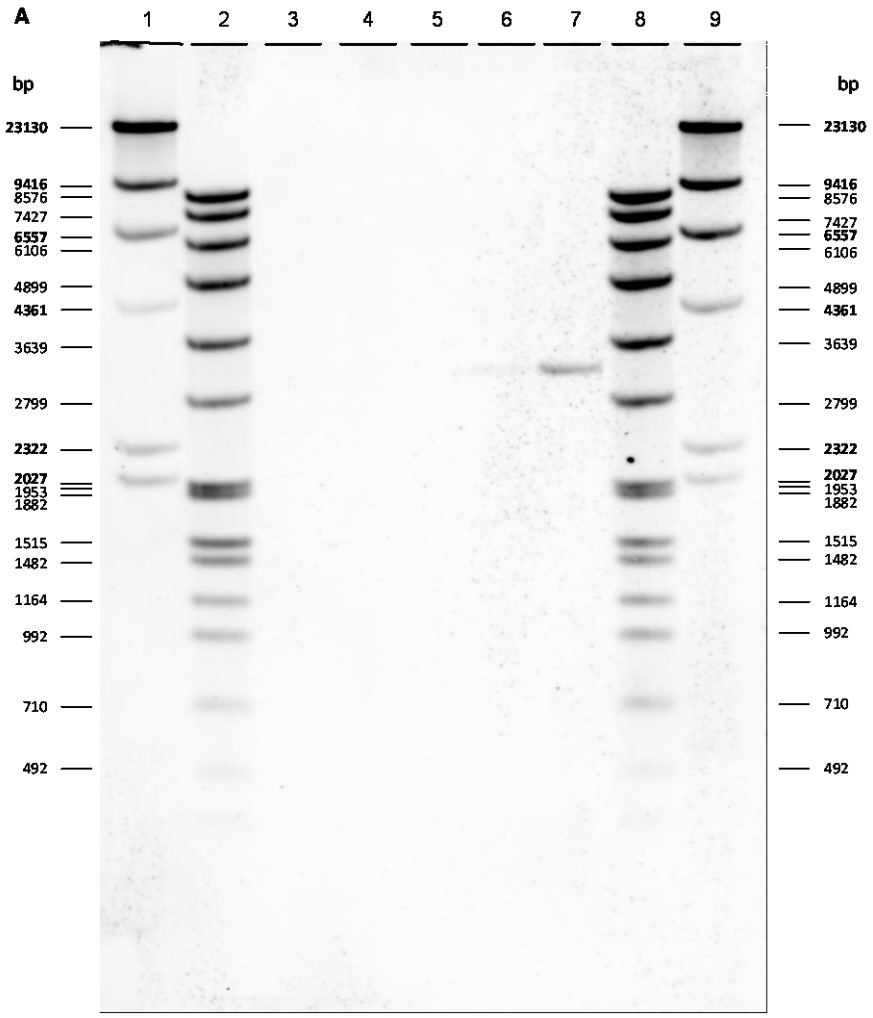


H1/THT063B/14-F4: Vector backbone probe P012-1

Figure 5.20. Hybridization performed with a vector backbone probe covering the ORI ColE1 region (P012) to assess the vector backbone presence in the T1 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (T1 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *PsiI*/*Sap*I and hybridized with a vector backbone probe (P012-1) and with the T-DNA probe (P009-1) (data not shown).

Lane 1: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3.5 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 3.5 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 3.5 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + 1/10th of an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 7: 3.5 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 8: 3.5 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3.5 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

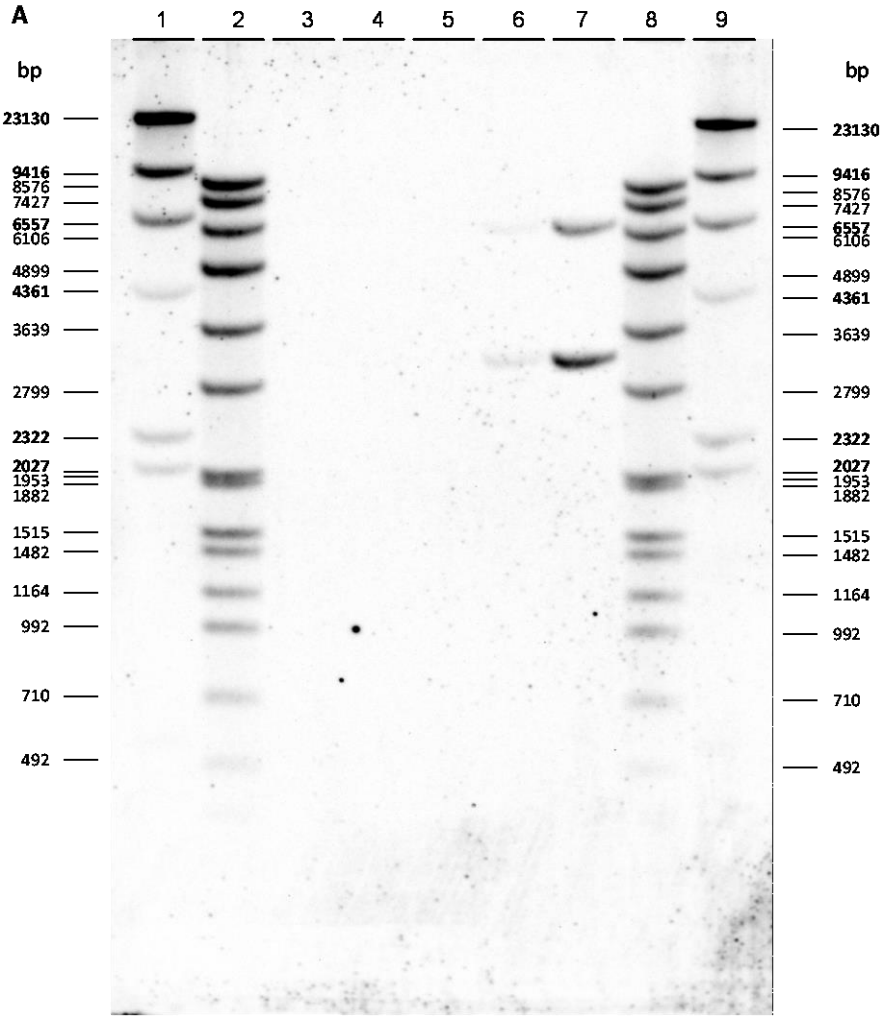


H1/THT063B/23-F1: Vector backbone probe P010-1

Figure 5.21. Hybridization performed with a vector backbone probe covering the aadA sequence (P010) to assess the vector backbone presence in the BC2F3 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (BC2F3 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *Psi*I/*Sap*I and hybridized with a vector backbone probe (P010-1) and with the T-DNA probe (P009-5) (data not shown).

Lane 1: 4 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 4 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 4 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 4 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 4 µg gDNA from non-GM counterpart - *Hin*cII digested + 1/10th of an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 7: 4 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 8: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 4 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

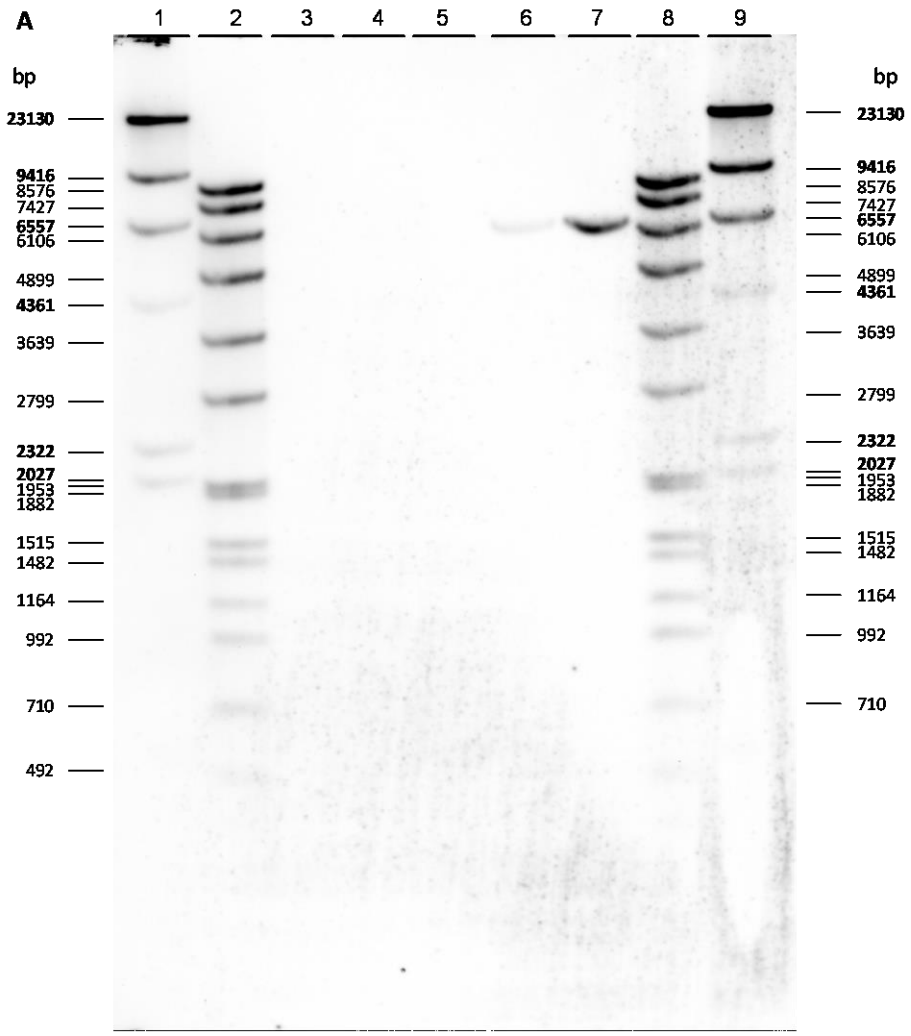


H2/THT063B/24-F4: Vector backbone probe P011-2

Figure 5.22. Hybridization performed with a vector backbone probe covering the ORI pVS1 region (P011) to assess the vector backbone presence in the BC2F3 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (BC2F3 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *PsiI*/*Sap*I and hybridized with a vector backbone probe (P011-2) and with the T-DNA probe (P009-2) (data not shown).

Lane 1: 4 µg gDNA from non-GM counterpart - *Hind*III digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 4 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 4 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 4 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 4 µg gDNA from non-GM counterpart - *Hinc*II digested + 1/10th of an equimolar amount of pTSIH09 - *Hinc*II digested
Lane 7: 4 µg gDNA from non-GM counterpart - *Hinc*II digested + an equimolar amount of pTSIH09 - *Hinc*II digested
Lane 8: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 4 µg gDNA from non-GM counterpart - *Hind*III digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)



H1/THT063B/25-F4: Vector backbone probe P012-3

Figure 5.23. Hybridization performed with a vector backbone probe covering the ORI ColE1 region (P012) to assess the vector backbone presence in the BC2F3 generation of cotton GHB811

gDNA was isolated from cotton GHB811 plants (BC2F3 generation) and from the non-GM counterpart plants. The gDNA samples were digested with restriction enzymes *Bsp*HI and *Psi*I/*Sap*I and hybridized with a vector backbone probe (P012-3) and with the T-DNA probe (P009-5) (data not shown).

Lane 1: 4 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 4 µg gDNA from cotton GHB811 - *Bsp*HI digested
Lane 4: 4 µg gDNA from cotton GHB811 - *Psi*I/*Sap*I digested
Lane 5: 4 µg gDNA from non-GM counterpart - *Bsp*HI digested
Lane 6: 4 µg gDNA from non-GM counterpart - *Hin*cII digested + 1/10th of an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 7: 4 µg gDNA from non-GM counterpart - *Hin*cII digested + an equimolar amount of pTSIH09 - *Hin*cII digested
Lane 8: 4 µg gDNA from non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 4 µg gDNA from non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

5.4. Inheritance of the insert

Genomic DNA from individual plants of three GHB811 cotton generations (F2, BC1F2, and BC2F2) was tested for the genotype of *hppdPfW336-1Pa* and *2mepsps* genes by polymerase chain reaction (PCR) analysis. The results from PCR analysis were used to calculate the segregation ratios of the genes contained within the GHB811 insert. Chi-square analysis of the segregation data for three generations was performed to test the hypothesis that the GHB811 cotton insert is inherited in a manner that is predictable according to Mendelian principles and is consistent with insertion into a single chromosomal locus within the cotton nuclear genome.

Plant samples were analyzed using gene-specific quantitative real-time PCR to determine the zygosity status of the *hppdPfW336-1Pa* and *2mepsps* genes. For each sample, two distinct sets of primer pairs amplified the target gene (*hppdPfW336-1Pa* or *2mepsps* gene) together with the endogenous reference gene (*adhC*) from cotton. For each sample, the copy number of the *hppdPfW336-1Pa* or *2mepsps* gene was determined relative to the one copy reference gene.

Chi-square analysis was performed for three generations of GHB811 cotton to confirm the segregation and stability of the GHB811 insert. The Chi-square analysis is based on testing the observed segregation ratio relative to the expected segregation ratio from Mendelian inheritance principles. For the F2, BC1F2 and BC2F2 generations of GHB811 cotton, the expected segregation ratio of homozygous, hemizygous and null segregate was 1:2:1. The χ^2 values were calculated with Microsoft Excel 2010 using the following equation.

$$\chi^2 = \sum \frac{|(\text{Observed} - \text{Expected})|^2}{\text{Expected}}$$

The results are summarized in Table 5.11 and Table 5.12.

Table 5.11. Observed versus expected genotype for the *2mepsps* gene in F2, BC1F2 and BC2F2 of GHB811 cotton as determined by PCR analysis

	F2		BC1F2		BC2F2	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Homozygous	19	21.25	24	23	45	57.25
Hemizygous	49	42.50	50	46	116	114.5
Null	17	21.25	18	23	68	57.25
X^2 Value	2.08		1.48		4.66	

* The critical value to reject the null hypothesis at the 5% confidence level is < 5.99 with two degrees of freedom.

Table 5.12. Observed versus expected genotype for the *hppdPfW336-1Pa* gene in F2, BC1F2 and BC2F2 of GHB811 cotton as determined by PCR analysis

	F2		BC1F2		BC2F2	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Homozygous	19	21.25	24	23	45	57.25
Hemizygous	49	42.50	50	46	116	114.5
Null	17	21.25	18	23	68	57.25
X^2 Value	2.08		1.48		4.66	

* The critical value to reject the null hypothesis at the 5% confidence level is < 5.99 with two degrees of freedom.

Segregation ratios determined for three generations of GHB811 cotton confirmed that the *hppdPfW336-1Pa* and *2mepsps* genes contained within the GHB811 insert are inherited in a predictable manner and as expected for a single insertion. These data are consistent with Mendelian principles and support the conclusion that the GHB811 event consists of a single insert integrated into a single chromosomal locus within the cotton nuclear genome.

5.5. DNA sequence of the transgenic and insertion loci

The DNA sequence of the cotton GHB811 transgenic locus and the corresponding insertion locus was determined.

Three overlapping PCR fragments were prepared for the determination of the GHB811 transgenic locus, using GHB811 gDNA as a template. To determine the GHB811 insertion locus, one fragment was amplified from gDNA extracted from the non-GM counterpart (Table 5.13). Sanger sequencing was performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Table 5.13. Overview of the sequencing fragments prepared

	Fragment ID	Template DNA	Primer pair	Length of final consensus sequence (bp)
GHB811 transgenic locus	FR-THT073-B-01	GHB811 gDNA	GLPA486 GLPA311	9320
	FR-THT073-B-01b(**)		GLPA696 GLPA311	
	FR-THT073-B-02		GLPA457 GLPA076	
	FR-THT073-B-03		GLPA485 GLPA487	
GHB811 insertion locus	FR-THT073-B-04	Coker 312 gDNA	GLPA486 GLPA487	2526
	FR-THT073-B-04b(**)		GLPA696 GLPA487	

(**) This fragment was amplified for a second time under different PCR conditions

The obtained consensus sequences of the transgenic and insertion loci were annotated by pairwise alignments using the Clone Manager software (Sci-Ed Central). An alignment between the GHB811 transgenic locus and the GHB811 insertion locus sequence was made to identify sequence regions of cotton origin within the GHB811 transgenic locus as well as the target site deletion (TSD) within the GHB811 insertion locus. The consensus sequence of the GHB811 transgenic locus was compared with the pTSIH09 sequence to identify the T-DNA region. Further sequence annotation within the T-DNA was performed by comparing the GHB811 transgenic locus sequence with each feature of the pTSIH09 T-DNA region.

Pairwise alignment between the GHB811 transgenic sequence and the GHB811 insertion locus sequence identified two regions sharing 100% pairwise sequence identity. These two regions are provided in Table 5.14.

Table 5.14. Regions of 100% pairwise sequence identity comparing the GHB811 transgenic and insertion loci

Region of homology	% matches	Length (bp)	GHB811 transgenic locus		GHB811 insertion locus	
			start	end	Start	end
Region A: 5' flanking sequence	100	1217	bp 1	bp 1217	bp 1	bp 1217
Region B: 3' flanking sequence	100	1296	bp 8033	bp 9328	bp 1231	bp 2526

Homology region A was identified as 5' flanking sequence on the GHB811 transgenic locus sequence and the GHB811 insertion locus sequence. Homology region B was identified as 3'

flanking sequence on the GHB811 transgenic locus sequence and the GHB811 insertion locus sequence.

In the GHB811 insertion locus sequence, 13 bp were observed which are not present in the GHB811 transgenic locus. These base pairs were deleted during the transformation process and are referred to as TSD.

Pairwise alignment between the GHB811 transgenic sequence and the pTSIH09 plasmid sequence identified three regions sharing 100% pairwise sequence identity which are provided in Table 5.15.

Table 5.15. Regions of 100% pairwise sequence identity comparing the GHB811 transgenic locus with pTSIH09

Region of homology:	% matches	Length (bp)	GHB811 transgenic locus		pTSIH09	
			start	end	start	end
Region A: T-DNA	100	6817	bp 1218	bp 8034	bp 24	bp 6840
Region B: ThistonAt	100	667	bp 7193	bp 7859	bp 749	bp 83
Region C: ThistonAt	100	667	bp 1277	bp 1943	bp 6665	bp 5999
Region D lox	100	34	bp 7864	bp 7897	bp 2796	bp 2829
Region E lox	100	34	bp 3390	bp 4023	bp 6670	bp 6703

Homology region A on the transgenic sequence which is 100 % identical to the T-DNA region of pTSIH09 was identified as T-DNA. The different features of the T-DNA were annotated as well. The four additional homologies result from the presence of two “ThistonAt” and two “lox” features within the T-DNA region.

Two base pairs at the 3’ end of the T-DNA region (bp 8033 to bp 8034) were identical to both the plasmid sequence pTSIH09 and the insertion locus. These base pairs were annotated as 3’ flanking sequence.

A schematic representation of the GHB811 transgenic locus in relation to the pTSIH09 plasmid is provided in Figure 5.24.

The results demonstrated that upon transformation, 13 bp from the GHB811 insertion locus were replaced by 6815 bp of inserted sequences. The flanking sequences obtained at the transgenic locus were identical to the homologous sequences obtained from the insertion locus. This demonstrates that the cotton GHB811 flanking sequences are of cotton origin within its original genomic organization. Annotation of the inserted sequences in the GHB811 transgenic locus sequence demonstrated that it corresponds to the complete T-DNA region of pTSIH09 and did not indicate any T-DNA rearrangements.

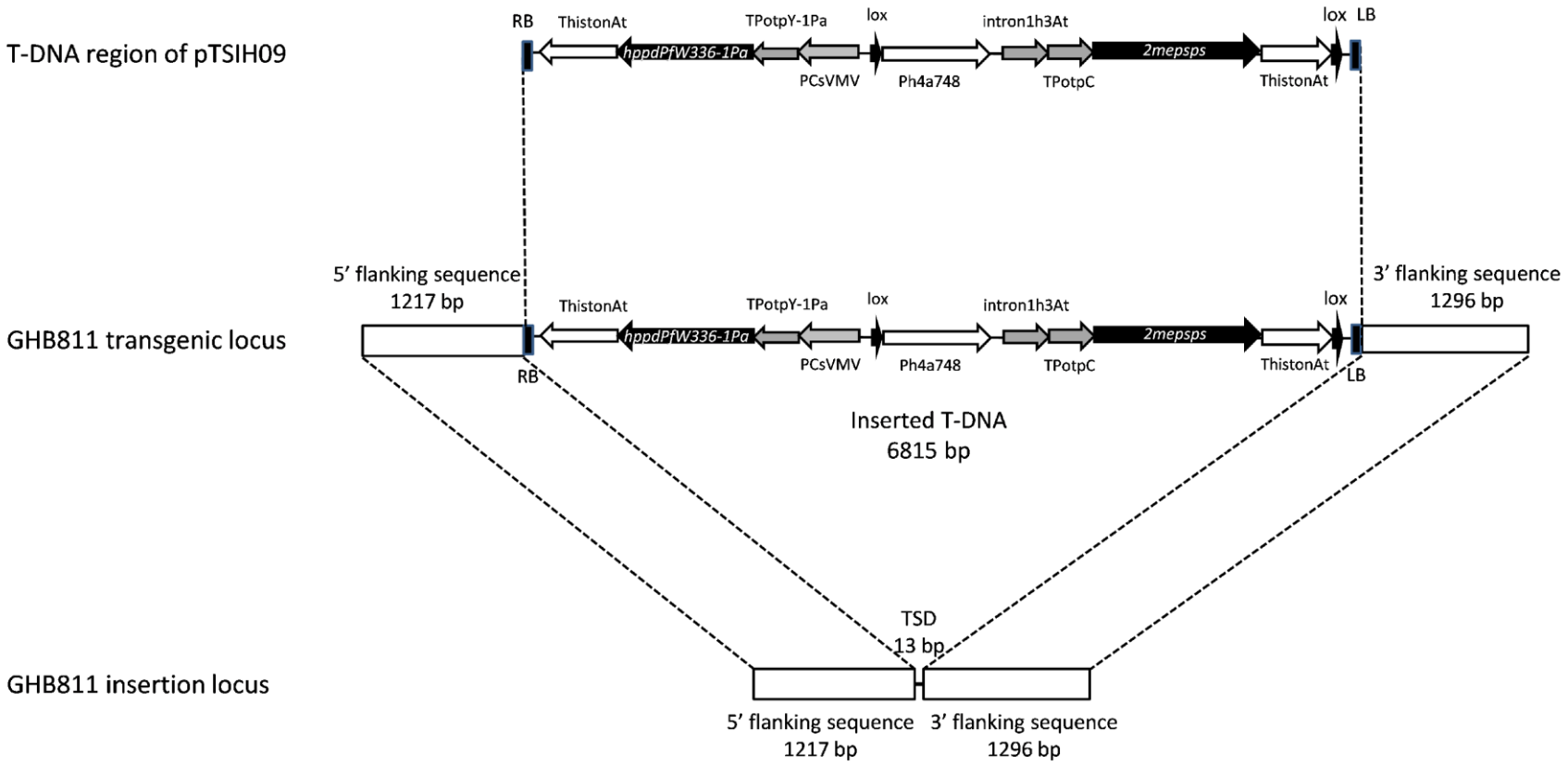


Figure 5.24. Schematic drawing of the GHB811 transgenic locus in relation with the GHB811 insertion locus and the T-DNA of transforming plasmid pTSIH09

5.6. Bioinformatics analyses of the transgenic and insertion loci

Bioinformatics analysis of the GHB811 cotton insertion locus

A bioinformatics analysis was performed on the GHB811 cotton insertion locus sequence, to identify the position of the insertion locus in the genome and to determine whether regulatory sequences or endogenous cotton genes were interrupted upon the insertion of T-DNA sequences.

The GHB811 transgenic locus, containing the inserted DNA together with the 5' and 3' flanking sequences, was used as query sequence. The Basic Local Alignment Search Tool (BLAST) searches were performed to find the location of the GHB811 cotton insertion locus in the cotton genome and to search for sequence similarities with known genes and proteins. BLAST analysis demonstrated that the insertion locus sequence originates from cotton chromosome A05.

The similarities between the GHB811 cotton insertion locus and sequences within the nucleotide collection and the Expressed Sequence Tag (EST) databases were identified using the BLASTn tool available on the NCBI website. In addition, A BLASTx search of the GHB811 cotton insertion locus sequence against the NCBI non-redundant protein database was performed. The results indicate that it is unlikely that the insertion of T-DNA sequences in the GHB811 cotton insertion locus interrupted or altered the transcriptional or translational activity of endogenous cotton genes.

Identification of Open Reading Frames and homology search of sequences with known allergens and toxins

A bioinformatics analysis was performed on the transgenic locus sequence of the GHB811 cotton to identify open reading frames (ORF).

The GHB811 transgenic locus, containing the inserted DNA together with the 5' and 3' flanking sequences, was used as query sequence. The GetORF search program was used to identify all ORF crossing a junction or overlapping the inserted DNA, between two translation stop codons, with a minimum size coding for 3 amino acids. This search identified 549 ORF.

In the next step, the translated amino acid sequences from the identified ORF with a minimum size of 30 amino acids were used as query sequences in homology searches to known allergens and toxins. After elimination of duplicates, they represented 126 unique sequences.

Two *in silico* approaches were used to evaluate the potential amino acid sequence identity with known allergens contained in the public allergen database AllergenOnline (www.allergenonline.org):

1. An 8-mer search was carried out to identify any short sequences of 8 amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll from the EMBOSS suite, which compared each ORF sequence with all known allergens present in the allergen database.

2. An overall identity search was carried out by using FASTA algorithm, which compared each complete query sequence with all protein sequences present in the AllergenOnline database. The scoring matrix was BLOSUM50. An E-value threshold of 1 was used. The criterion indicating potential relevant identity to an allergen was ≥35% identity over at least 80 amino acids for sequences of ≥80 amino acids, or ≥35% recalculated over a hypothetical 80 amino acid window for sequences of <80 amino acids.

In addition, each query sequence was evaluated for potential identity with known toxins. An overall identity search was carried out by using FASTA algorithm with all protein sequences present in the NCBI non-redundant database, using the BLOSUM50 scoring matrix. An E-value threshold of 0.1 was used for pre-selecting the most identical proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the potential polypeptide.

There was no match sharing 100% identity to allergenic proteins using the 8-mer search. The overall search showed no biologically relevant identity between the query sequences and any known allergenic proteins.

In addition, no biologically relevant identities were found with any toxic protein from the NCBI non-redundant database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the potential ORF polypeptides.

6. Characterization of the introduced proteins

6.1. Identity and function of the 2mEPSPS protein

The coding sequence of 5-enol pyruvylshikimate-3-phosphate synthase (*epsps*) gene was isolated from maize (*Zea Mays* L.) Two amino acids were substituted (threonine by isoleucine at position 102 and proline by serine at position 106) (Lebrun *et al*., 1997). These modifications confer to the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants expressing this modified protein become tolerant to glyphosate herbicides (Lebrun *et al*., 1997). The modified protein is designated as 2mEPSPS.

Background information and history of safe use

5-enolpyruvylshiklmate-3-phosphate synthase (EPSPS) catalyzes the penultimate step of the shikimate pathway, which is responsible for the synthesis of aromatic amino acids and other aromatic compounds in plants, fungi and microorganisms including apicomplexan parasites (Herrmann,1995). As such, it has been shown that EPSPS enzymes are ubiquitous in nature and are present in food and feed derived from plant and microbial sources. No health-related adverse effects have been associated with these proteins.

The *2mepsps* gene was generated by introducing mutations into the *epsps* gene from maize (*Z. mays* L.) that result in two amino acid substitutions. The modified EPSPS (2mEPSPS) enzyme has a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of glyphosate herbicides (Lebrun *et al*., 1997). Since the 2mEPSPS protein is derived from maize and has only two amino acid modifications, the safety profile of the novel protein is expected to remain unchanged relative to its wild-type counterpart.

In conclusion, EPSPS proteins are present in food and feed from plant and microbial sources with good safety records. Therefore, EPSPS proteins have a history of safe use.

Biochemistry and mode of action

5-enolpyruvylshiklmate-3-phosphate synthase (EPSPS) is an enzyme that catalyzes the condensation of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimate-3-phosphate and phosphate in the shikimate pathway, which is responsible for the synthesis of aromatic amino acids and other aromatic compounds in plants, fungi and microorganisms including apicomplexan parasites (Figure 6.1).

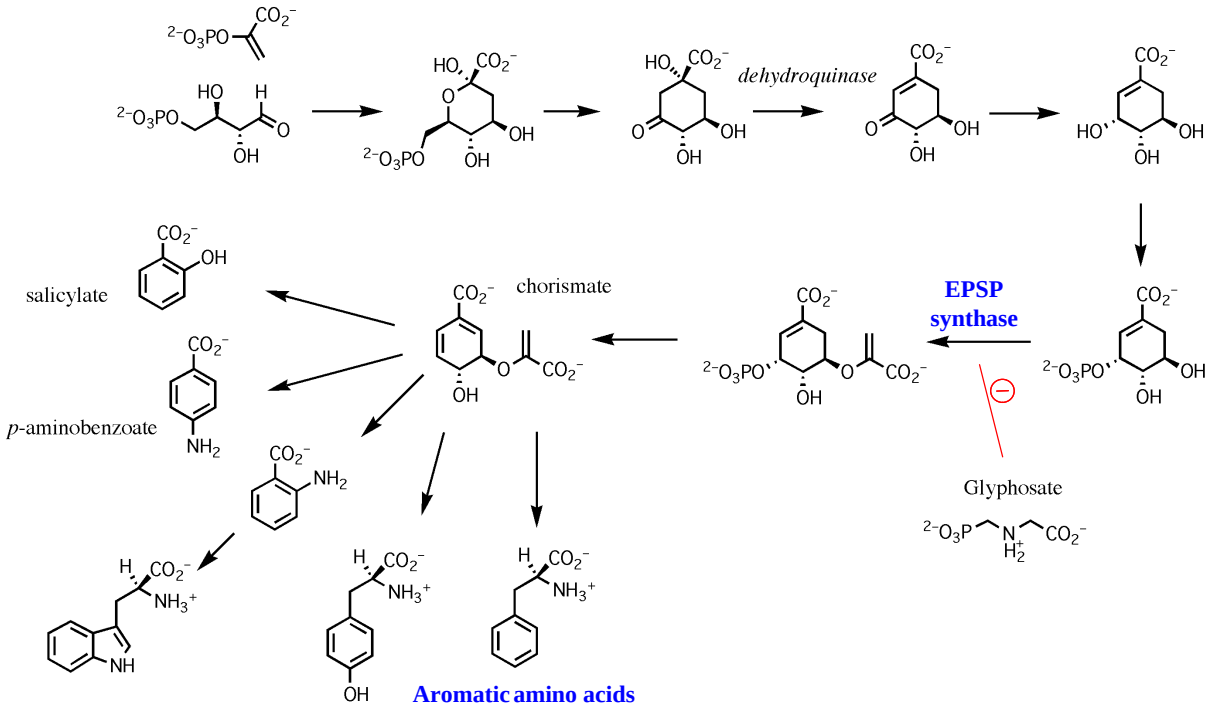


Figure 6.1. The shikimate pathway

Glyphosate inhibits EPSPS enzyme and shuts down the shikimate pathway, leading to plant death. The modified EPSPS (2mEPSPS) enzyme has a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of glyphosate herbicides (Lebrun *et al*., 1997).

6.2. Identity and function of the HPPD W336 Protein

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32. One amino acid was substituted (glycine at position 336 with tryptophan) to improve the tolerance against HPPD inhibitors. The modified protein is designated as HPPD W336 (Boudec *et al*., 2001).

Background information and history of safe use

The *hppd* gene was isolated from the bacterium *Pseudomonas fluorescens*, strain A32. *P. fluorescens* is a Gram-negative, rod-shaped, motile, asporogenous, aerobic bacterium. *P. fluorescens*, is ubiquitous in the environment, including soil, water and food (OECD, 1997). It has many beneficial uses in agriculture, human health and bioremediation. It is not described as allergenic, toxic or pathogenic to healthy humans and animals and has an overall history of safe use.

HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession

number Q22633), mouse (*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD proteins have been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920), barley (*Hordeum vulgare* Accession number O48604), pork (*Sus scrofa*, Accession number Q02110)) and beef (*Bos Taurus*, Accession number Q5EA20).

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD proteins have a history of safe use.

Biochemistry and mode of action

The biochemical pathways in which HPPD is involved differ between plants and non-photosynthetic organisms. In bacteria and animals, it merely serves catabolic purposes by catalyzing the first committed step in tyrosine degradation that in the end yields energetically exploitable glucogenic and ketogenic products (Brownlee *et al*., 2004). In plants, however, it is also involved in several anabolic pathways; its reaction product homogentisate (2,5-dihydroxyohenylacetate) being the aromatic precursor of tocopherol, tocotrienols and plastoquinone, which are essential to the photosynthetic transport chain and antioxidative systems (Fritze *et al*., 2004). Figure 6.2 shows a diagram of the different metabolic pathways in which HPPD is involved in plants and non-photosynthetic organisms.

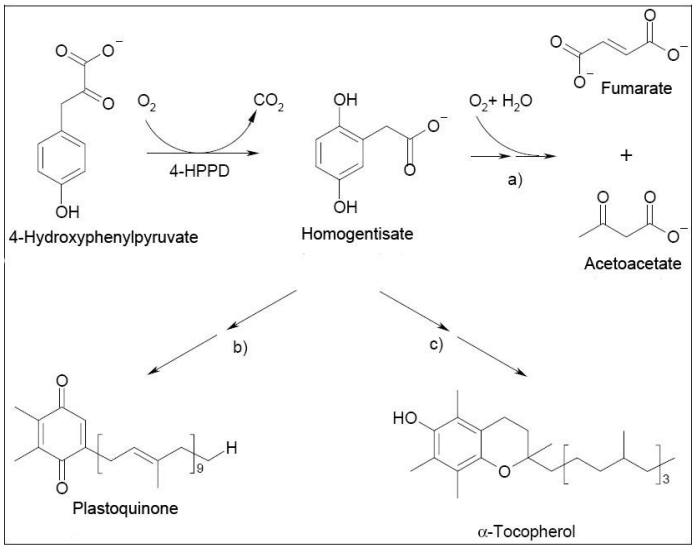


Figure 6.2. Biochemical pathways of HPPD proteins

a) catabolism of tyrosine, b) biosynthesis of plastoquinone (plants)
c) biosynthesis of tocopherol and tocotrienols (plants)

HPPD enzymes require a α-keto acid and molecular oxygen to oxidize or oxygenate a third molecule. The activity of HPPD is suppressed by benzoylisoxazoles bleaching herbicides, such as isoxaflutole (IFT), and by β-triketones such as sulcotrione and mesotrione (Pallett *et al*., 2001; Dayan *et al*., 2007).

The inhibitor of HPPD is the diketonitrile (DKN) derivative of isoxaflutole (IFT) formed by the opening of the isoxazole ring. DKN is formed rapidly in plants following uptake of IFT by roots and shoots. HPPD enzyme inhibition results in the disruption of the biosynthesis of carotenoids, which destabilizes photosynthesis and leads to bleaching of the foliage and death of the plant (Figure 6.3).

In order to create a form of the HPPD enzyme with tolerance to IFT herbicide, a single amino acid substitution, glycine (G) to tryptophan (W) at position 336, was introduced to the native HPPD protein from *Pseudomonas fluorescens* (Boudec *et al*., 2001) resulting the modified IFT-tolerant HPPD W336 protein.

Several different HPPD variants, including the wild type HPPD and modified HPPD W336 enzymes were tested for their activity in the presence or absence of the inhibitor IFT. When compared to the wild type HPPD enzyme, HPPD W336 enzyme was significantly less inhibited by IFT (Fischer, 2008). Comparison of the level of alpha tocopherol, a HPPD enzyme pathway metabolite, between GHB811 cotton (in the presence or absence of IFT), the non-GM counterpart, and commercial reference varieties showed no biologically relevant differences (Section 7. Compositional Analysis). Thus expression of HPPD W336 in GHB811 cotton does not affect the metabolism of other plant metabolites compared to plants expressing only the native HPPD enzyme.

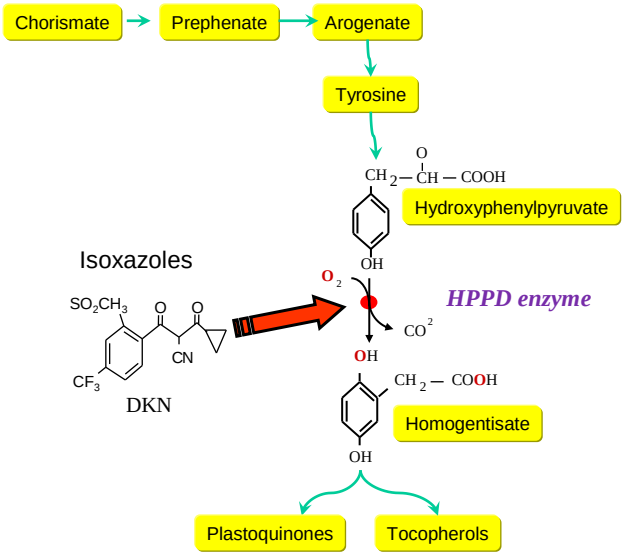


Figure 6.3. Interaction of HPPD and isoxazole herbicides

6.3. Expression levels of the introduced proteins

Protein expression levels of HPPD W336 and 2mEPSPS were determined by Enzyme-Linked immunosorbent assay (ELISA) in field-grown cotton matrices from GHB811 plants treated and not treated with trait-specific herbicides grown at three field trials in the United States in 2015.

Protein expression analysis was conducted on tissue samples harvested from plants grown in the United States in 2015. Field sites were located in areas representative of the commercial production of cotton in the United States and sampled throughout the growing season for different tissues (Mississippi, Northa Carolina and Texas).

There were two plots of GHB811 included at each site. One plot was treated with trait-specific herbicide while the other plot was not treated. The isoxaflutole application to the treated GHB811 entry was made at a rate of 104.9 to 106.6 g ai/ha before emergence (BBCH 00). The glyphosate application was made at a rate of 1104 to 1123 g ai/ha at the seven to eight leaf growth stage (BBCH 17 - 18). All entries were of the Coker 312 background. The matrices analyzed are summarized in Table 6.1.

Table 6.1. Plant matrices analyzed for 2mEPSPS and HPPD W336 expression

Growth Stage[1]	Matrix	Sample Description[2]	Samples per Plot
4-6 leaf (BBCH14-16)	Leaf	All true leaves[3].	10
	Root	All roots.	10
Square initiation (BBCH 51-55)	Leaf	All true leaves.	5
Flowering (BBCH 60-69)	Pollen	Composite pollen	10 (GHB811 plots) 20 (non-GM Coker 312 plots)
2 weeks after first flower (BBCH 60-67)	Leaf	All true leaves	5
	Square	Composite of 6 pre-candle squares[4].	5
	Boll	Composite 4 - 6 immature bolls.	5
	Whole Plant	All above ground material.	5
Maturity (BBCH 83-97)	Fuzzy Seed	All open bolls.	5

[1]The BBCH-scale is a system for a uniform coding of phenologically similar growth stages of mono- and dicotyledonous plant species.
[2]A sample represents all of the indicated martrix type from one plant, with the exception of pollen which was a composite sample of multiple plants per plot to collect a minimum of 1 mL pollen per sample.
[3]True leaves in this study are defined as all leaves except cotyledons.
[4]Pre-candle squares in this study are defined as the immature floral structure including bracts and calyx, at its largest pre-bloom size.

The quantitation of 2mEPSPS protein in leaf, root, pollen square, boll, whole plant and fuzzy seed samples was conducted with a validated 2mEPSPS-specific ELISA method using the EnviroLogix QualiPlate™ Kit for 2mEPSPS. The quantitation of HPPD W336 protein in leaf, root, pollen, square, boll, whole plant and fuzzy seed samples was conducted with a validated HPPD W336-specific ELISA method using the EnviroLogix QuantiPlateTM Kit.

Expression of 2mEPSPS in cotton matrices

The level of 2mEPSPS protein in not treated and treated GHB811 cotton leaf, root, square, boll, whole plant and fuzzy seed matrices ranged from 76.36 to 1762.54 µg/g DW and 86.67 to 1685.85 µg/g DW, respectively (Table 6.2). The 2mEPSPS protein concentrations in not treated and treated GHB811 cotton pollen ranged from 12.86 to 33.47 µg/g FW and 21.42 to 33.15 µg/g FW, respectively (Table 6.2).

Leaf at BBCH 60-67 and BBCH 51-55 growth stages demonstrated the highest mean 2mEPSPS protein expression levels (Table 6.2). Mean (±SD) 2mEPSPS expression levels in not treated and treated leaf at BBCH 60-67 of GHB811 cotton was 1422.12 ± 206.41 µg/g DW and 1267.95 ± 247.75 µg/g DW, respectively. Mean (±SD) 2mEPSPS expression levels in not treated and treated leaf at BBCH 51-55 of GHB811 cotton was 1344.37 ± 224.96 µg/g DW and 1269.39 ± 175.42 µg/g DW, respectively.

Fuzzy seed demonstrated the lowest mean 2mEPSPS protein expression in all matrices reported on a DW basis (Table 6.2). Mean (± SD) 2mEPSPS expression levels in not treated and treated fuzzy seed of GHB811 cotton was 145.11 ± 37.86 µg/g DW and 150.88 ± 27.87 µg/g DW, respectively.

The mean 2mEPSPS concentrations for not treated and treated pollen were 24.69 ± 6.60 µg/g FW and 27.68 µg/g ± 3.47 µg/g FW respectively.

Expression of HPPD W336 in Cotton Matrices

The level of HPPD W336 expression in not treated and treated GHB811 cotton leaf, root, square, boll, whole plant and fuzzy seed matrices ranged from 10.91 to 1673.89 µg/g DW and 11.01 to 1402.82 µg/g DW, respectively (Table 6.3). The HPPD W336 protein concentrations in not treated and treated GHB811 cotton pollen ranged from <LLOQ to 0.69 µg/g FW and <LLOQ to 0.68 µg/g FW, respectively (Table 6.3).

Leaf at BBCH 51-55 growth stage demonstrated the highest mean HPPD W336 protein expression levels (Table 6.3). Mean (± SD) HPPD W336 expression levels in not treated and treated leaf at BBCH 51-55 of GHB811 cotton was 1043.64 ± 322.96 µg/g DW and 956.75 ± 204.79 µg/g DW, respectively.

Root demonstrated the lowest mean HPPD W336 protein expression levels in all matrices reported on a DW basis (Table 6.3). Mean (± SD) HPPD W336 expression levels in not treated and treated root of GHB811 cotton was 22.12 ± 8.37 µg/g DW and 25.42 ± 10.98 µg/g DW, respectively.

Mean (± SD) HPPD W336 expression levels in not treated and treated fuzzy seed of GHB811 cotton was 29.61 ± 14.96 µg/g DW and 27.01 ± 9.78 µg/g DW, respectively.

The HPPD W336 concentrations for majority of the not treated and treated pollen samples were below LLOQ (Table 6.3).

Table 6.2. Expression of 2mEPSPS in cotton matrices harvested from treated and not treated GHB811 grown at three sites

Matrix	BBCH Growth Stage	Entry	2mEPSPS (µg/g DW)				2mEPSPS (µg/g FW)			
			Mean	SD	Min	Max	Mean	SD	Min	Max
Leaf	14-16	B	968.03	520.32	211.22	1423.94	161.89	83.89	36.55	236.36
		C	874.63	353.68	347.43	1418.35	144.25	43.41	64.31	193.40
Root	14-16	B	169.25	46.94	97.44	249.81	24.56	4.22	16.16	30.28
		C	163.76	33.16	118.30	218.48	23.08	3.71	15.02	28.60
Leaf	51-55	B	1344.37	224.96	840.89	1651.21	255.91	62.43	174.16	380.38
		C	1269.39	175.42	1029.95	1685.85	252.37	48.53	176.49	342.72
Leaf	60-67	B	1422.12	206.41	1117.38	1762.54	307.05	42.53	238.76	383.67
		C	1267.95	247.75	756.49	1600.36	273.05	59.69	156.17	351.98
Pollen	60-69	B	NA	NA	NA	NA	24.69	6.60	12.86	33.47
		C	NA	NA	NA	NA	27.68	3.47	21.42	33.15
Square	60-67	B	591.00	53.34	485.64	689.39	126.23	14.92	105.64	151.38
		C	506.64	84.21	381.81	659.10	106.20	14.36	78.39	124.97
Bolls	60-67	B	474.77	65.86	360.31	575.47	80.29	18.08	58.02	122.56
		C	437.00	61.85	318.89	522.18	71.26	9.67	55.93	86.08
Whole Plant	60-67	B	788.13	128.73	595.67	1080.81	182.13	47.46	127.19	276.04
		C	795.81	132.72	611.73	1065.80	176.28	48.95	116.95	290.24
Fuzzy Seed	83-97	B	145.11	37.86	76.36	221.42	129.79	38.41	65.07	205.88
		C	150.88	27.87	86.67	198.93	132.94	20.38	80.83	162.76

Entry B = GHB811 (not treated); Entry C = GHB811 (treated).
Mean and standard deviation (SD) for each entry was based on the total sample population (N=12).
NA = Not Applicable. Pollen samples were analyzed on fresh tissue only.

Table 6.3. Expression of HPPD W336 in cotton matrices harvested from treated and not treated GHB811 grown at three sites

Matrix	BBCH Growth Stage	Entry	HPPD W336 (µg/g DW)				HPPD W336 (µg/g FW)			
			Mean	SD	Min	Max	Mean	SD	Min	Max
Leaf	14-16	B	668.06	478.48	136.50	1337.49	116.29	87.24	23.11	244.29
		C	808.10	403.34	376.72	1402.82	142.10	72.18	58.71	231.78
Root	14-16	B	22.12	8.37	12.76	43.01	3.40	1.52	1.66	6.62
		C	25.42	10.98	11.10	46.06	3.59	1.48	1.55	5.89
Leaf	51-55	B	1043.64	322.96	717.12	1673.89	198.51	69.61	124.77	328.42
		C	956.75	204.79	722.63	1232.40	188.78	40.22	124.84	245.22
Leaf	60-67	B	862.75	208.08	515.57	1225.60	184.73	36.97	119.55	250.74
		C	781.28	164.18	563.26	1013.20	166.51	31.37	116.28	221.29
Pollen	60-69	B	NA	NA	NA	NA	<LLOQ	ND	<LLOQ	0.69
		C	NA	NA	NA	NA	<LLOQ	ND	<LLOQ	0.68
Square	60-67	B	304.97	24.47	269.07	337.78	65.30	8.67	51.51	83.16
		C	284.52	34.38	235.73	365.42	60.88	14.69	44.51	95.87
Bolls	60-67	B	181.03	37.20	116.55	241.68	30.59	8.34	20.77	46.27
		C	125.62	34.32	70.03	193.68	20.29	4.50	12.28	27.37
Whole Plant	60-67	B	308.52	93.13	159.44	433.89	68.00	13.34	43.84	87.13
		C	297.03	73.31	182.34	399.34	63.21	9.37	44.81	77.78
Fuzzy Seed	83-97	B	29.61	14.96	10.91	62.33	26.45	13.65	9.30	55.96
		C	27.01	9.78	11.01	43.85	23.82	8.46	10.27	39.46

Entry B = GHB811 (not treated); Entry C = GHB811 (treated).
Mean and standard deviation (SD) for each entry was based on the total sample population (N=12).
NA = Not Applicable. Pollen samples were analyzed on fresh tissue only.
ND = Not Determined. SD for HPPD W336 expression levels were not determined, since only 1 sample from each entry had a quantifiable value (> Lower Limit of Quantitation (LLOQ)).

6.4. Comparability of *in planta* and *in vivo* proteins

For the safety assessment of GM crops, certain safety tests and studies require large amounts of protein. The expression levels of the 2mEPSPS and HPPD W336 in GHB811 were too low to allow for purification of sufficient quantities of the two proteins directly from GHB811 for use in the safety assessment studies. Therefore, the 2mEPSPS and HPPD W336 proteins were produced in a high-expressing recombinant host organism, *E.coli*. and the proteins produced by *E.coli* were engineered to match the amino acid sequences of their counterparts expressed in GHB811. The equivalence of GHB811 cotton-produced and bacterially-produced proteins were examined to ensure that the proteins from the two host sources were equivalent so that the bacterially-produced proteins could be used as a surrogate in the studies.

6.4.1. The equivalence of GHB811-purified and microbially-produced 2mEPSPS proteins

A purification of 2mEPSPS protein was performed from the GHB811 cotton leaf matrix using affinity chromatography. GHB811 cotton-purified 2mEPSPS protein was characterized and evaluated for equivalence with bacterially-produced 2mEPSPS protein based on a panel of analytical tests and assays, including densitometry analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectroscopy; and N-terminal sequence analysis.

Assessment and comparison of the apparent molecular mass

The GHB811 cotton-purified 2mEPSPS protein and the bacterially-produced 2mEPSPS protein were compared side by side by means of an SDS-PAGE analysis (Figure 6.4). Additionally, the bacterially-produced 2mEPSPS protein was spiked into the protein extract sample resulting from treatment of non-GM cotton variety Coker 312, which was subjected to the same affinity purification procedure as the plant-purified 2mEPSPS protein sample (*i.e.* treated non-GM counterpart) to allow comparison in a similar cotton plant matrix.

A specific, predominant band was observed for both samples, which corresponds to the expected molecular mass of the 2mEPSPS protein (47.4 kDa). This demonstrated that the apparent molecular mass of the GHB811 cotton-purified and the bacterially-produced 2mEPSPS protein are comparable. The treated non-GM counterpart negative control showed some non-specific background staining derived from the plant matrix.

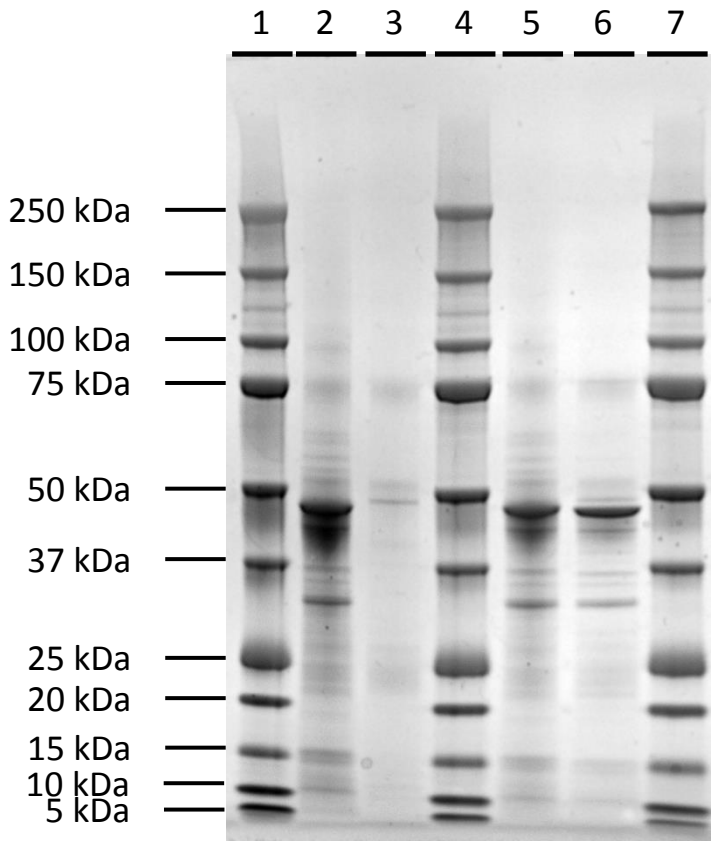


Figure 6.4. Apparent molecular mass assessment and comparison of GHB811 cotton-purified 2mEPSPS protein and the bacterially-produced 2mEPSPS protein

Both GHB811 cotton-purified and bacterially-produced 2mEPSPS protein samples were loaded on a Criterion XT Bis-Tris 4-12 % gel and SDS-PAGE gel electrophoresis was performed in 1x MOPS gel running buffer, followed by Coomassie staining.

Loading order :
Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 1 µg of 2mEPSPS protein of the GHB811 cotton-purified sample 16-RSTHN035-A-02
Lane 3: 1.78 µL of the non-GM counterpart derived negative control sample 16-RSTHN035-A-05
Lane 4: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 5: 1 µg of 2mEPSPS protein of the GHB811 cotton-purified sample 16-RSTHN035-A-02
Lane 6: 1 µg of bacterially-produced 2mEPSPS protein (batch 1417_2mEPSPS) spiked in 1.78 µL of non-GM counterpart derived negative control sample 16-RSTHN035-A-05 (16-RSTHN035-A-08)
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)

Assessment and comparison of the immuno-reactivity

The GHB811-purified 2mEPSPS protein and the bacterially-produced 2mEPSPS protein were compared side by side by means of western blot analysis (Figure 6.5).

Using a 2mEPSPS-specific polyclonal antibody, a signal corresponding to the expected molecular mass of the 2mEPSPS protein was detected for both samples. A very weak band of a comparable size was observed for the crude extract of the non-GM counterpart, which most likely corresponds to the cotton endogenous EPSPS protein.

The obtained results confirmed the immuno-reactivity of the GHB811-purified 2mEPSPS protein and the comparability to the bacterially-produced 2mEPSPS protein.

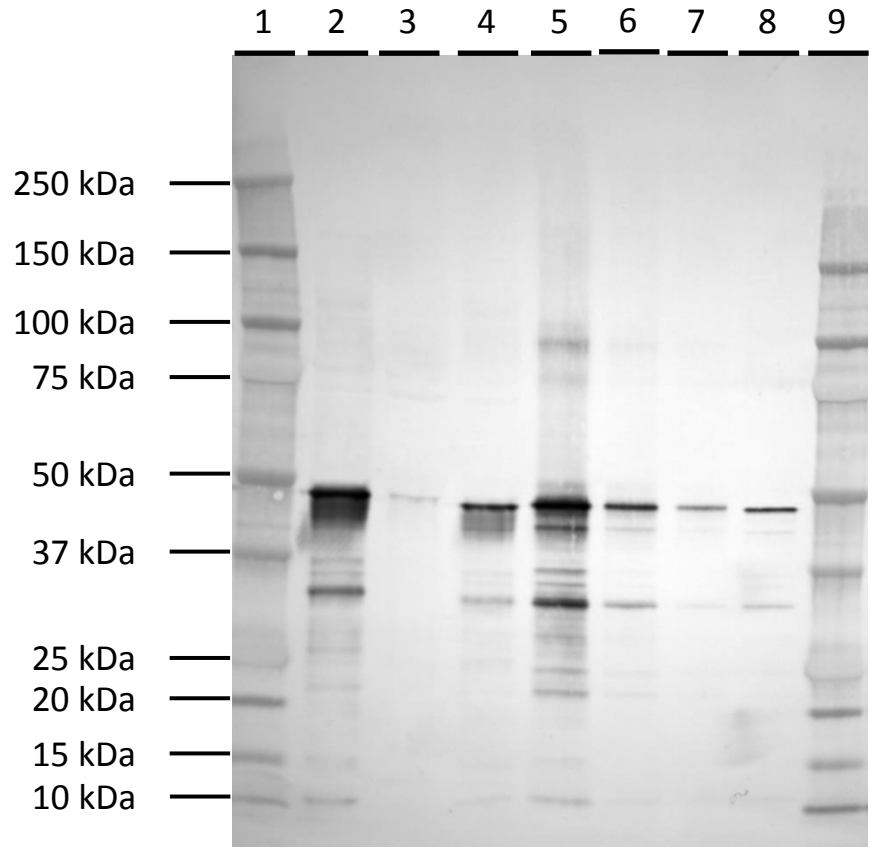


Figure 6.5. Assessment and comparison of immuno-reactivity of GHB811 cotton-purified 2mEPSPS protein and bacterially-produced 2mEPSPS protein

Both plant-purified and bacterially-produced 2mEPSPS protein samples were loaded on a Criterion XT Bis-Tris 4-12% gel and SDS-PAGE gel electrophoresis was performed in 1x MOPS gel running buffer. After semi-dry blotting, the proteins were visualized by colorimetric detection using a 1:5,000 dilution of the rabbit anti-2mEPSPS antibody (batch 1323_2mEPSPS_Ab) as primary antibody and a 1:7,000 dilution of the goat anti-rabbit antibody conjugated to Alkaline Phosphatase as the secondary antibody, followed by NBT BCIP substrate addition.

Loading order :
Lane 1: 5 µL of Precision Plus Protein™ Dual Xtra Standards
Lane 2: 10 µL of crude protein extract from GHB811 cotton (16-RSTHN035-A-00)
Lane 3: 10 µL of crude protein extract from the non-GM counterpart (16-RSTHN035-A-03)
Lane 4: 40 ng 2mEPSPS protein spiked into 10 µL of crude extract from the non-GM counterpart (16-RSTHN035-A-06)
Lane 5: 40 ng of plant-purified 2mEPSPS protein from GHB811 cotton (16-RSTHN035-A-02)
Lane 6: 10 ng of plant-purified 2mEPSPS protein from GHB811 cotton (16-RSTHN035-A-02)
Lane 7: 4 ng of plant-purified 2mEPSPS protein from GHB811 cotton (16-RSTHN035-A-02)
Lane 8: 10 ng of bacterially-produced 2mEPSPS protein (batch 1417_2mEPSPS)
Lane 9: 5 µL of the Precision Plus Protein™ Dual Xtra Standards

Assessment and comparison of the glycosylation status

The results of the glycostaining analysis are shown in Figure 6.6.

The glycosylated proteins of the horseradish peroxidase positive control and the alpha-one acidic glycoprotein of the glycoprotein mix were visualized as bright bands on the gel, while for the 2mEPSPS protein samples, no signal was observed (Figure 6.6, panel A).

The presence of sufficient 2mEPSPS protein on the gel was demonstrated by staining the gels with Coomassie after the glyco-staining procedure (Figure 6.6, panel B).

The absence of glycosylation was demonstrated for both the GHB811-purified 2mEPSPS protein and the bacterially-produced 2mEPSPS protein. Consequently, both 2mEPSPS protein samples have a comparable glycosylation status.

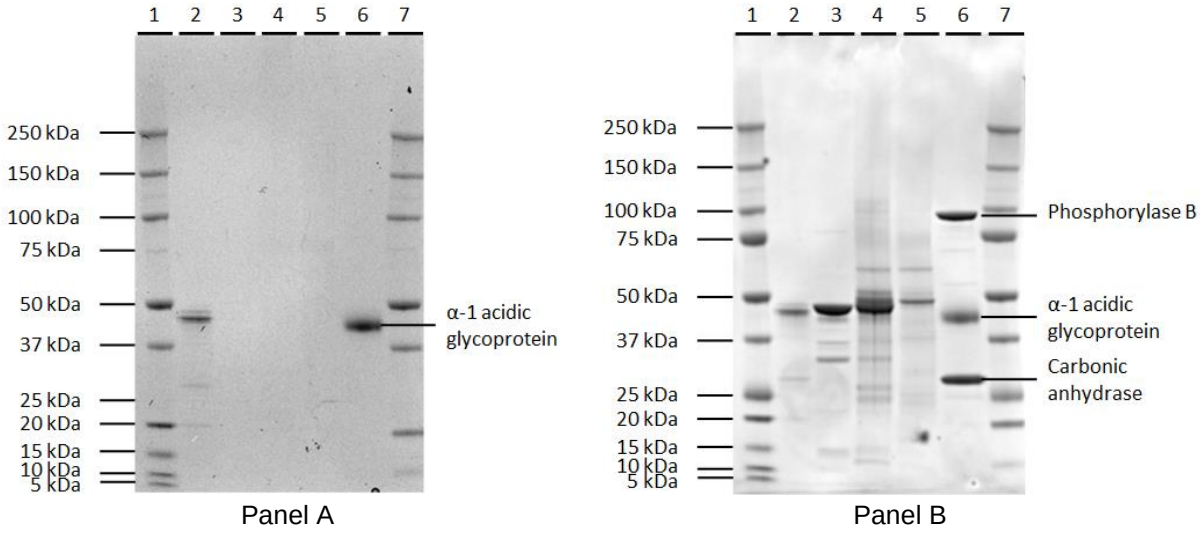


Figure 6.6. Assessment and comparison of the of the glycosylation status of the GHB811 cotton-purified 2mEPSPS protein and the bacterially-produced 2mEPSPS

One Criterion XT Bis-Tris 4-12 % SDS-PAGE gel was prepared and cut into two pieces, each part containing 3 µg 2mEPSPS protein of both the bacterially-produced 2mEPSPS protein batch 1417_2mEPSPS and the GHB811 cotton-purified 2mEPSPS protein together with the appropriate positive and negative controls to assess the glycosylation status.

Panel A shows the result of the staining using the Glycoprotein Detection Kit (Sigma) to demonstrate the absence of glycosylation of both the 2mEPSPS protein samples. For the second set of samples, a glycostaining was performed in which the oxidation step in the procedure was omitted to demonstrate the absence of any non-specific binding (data not shown).
Panel B shows a Coomassie staining of the SDS-PAGE gel to demonstrate the presence of the proteins on the gel.

Loading order of Panel A and B:
Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards
Lane 2: 45.45 pmoles of Horseradish Peroxidase (positive control)
Lane 3: 3 µg of bacterially-produced 2mEPSPS protein batch 1417_2mEPSPS
Lane 4: 3 µg of GHB811 cotton-purified 2mEPSPS protein of sample 16-RSTHN035-A-01
Lane 5: 7.83 µL of the non-GM counterpart derived negative control sample (16-RSTHN035-A-04)
Lane 6: 45.45 pmoles of Glycoprotein mix
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards

Assessment of the intact molecular mass and peptide mapping

The intact molecular mass was determined using the UPLC-UV-MS and the peptide mapping was established using UPLC-UV-MS^E analysis for the GHB811-purified 2mEPSPS protein.

The determined intact molecular mass allowed the identification of two intact molecular masses. The first and major molecular mass of 47551.5 Da corresponds to the theoretical molecular mass of a 2mEPSPS protein with an N-terminal cysteinic sulfinic acid, derived from the transit peptide (47551.0 Da). The second, minor molecular mass of 47284.9 Da corresponds to the theoretical molecular mass of an N-terminal des-Methionine (mature form of the 2mEPSPS protein minus the initial methionine residue, desMet) (47284.7 Da).

Peptides resulting from a trypsin digest of the GHB811-purified 2mEPSPS protein were analysed. Figure 6.7 provides an overview of the mapped peptides against the theoretical amino acid sequence of the 2mEPSPS protein. A coverage of 89 % was determined, which confirms the identity of the 2mEPSPS protein.

The intact molecular mass was determined and the peptide mapping was established using LC-UV-MS analysis for bacterially-produced 2mEPSPS protein.

The determined intact molecular mass of 47288 Da confirmed the theoretical molecular mass of 47284 Da corresponding with the mass of the 2mEPSPS protein minus the methionine residue (desMet).

Peptides resulting from a trypsin digest of bacterially-produced 2mEPSPS protein were analysed using LC-UV-MS. Figure 6.8 provides an overview of the mapped peptides against the theoretical amino acid sequence of the 2mEPSPS protein. A coverage of 95.5 % was determined, which confirms the identity of 2mEPSPS protein.

Figure 6.7. Schematic overview of the coverage of the theoretical 2mEPSPS sequence by the tryptic peptides from the cotton GHB811-purified 2mEPSPS protein

Black and white bars represent the N-terminal peptides corresponding respectively to the desMet 2mEPSPS protein and the 2mEPSPS protein with an N-terminal cysteinic sulfinic acid, derived from the transit peptide.

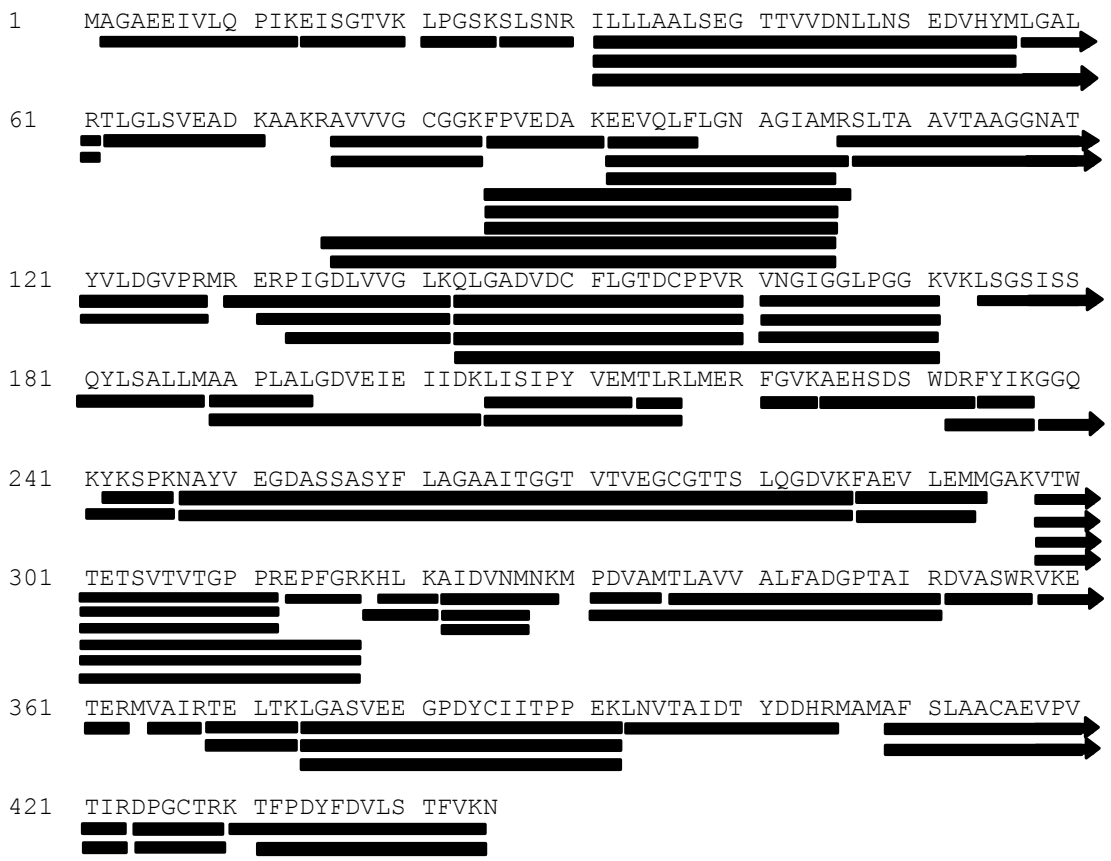


Figure 6.8. Schematic overview of the detected peptides derived from tryptic digestion of bacterially-produced 2mEPSPS

Assessment of the N-terminal sequence

The N-terminal sequence of the GHB811 cotton-purified 2mEPSPS protein was determined by Edman degradation. The obtained data for the GHB811 cotton-purified sample suggested the presence of two N-termini. The obtained sequence result could be resolved as AGAEEIVLQP, corresponding to the desMet N-terminus (*i.e.* N-terminus without methionine) of the 2mEPSPS protein, and sequence XMAGAEEIVL, potentially corresponding to incomplete cleavage of the transit peptide of the 2mEPSPS protein. This confirms the results observed with the intact molecular mass determination.

The N-terminal sequence of bacterially-produced 2mEPSPS protein was determined by Edman degradation. The obtained sequence result could be resolved as AGAEEIVLQP, corresponding to the desMet N-terminus (*i.e.* N-terminus without methionine) of the 2mEPSPS protein. This confirms the results observed with the intact molecular mass determination.

Conclusion

The equivalence of the GHB811 cotton-purified 2mEPSPS protein with bacterially-produced 2mEPSPS protein was demonstrated based on a panel of analytical tests and assays, including densitometry analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectroscopy; and N-terminal sequence analysis. In addition to the des-Methionine 2mEPSPS protein (N-terminus without methionine), the GHB811 cotton-purified sample contained a second 2mEPSPS-derived structure with two additional amino acid residues at the N-terminus, potentially corresponding to incomplete cleavage of the transit peptide of the 2mEPSPS protein. This form did not have any impact on the observed characteristics for the GHB811 cotton-purified 2mEPSPS protein.

6.4.2. The equivalence of GHB811-purified and micorobially-produced HPPD W336 proteins

A purification of HPPD W336 protein were performed from the GHB811 cotton leaf matrix using affinity chromatography. GHB811 cotton-purified HPPD W336 protein was characterized and evaluated the equivalence with bacterially-produced HPPD W336 protein based on a panel of analytical tests and assays, including densitometry analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectroscopy; and N-terminal sequence analysis.

Assessment and comparison of the apparent molecular mass

The GHB811 cotton-purified HPPD W336 protein and the bacterially-produced HPPD W336 protein were compared side by side by means of an SDS-PAGE analysis (Figure 6.9). Additionally, the bacterially-produced HPPD W336 protein was spiked into the protein extract sample resulting from treatment of non-GM cotton variety Coker 312, which was subjected to the same affinity purification procedure as the plant-purified HPPD W336 protein sample (*i.e.* treated non-GM counterpart) to allow comparison in a similar cotton plant matrix.

A specific, predominant band was observed for both samples, which corresponds to the expected molecular mass of the HPPD W336 protein (40.3 kDa). This demonstrated that the apparent molecular mass of the GHB811 cotton-purified and the bacterially-produced HPPD W336 protein are comparable. The treated non-GM counterpart negative control showed some non-specific background staining derived from the plant matrix.

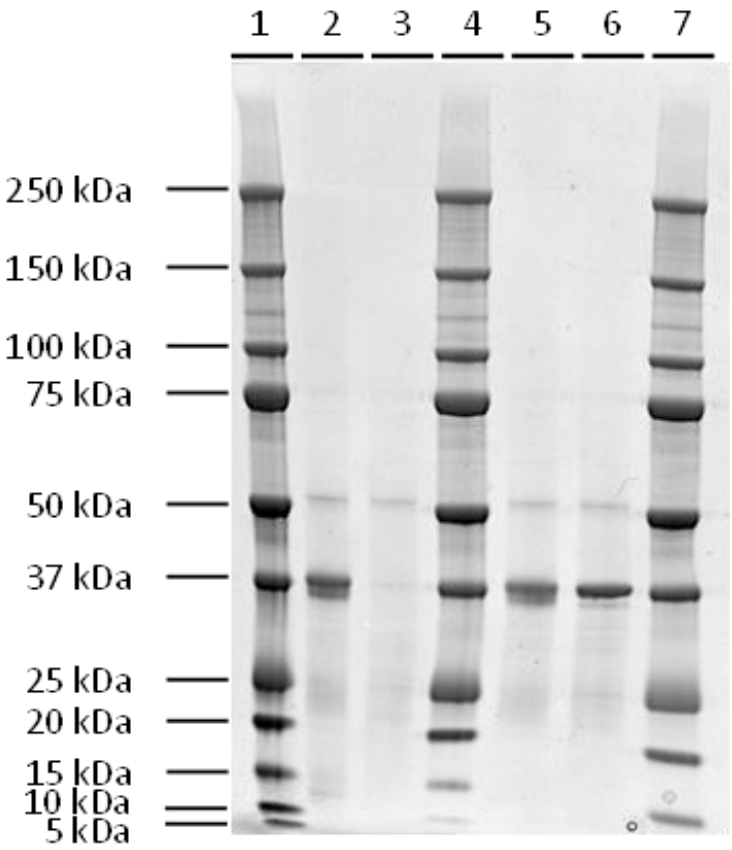


Figure 6.9. Apparent molecular mass assessment and comparison of GHB811 cotton-purified HPPD W336 protein and the bacterially-produced HPPD W336 protein

Both GHB811 cotton-purified and bacterially-produced HPPD W336 protein samples were loaded on a Criterion XT Bis-Tris 4-12 % gel and SDS-PAGE gel electrophoresis was performed in 1x MOPS gel running buffer, followed by Coomassie staining.

Loading order :
Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 1 µg of HPPD W336 protein of the GHB811 cotton-purified sample 16-RSTHN035-B-01
Lane 3: 5 µL of the non-GM counterpart derived negative control sample 16-RSTHN035-B-03
Lane 4: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 5: 1 µg of HPPD W336 protein of the GHB811 cotton-purified sample 16-RSTHN035-B-01
Lane 6: 1 µg of bacterially-produced HPPD W336 protein (batch 1411_HPPD W336) spiked in 5 µL of non-GM counterpart derived negative control sample 16-RSTHN035-B-03 (16-RSTHN035-B-05)
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)

Assessment and comparison of the immuno-reactivity

The GHB811 cotton-purified HPPD W336 protein and the bacterially-produced HPPD W336 protein were compared side by side by means of a western blot (Figure 6.10).

Using a HPPD W336-specific polyclonal antibody, a signal corresponding to the expected molecular mass of the HPPD W336 protein was detected for both samples. A very weak band of a slightly lower size was observed for the crude extract of the non-GM counterpart, which is due to cross-reactivity of the used polyclonal antibody batch to the plant matrix.

The obtained results confirmed the immuno-reactivity of the GHB811 cotton-purified HPPD W336 protein and the comparability to the bacterially-produced HPPD W336.

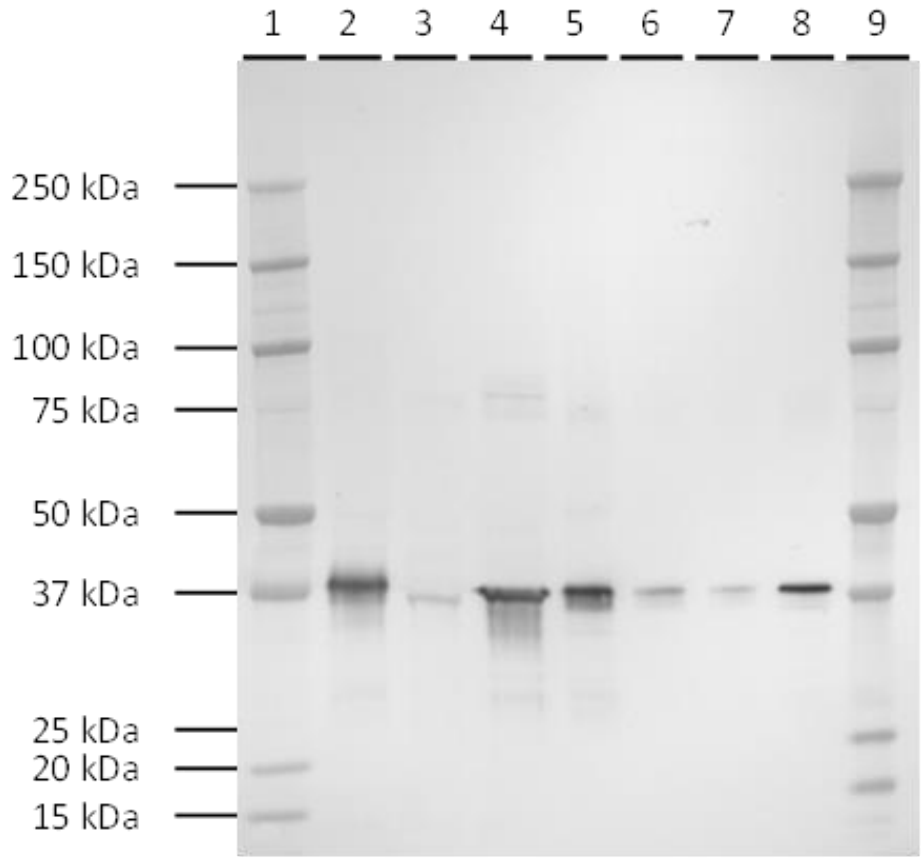


Figure 6.10. Assessment and comparison of immuno-reactivity of GHB811 cotton-purified HPPD W336 protein and bacterially-produced HPPD W336 protein

Both plant-purified and bacterially-produced HPPD W336 protein samples were loaded on a Criterion XT Bis-Tris 4-12% gel and SDS-PAGE gel electrophoresis was performed in 1x MOPS gel running buffer. After semi-dry blotting, the proteins were visualized by colorimetric detection using a 1:10,000 dilution of the rabbit anti-HPPD W336 antibody (batch 1227_HPPD W336_Ab) as primary antibody and a 1:7,000 dilution of the goat anti-rabbit antibody conjugated to Alkaline Phosphatase as the secondary antibody, followed by NBT and BCIP substrate addition.

Loading order :
Lane 1: 5 µL of Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 10 µL of crude protein extract from GHB811 cotton (16-RSTHN035-B-00)
Lane 3: 10 µL of crude protein extract from the non-GM counterpart (16-RSTHN035-B-02)
Lane 4: 40 ng HPPD W336 protein spiked into 10 µL of crude extract from the non-GM counterpart (16-RSTHN035-B-04)
Lane 5: 40 ng of plant-purified HPPD W336 protein from GHB811 cotton (16-RSTHN035-B-01)
Lane 6: 10 ng of plant-purified HPPD W336 protein from GHB811 cotton (16-RSTHN035-B-01)
Lane 7: 4 ng of plant-purified HPPD W336 protein from GHB811 cotton (16-RSTHN035-B-01)
Lane 8: 10 ng of bacterially-produced HPPD W336 protein (batch 1411_HPPD W336)
Lane 9: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)

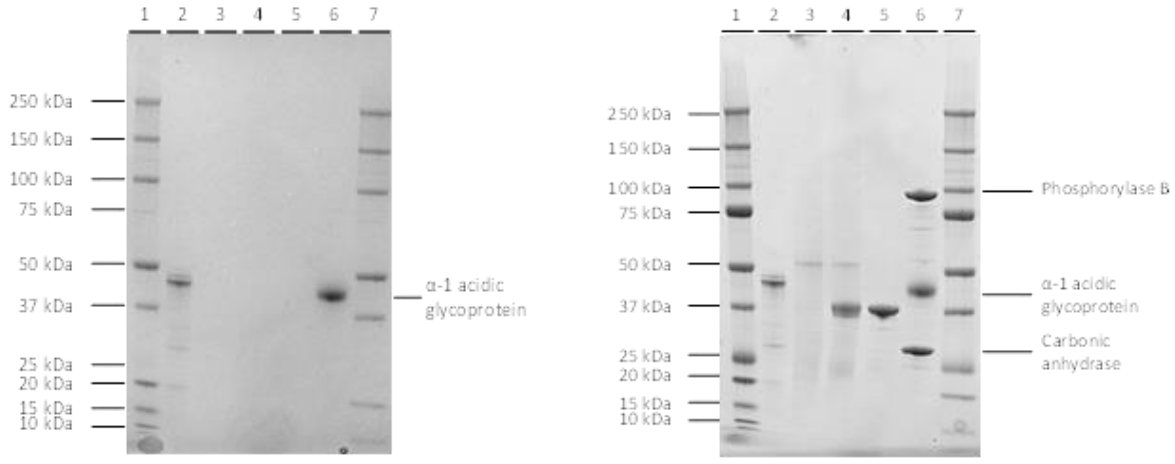
Assessment and comparison of the glycosylation status

The results of the glycostaining analysis are shown in Figure 6.11.

The glycosylated proteins of the horseradish peroxidase positive control and the alpha-one acidic glycoprotein of the glycoprotein mix were visualized as bright bands on the gel, while for the HPPD W336 protein samples, no signal was observed (Figure 6.11, panel A).

The presence of sufficient HPPD W336 protein on the gel was demonstrated by staining the gels with Coomassie after the glyco-staining procedure (Figure 6.11, panel B).

The absence of glycosylation was demonstrated for both the GHB811 cotton-purified HPPD W336 protein and the bacterially-produced HPPD W336 protein. Consequently, both HPPD W336 protein samples have a comparable glycosylation status.



Panel A Panel B

Figure 6.11. Assessment and comparison of the of the glycosylation status of the GHB811 cotton-purified HPPD W336 protein and the bacterially-produced HPPD W336 protein

One Criterion XT Bis-Tris 4-12 % SDS-PAGE gel was prepared and cut into two pieces, each part containing 2 µg HPPD W336 protein of both the bacterially-produced HPPD W336 protein batch 1411_HPPD W336 and the GHB811 cotton-purified HPPD W336 protein together with the appropriate positive and negative controls to assess the glycosylation status.

Panel A shows the result of the staining using the Glycoprotein Detection Kit (Sigma) to demonstrate the absence of glycosylation of both the HPPD W336 protein samples. For the second set of samples, a glycostaining was performed in which the oxidation step in the procedure was omitted to demonstrate the absence of any non-specific binding (data not shown).
Panel B shows a Coomassie staining of the SDS-PAGE gel to demonstrate the presence of the proteins on the gel.

Loading order of Panel A and B:
Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 45.45 pmoles of Horseradish Peroxidase (positive control)
Lane 3: 10 µL of the non-GM counterpart derived negative control sample (16-RSTHN035-B-03)
Lane 4: 2 µg of GHB811 cotton-purified HPPD W336 protein of sample 16-RSTHN035-B-01
Lane 5: 2 µg of bacterially-produced HPPD W336 protein batch 1411_HPPD W336
Lane 6: 45.45 pmoles of Glycoprotein mix
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)

Assessment of the intact molecular mass and peptide mapping

The intact molecular mass was determined using the UPLC-UV-MS and the peptide mapping was established using UPLC-UV-MS^E analysis for the GHB811-purified HPPD W336 protein.

The determined intact molecular mass allowed the identification of two intact molecular masses. The first and major molecular mass (40,446.8 Da) corresponds to a HPPD W336 protein with an N-terminal cysteinic sulfinic acid, derived from the transit peptide (40,447.2 da). The second, minor molecular mass (40,179.9 Da) corresponds to an N-terminal des-Methionine (mature form of the protein minus the initial methionine residue, desMet) HPPD W336 protein (40,180.8 Da).

Peptides resulting from a trypsin digest of the GHB811 cotton-purified HPPD W336 protein were analysed. Figure 6.12 provides an overview of the mapped peptides against the theoretical amino acid sequence of the HPPD W336 protein. A coverage of 98.6 % was determined, which confirmed the identity of the HPPD W336 protein.

The intact molecular mass was determined and the peptide mapping was established using LC-UV-MS analysis for bacterially-produced HPPD W336 protein.

The determined intact molecular mass (40181.0 Da) confirms the theoretical molecular mass of the protein of 40180.8 Da corresponding with the mass of the HPPD W336 protein minus the methionine residue (desMet).

Peptides resulting from a trypsin digest of bacterially-produced HPPD W336 protein were analysed using LC-UV-MS. Figure 6.13 provides an overview of the mapped peptides against the theoretical amino acid sequence of the HPPD W336 protein. A coverage of 96.1 % was determined, which confirms the identity of HPPD W336 protein.

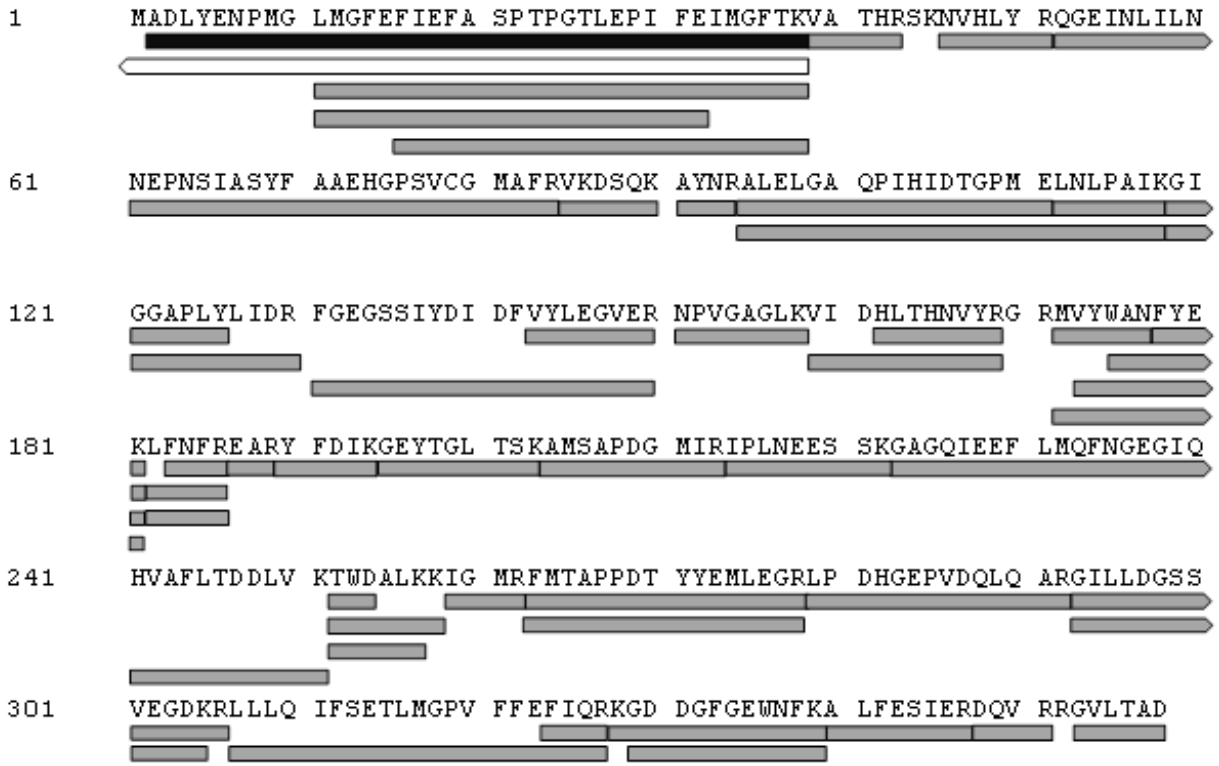


Figure 6.12. Schematic overview of the coverage of the theoretical HPPD W336 sequence by the tryptic peptides from the cotton GHB811-purified HPPD W336 protein detected by UPLC-UV-MS^{E}

Black and white bars represent the N-terminal peptides corresponding respectively to the desMet HPPD W336 protein and the HPPD W336 protein with an N-terminal cysteinic sulfinic acid, derived from the transit peptide.

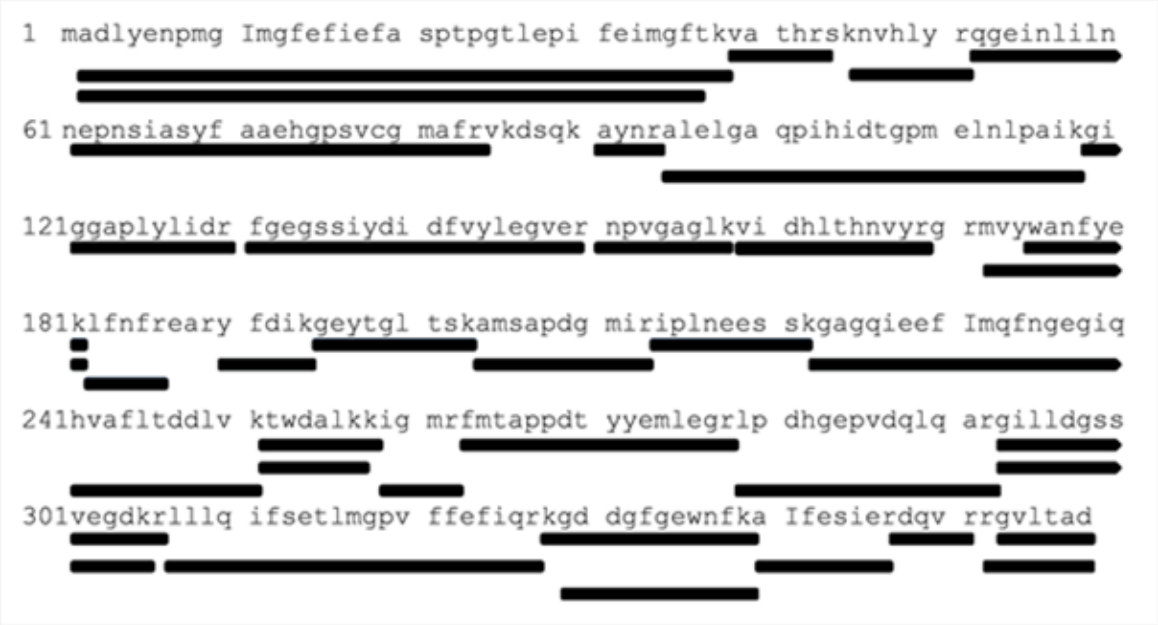


Figure 6.13. Schematic overview of the tryptic peptides detected.
Mapping of the peptides derived from the DTT reduced and trypsin digested HPPD W336 protein sample batch 1411_HPPD W336 against the theoretical amino acid sequence of the HPPD W336 protein

Assessment of the N-terminal sequence

The N-terminal sequence of the GHB811 cotton-purified HPPD W336 protein was determined by Edman degradation. The obtained data for the GHB811 cotton-purified sample suggested the presence of two N-termini. The obtained sequence result could be resolved as ADLYENPMGL, corresponding to the desMet N-terminus (*i.e.* N-terminus without methionine) of the HPPD W336 protein, and sequence XMADLYENPM, potentially corresponding to incomplete cleavage of the transit peptide of the HPPD W336 protein. These results support the data obtained within the intact molecular mass determination.

The N-terminal sequence of bacterially-produced HPPD W336 protein was determined by Edman degradation. The obtained N-terminal sequence (ADLYENPMGL) corresponds to the desMet N-terminus (*i.e.* N-terminus without methionine) of the HPPD W336 protein. This confirms the results observed with the intact molecular mass determination.

Conclusion

The equivalence of the GHB811 cotton-purified HPPD W336 protein with bacterially-produced HPPD W336 protein was demonstrated based on a panel of analytical tests and assays, including densitometry analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectroscopy; and N-terminal sequence analysis. In addition to the des-Methionine HPPD W336 protein, the GHB811 cotton-purified sample contained a second HPPD W336-derived structure with two additional amino acid residues at the N-terminus, potentially corresponding to incomplete cleavage of the transit peptide of the HPPD W336 protein. This form did not have any impact on the observed characteristics for the GHB811 cotton-purified HPPD W336 protein.

6.5. Digestibility and stability of the introduced proteins

6.5.1. Digestibility and stability of the 2mEPSPS protein

***In vitro* digestibility in human simulated gastric fluid**

The 2mEPSPS protein was tested for digestibility in human simulated gastric fluid (SGF) containing pepsin at pH 1.2 for incubation times from 0.5 to 60 minutes.

The 2mEPSPS protein solution was incubated at 37°C with SGF (a pepsin solution at pH 1.2) and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Appropriate controls included the test protein at pH 1.2 without pepsin and SGF without the test protein.

The 2mEPSPS protein was degraded very rapidly in human simulated gastric fluid, within 30 seconds of incubation, in presence of pepsin, at pH 1.2.

***In vitro* digestibility in human simulated intestinal fluid**

The 2mEPSPS protein was tested for digestibility in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes.

The 2mEPSPS protein solution was incubated at 37°C with SIF (a porcine pancreatin solution at pH 7.5) and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the 2mEPSPS protein and potential stable protein fragments by western blot and Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Appropriate controls included the 2mEPSPS without pancreatin and SIF without 2mEPSPS protein.

The 2mEPSPS protein was degraded very rapidly with no fragment protein visible within 30 seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

***In vitro* stability to heat by SDS-PAGE and western blot**

The 2mEPSPS protein was tested for heat stability using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses.

The 2mEPSPS protein solution was incubated for 30 minutes at 4°C, 25°C, 37°C, 55°C, 75°C, and 95°C. Next, the samples were centrifuged. An uncentrifuged sample (UC), the supernatant (S) and the resuspended pellet (P) were analyzed by SDS-PAGE and western blot analyses. The 2mEPSPS protein treated at 4°C was used for comparison of the other temperature-treated samples.

After temperature-treatments at 25°C and 37°C the majority of 2mEPSPS remained in the supernatant as soluble protein. After temperature-treatment at 55°C and above, 2mEPSPS began to appear as insoluble protein, with minor degradation and aggregation of the protein possibly occurring at the elevated temperatures.

***In vitro* stability to heat by activity assay**

The 2mEPSPS protein was tested for heat stability using the EPSPS quantitative activity assay.

The 2mEPSPS protein solution was incubated for 30 minutes at 4°C, 25°C, 37°C, 55°C, 75°C, and 95°C. The specific activity of each temperature-treated 2mEPSPS protein was then measured by the EPSPS quantitative activity assay. The specific activity for the 2mEPSPS treated at 4°C was used for comparison of the other temperature-treated samples.

The results of the EPSPS quantitative activity assay analysis on the temperature treated 2mEPSPS protein are shown in Table 6.4. There was no decrease of the mean specific activity of 2mEPSPS after treatment at 25°C and 37°C. After treatment at 55°C there was a decrease in the mean specific activity of 2mEPSPS. There was no residual specific activity detected for 2mEPSPS after treatment at 75°C and above.

Table 6.4. Specific activity of 2mEPSPS after temperature-treatment

Temperature treatment (ºC)	Mean 2mEPSPS Specific Activity (U/mg)	% 2mEPSPS Activity Remaining
4ºC	4.987	100.0
25ºC	4.942	99.1
37ºC	5.120	102.7
55ºC	0.063	1.3
75ºC	Not Active	Not Active
95ºC	Not Active	Not Active

6.5.2. Digestibility and stability of the HPPD W336 protein

***In vitro* digestibility in human simulated gastric fluid**

The HPPD W336 protein was tested for digestibility in human simulated gastric fluid (SGF) containing pepsin at pH 1.2 for incubation times from 0.5 to 60 minutes.

The HPPD W336 protein was incubated at 37°C in SGF and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and by western blot.

The HPPD W336 protein was digested within 30 seconds of incubation in SGF, in presence of pepsin, at pH 1.2.

***In vitro* digestibility in human simulated intestinal fluid**

The HPPD W336 protein was tested for digestibility in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes.

The HPPD W336 protein was incubated at 37°C with SIF and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solutions were analyzed for presence of the HPPD W336 protein and potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by western blot. Appropriate controls included the HPPD W336 without pancreatin and SIF without HPPD W336 protein.

The HPPD W336 protein was degraded within 30 seconds of incubation with SIF, in presence of pancreatin, at pH 7.5. There were no stable protein fragments visible.

***In vitro* stability to heat by SDS-PAGE and western blot**

The HPPD W336 protein was tested for heat stability using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses.

The HPPD W336 protein was incubated at 60, 75 or 90 °C for 10, 30 or 60 minutes. The HPPD W336 protein treated at 4°C was used for comparison of the other temperature-treated samples.

The heat treated protein samples were examined with Coomassie blue stained-SDS-PAGE or western blot using a specific polyclonal rabbit anti-HPPD W336 protein antibody. Upon heat treatments, similar results are obtained either by the western blot or the Coomassie blue stained-SDS-PAGE.

After heat treatments, there were no visible changes to the HPPD W336 band at 60, 75 or 90°C from 10 to 60 minutes with intensities similar to the unheated sample.

***In vitro* stability to heat by activity assay**

The HPPD W336 protein was tested for heat stability using the enzyme activity assay. For enzymatic activity, an absorbance assay was developed which monitored the disappearance of substrate (4-hydroxyphenylpyruvate (HPP)) after derivatisation with 2,4-

Dinitrophenylhydrazine (DNP). To assess the temperature stability of the HPPD W336 protein, the protein was incubated at 45°C, 60°C and 95°C for 2.5, 5, 10, 20 and 60 minutes. Subsequently the activity of the protein was assessed under standard conditions (room temperature).

The results of the HPPD enzymatic activity assay analysis on the heat treated HPPD W336 protein are shown in Figure 6.14. The activity drops below 50% after the protein was incubated at 45°C for 20 minutes. At more elevated temperatures (60°C and 95°C) HPPD activity is abolished after 2.5 minutes.

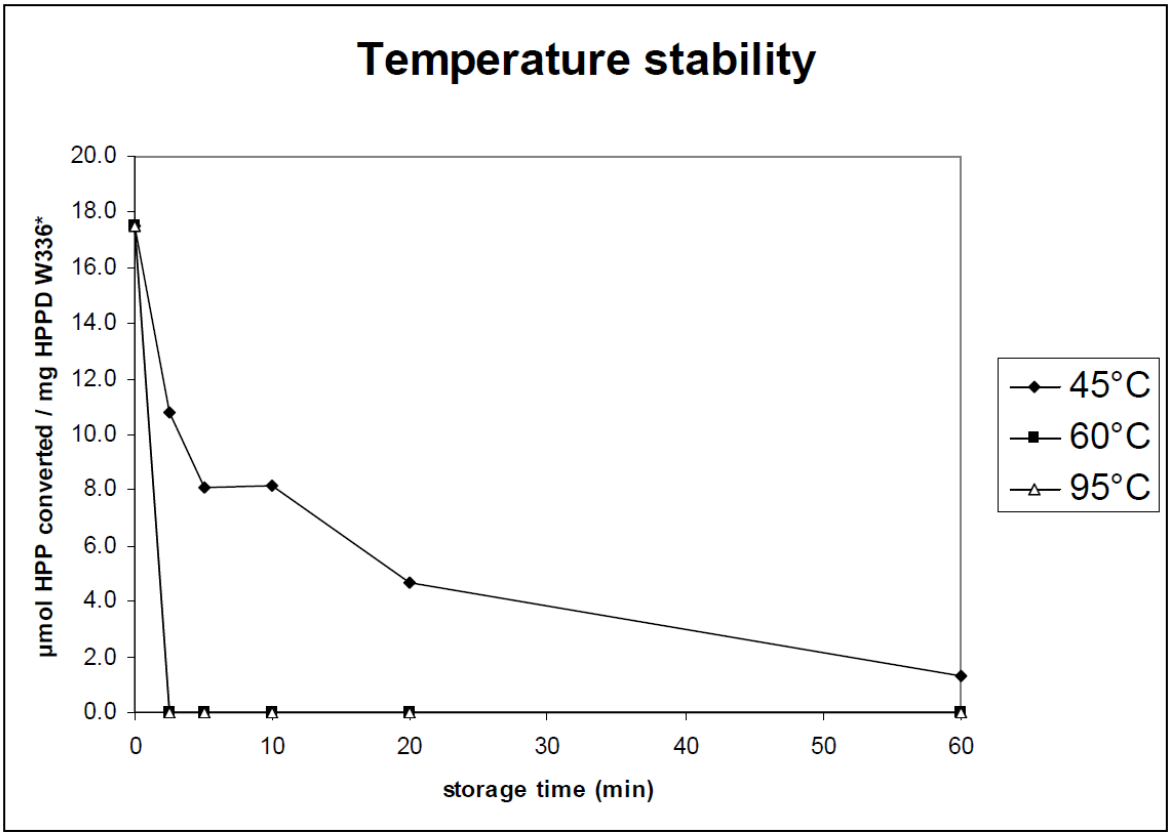


Figure 6.14. The effect of temperature on the activity of the HPPD W336 enzyme

6.6. Homology of the introduced proteins with known toxins and allergens

2mEPSPS

The potential amino acid sequence homology of the double mutated maize 5-enol pyruvylshikimate-3-phosphate synthase (2mEPSPS) protein with known allergens and known toxins was evaluated by using several *in silico* approaches.

This search evaluated the potential amino acid sequence identity of the query protein with known allergens by using two *in silico* approaches.

1. An overall identity search was carried out to compare the complete query sequence with all protein sequences present in the public allergen database AllergenOnline (www.allergenonline.org). The FASTA algorithm was used, with the BLOSUM50 scoring matrix and an E-value threshold of 10. The criterion indicating potential allergenicity was ≥35% identity over at least 80 consecutive amino acids with an allergenic protein.

2. An 8-mer search was carried out to identify any short sequences of 8 amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll from the EMBOSS suite, which compared the query sequence with all known allergens present in the allergen database.

Furthermore, this study considered the potential N-glycosylation sites by searching their known consensus sequences, potentially found in allergenic proteins.

In addition, two *in silico* approaches based on the FASTA algorithm associated with the BLOSUM50 scoring matrix were used to evaluate the potential amino acid sequence identity of the query protein with known toxins:

1. An overall identity search with all protein sequences present in the NCBI non-redundant database. An E-value threshold of 0.1 was used for pre-selecting the most similar proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the query protein.

2. An overall identity search with all protein sequences present in the in-house Bayer toxin database. An E-value threshold of 10 was used for pre-selecting the most identical toxins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the potential toxic properties of the query protein.

The overall identity search showed no biologically relevant identity between the query protein and any known allergenic proteins. In addition, the 8-mer search showed no 100% identity with known allergenic proteins.

Two potential N-glycosylation sites were identified on the amino acid sequence of the query protein. However, the presence of these sites is neither necessarily predictive of a potential glycosylation of the protein *in planta* nor of a potential allergenicity.

As expected, the overall homology search against the general protein database showed that most of the matches corresponded to EPSPS sequences from various organisms. There is no record of potential toxicity associated with these proteins. Furthermore, no identities were found with any toxic proteins from the Bayer toxin database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the 2mEPSPS protein.

HPPD W336

The potential amino acid sequence homology of the single mutated 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein with known allergens and known toxins was evaluated by using several *in silico* approaches.

This search evaluated the potential amino acid sequence identity of the query protein with known allergens by using two *in silico* approaches.

1. An overall identity search was carried out to compare the complete query sequence with all protein sequences present in the public allergen database AllergenOnline (www.allergenonline.org). The FASTA algorithm was used, with the BLOSUM50 scoring matrix and an E-value threshold of 1. The criterion indicating potential allergenicity was ≥35% identity over at least 80 consecutive amino acids with an allergenic protein.

2. An 8-mer search was carried out to identify any short sequences of 8 amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll from the EMBOSS suite, which compared the query sequence with all known allergens present in the allergen database.

Furthermore, this study considered the potential N-glycosylation sites by searching their known consensus sequences, potentially found in allergenic proteins.

In addition, two *in silico* approaches based on the FASTA algorithm associated with the BLOSUM50 scoring matrix were used to evaluate the potential amino acid sequence identity of the query protein with known toxins:

1. An overall identity search with all protein sequences present in the NCBI non-redundant database. An E-value threshold of 0.1 was used for pre-selecting the most similar proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the query protein.

2. An overall identity search with all protein sequences present in the in-house Bayer toxin database. An E-value threshold of 10 was used for pre-selecting the most identical toxins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the potential toxic properties of the query protein.

The overall identity search showed no biologically relevant identity between the query protein and any known allergenic proteins. In addition, the 8-mer search showed no 100% identity with known allergenic proteins.

No potential N-glycosylation sites were identified on the amino acid sequence of the query protein.

As expected, the overall homology search against the general protein database showed that, in most cases, the HPPD W336 protein matched with other HPPD proteins from various origins, which have safety records. In addition, no significant similarities were found with any toxic protein from the Bayer toxin database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the HPPD W336 protein.

6.7. Acute toxicity study in the mouse

2mEPSPS

Groups of 6 male and 6 female C57BL/6J mice were administered the 2mEPSPS protein (batch number 1417_2mEPSPS) by oral gavage at the limit dose level of 2000 mg/kg body weight. Similarly constituted groups of 6 male and 6 female mice received vehicle alone, administered in the same manner, and acted as controls. All animals were observed for clinical signs daily for fifteen days while their body weights and food consumption were measured weekly. At the termination of the study period, the animals were weighed and subjected to a necropsy, including a macroscopic examination. The tissues were retained for possible microscopic examinations.

There were no mortalities, no treatment-related clinical signs, no effects on the body weight and food consumption parameters as well as no macroscopic changes at necropsy, in C57BL/6J mice after an acute oral administration of the 2mEPSPS protein at 2000 mg/kg body weight.

In conclusion, the treatment with the 2mEPSPS protein at 2000 mg/kg body weight via the oral route did not produce any signs of systemic toxicity in the male and female C57BL/6J mice.

HPPD W336

Groups of 6 male and 6 female C57BL/6J mice were administered the HPPD W336 protein by oral gavage at the limit dose level of 2000 mg/kg body weight. Similarly constituted groups of 6 male and 6 female mice received vehicle alone, administered in the same manner, and acted as controls. All animals were observed for clinical signs daily for fifteen days while their body weights and food consumption were measured weekly. At the termination of the study period, the animals were weighed and subjected to a necropsy, including a macroscopic examination. The tissues were retained for possible microscopic examinations.

There were no mortalities, no treatment-related clinical signs, no effects on the body weight and food consumption parameters as well as no macroscopic changes at necropsy, in C57BL/6J mice after an acute oral administration of the HPPD W336 protein at 2000 mg/kg body weight.

In conclusion, the treatment with the HPPD W336 protein at 2000 mg/kg body weight via the oral route did not produce any signs of systemic toxicity in the male and female C57BL/6J mice.

7. Compositional analysis of key components

Composition analyses were conducted to determine levels of key nutrients and anti-nutrients of GHB811 cotton and compare those results to the non-GM counterpart and non-GM reference cotton varieties.

7.1. Field productions

Composition analysis was conducted on samples collected from 8 field trials completed in 2014 and 2015 located in U.S. cotton production areas (Table 7.1).

Table 7.1. Field trial sites for compositional analyses sample production

Year	Site Code	Nearest Town or City	State	County or Parish
2014	03	Kerman	California	Fresno
	07	Chula	Georgia	Tift
	09	Cheneyville	Louisiana	Rapides
	10	Greenville	Mississippi	Washington
	11	Elko	South Carolina	Barnwell
2015	15	Wall	Texas	Tom Green
	17	Hertford	North Carolina	Perquimans
	21	Edmonson	Texas	Hale

In addition to the GHB811 cotton and its non-GM counterpart, seven reference varieties that represent the natural variability existing in cotton were included in this study to provide reference ranges for the composition assessment. Each field trial site planted three of the seven reference varieties. The entries included are presented in Table 7.2.

Table 7.2. Description of entries for compositional analyses sample production

Entry ID	Description	Background	Trait-Specific Herbicide Treatment	Seed Lot Number (Year)	Sites
A	Non-GM Counterpart (Coker 312)	Coker 312	Not Treated	12PRGH050001 (2014) 14SHGH500001 (2015)	All
J	GHB811	Coker 312	Not Treated	13WAGH01252 (2014) 14SHGH000603 (2015)	All
K	GHB811	Coker 312	Treated	13WAGH01252 (2014) 14SHGH000603 (2015)	All
B	FM958	Non-GM	Not Treated	12LUGH000332 (2014)	03
				14SHGH500002 (2015)	15, 21
C	FM989	Non-GM	Not Treated	12LUGH000334 (2014)	03
				14SHGH500004 (2015)	15, 21
D	ST457	Non-GM	Not Treated	12LUGH000336 (2014)	07, 09, 10, 11
				14SHGH500005 (2015)	17
E	DP399	Non-GM	Not Treated	14LUGH000002 (2014 and 2015)	07, 09, 10, 11, 17
F	ST468	Non-GM	Not Treated	13WAGH03142 (2014)	07, 09, 10, 11
				14SHGH500007 (2015)	17
G	Acala Maxxa	Non-GM	Not Treated	13WAGH02234 (2014)	03
N	FM966	Non-GM	Not Treated	14SHGH500003 (2015)	15, 21

Entries were replicated four times in a randomized complete block design at field trial sites as shown in Table 7.2. The entries were randomly assigned to plots at each field trial site independently by the eStudy™ electronic notebook software.

Conventional herbicide management (CHM) was applied to all entries. The GHB811 cotton plots treated with trait-specific herbicides (Entry K) received one application of isoxaflutole at a rate of 100.3 to 115.2 grams active ingredient per hectare (g ai/ha) at BBCH Growth Stage BBCH 00–13 and one application of glyphosate at a rate of 1067 to 1222 g ai/ha at BBCH Growth Stage 16–19.

Seed cotton samples were harvested, without bias, from all plots at crop maturity and ginned to produce fuzzy seed for composition analysis. Samples were shipped frozen to Bayer CropScience LP, Morrisville, North Carolina where they were placed in frozen storage (-5º C or lower).

7.2. Composition Analysis

The composition analysis of the cotton fuzzy seed samples was conducted at EPL Bio Analytical Services (Niantic, IL). The samples were pre-ground and then completely homogenized using an Ultra Centrifugal Mill. They were maintained at a temperature of approximately -20 °C for the duration of the experimental phase, except when removed from the freezer for homogenization, sample preparation or analysis.

Composition analytes, units and EPL Bio Analytical Services method mnemonics are presented in Table 7.3.

Table 7.3. Composition analytes, units and methods for cotton fuzzy seed

Analyte	Units	EPL Method
Proximates and Fiber		
Moisture	% FW	NC-4
Ash	% FW, DW	NC-2
Carbohydrates		Calculated (NC-494)
Crude Fat		NC-230
Crude Protein		NC-20
Acid Detergent Fiber		NC-3
Neutral Detergent Fiber		NC-9
Total Dietary Fiber		NC-359
Amino Acids		
Alanine, Arginine, Aspartic Acid, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine	% FW, DW	NC-58
Cystine, Methionine		NC-279
Tryptophan		NC-22
Fatty Acids		
C8:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3,C18:4, C20:0, C20:1, C20:2, C20:3, C20:4, C20:5, C22:0, C22:1, C22:5 n-3,C22:5 n-6, C22:6, C24:0	% FW, DW, % Total Fatty Acids	NC-319
Minerals and Alpha Tocopherol		
Calcium, Copper, Iron, Magnesium, Manganese, Phosphorus, Potassium, Sodium, Zinc	mg/kg FW, DW	NC-60
Alpha Tocopherol (Vitamin E)	mg/kg FW, DW	NC-346
Anti-nutrients		
Free Gossypol	% FW, DW	NC-37
Total Gossypol		NC-36
Cyclopropenoid Fatty Acids		
Dihydrosterculic Acid	% FW, DW % Total Fatty Acids	NC-231
Sterculic Acid		
Malvalic Acid		

7.3. Statistical analysis

Composition data generated from eight sites were used for statistical analysis. The data were read into SAS and statistical analyses were performed using SAS version 9.3.

Analytes, for which more than one third of the values were less than the limit of quantitation (< LOQ), were excluded from statistical evaluation (i.e., ANOVA and mean comparisons) and discussion. These analytes are presented in Table 7.4. For six analytes, Tyrosine, C17:1 Heptadecenoic Acid, C20:1 Eicosenoic Acid, C24:0 Lignoceric Acid, Malvalic Acid and Sterculic Acid, less than one third of the values were below LOQ. In these cases a value equal to half the LOQ (dry weight basis) was substituted for the purpose of statistical analysis, including the calculation of means.

Minimum and maximum values are presented in Table 7.5 – Table 7.9 for analytes where some values are above LOQ, but there is insufficient data for statistical analysis.

Combined-site analysis

Composition parameters were analyzed for each characteristic combined over all sites with a mixed model analysis of variance:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta_{k(j)} + \varepsilon_{ijk}$$

where Y_{ijk} is the individual value measurement, μ the overall mean, α_i the fixed effect associated with entry, β_j the random effect associated with site, $\alpha\beta_{ij}$ the random effect for the interaction of entry by site, $\delta_{k(j)}$ the random effect associated with block nested within site and ε_{ijk} the random error. Based on the mixed model, entry differences (A vs J and A vs K) were estimated and the *p*-values (pairwise *t*-test under the mixed model) were provided.

Studentized residuals based on the mixed model were visually checked for model assumptions, including normality. Overall no serious departure from model assumptions was observed for majority of the parameters. A small proportion of the parameters show a moderate departure from normality, in which situation the use of the mixed model is still valid based on the robustness of analysis of variance to moderate departures from normality of the error distribution (Schabenberger and Pierce*,* 2002; Jacqmin-Gadda *et al.,* 2007).

Data for the combined-site analysis for each of 54 quantifiable analytes measured for cotton fuzzy seed included mean and standard deviation for Entry A, Entry J and Entry K and the minimum and maximum values for the seven cotton reference varieties (Entries B–G, N) as well as the range of means for reference varieties. Also included are tolerance intervals calculated for each analyte based on the reference varieties over all combined sites. The tolerance intervals are specified to contain 99% of the population with 95% confidence. In addition, pairwise comparisons were made between the non-GM counterpart (Entry A) and the GM variety not treated with trait-specific herbicides (Entry J) and between the non-GM counterpart (Entry A) and the GM variety treated with trait-specific herbicides (Entry K). A significant difference was noted when the t-test p-value between the comparators was <0.05.

Table 7.4. Parameters with values below the limit of quantitation (LOQ)

Parameter	Number of Values >= LOQ	Number of Values < LOQ	LOQ value	Unit	Excluded from analysis
Tyrosine	189	3	0.313	% DW	No
C8:0 Caprylic Acid	0	192	0.0197	% Total Fatty Acids	Yes
C10:0 Capric Acid	0	192	0.0200	% Total Fatty Acids	Yes
C12:0 Lauric Acid	1	191	0.00505	% Total Fatty Acids	Yes
C14:1 Myristoleic Acid	0	192	0.0203	% Total Fatty Acids	Yes
C15:0 Pentadecanoic Acid	0	192	0.0204	% Total Fatty Acids	Yes
C15:1 Pentadecenoic Acid	0	192	0.0204	% Total Fatty Acids	Yes
C17:1 Heptadecenoic Acid	175	17	0.00513	% Total Fatty Acids	No
C18:4 Stearidonic Acid	46	146	0.00514	% Total Fatty Acids	Yes
C20:1 Eicosenoic Acid	191	1	0.00517	% Total Fatty Acids	No
C20:2 Eicosadienoic Acid	2	190	0.00517	% Total Fatty Acids	Yes
C20:3 Eicosatrienoic Acid	0	192	0.0207	% Total Fatty Acids	Yes
C20:4 Arachidonic Acid	0	192	0.0207	% Total Fatty Acids	Yes
C20:5 Eicosapentaenoic Acid	2	190	0.00516	% Total Fatty Acids	Yes
C22:1 Erucic Acid	0	192	0.0207	% Total Fatty Acids	Yes
C22:5 N3 Docosapentaenoic Acid	111	81	0.00518	% Total Fatty Acids	Yes
C22:5 N6 Docosapentaenoic Acid	103	89	0.00518	% Total Fatty Acids	Yes
C22:6 Docosahexaenoic Acid	0	192	0.00518	% Total Fatty Acids	Yes
C24:0 Lignoceric Acid	142	50	0.0104	% Total Fatty Acids	No
Malvalic Acid	178	14	0.00476	% Total Fatty Acids	No
Sterculic Acid	176	16	0.00477	% Total Fatty Acids	No

7.4. Results of compositional analysis

Proximates and fiber in cotton fuzzy seed (Table 7.5)

No significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for moisture, ash, carbohydrates, crude fat, acid detergent fiber, and total dietary fiber.

No significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated with trait-specific herbicides (Entry J) for neutral detergent fiber. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for crude protein. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton treated with trait-specific herbicides (Entry K) for neutral detergent fiber.

However, the means for all entries, for all proximates and fiber, were within the range of means of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Amino acids in cotton fuzzy seed (Table 7.6)

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated with trait-specific herbicides (Entry J) for cystine and methionine. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton treated with trait-specific herbicides (Entry K) for cystine and methionine.

However, the means for all entries, for all amino acids, were within the range of means of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Fatty acids in cotton fuzzy seed (Table 7.7)

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for myristic, palmitic, heptadecanoic, heptadecenoic, oleic, linoleic, linolenic, eicosenoic, behenic and lignoceric acids.

No significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated with trait-specific herbicides (Entry J) for arachidic acid. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for palmitoleic and stearic acids. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton treated with trait-specific herbicides (Entry K) for arachidic acid.

However, the means for arachidic acid, palmitoleic acid and stearic acid were within the range of means of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Minerals and alpha tocopherol in cotton fuzzy seed (Table 7.8)

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc.

Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for alpha tocopherol.

The means for non-GM Coker 312 (Entry A) and both GHB811 entries (Entry J and K), for alpha tocopherol, were outside and above the range of means of the reference varieties.However, literature ranges for alpha tocopheral levels in fuzzy seed of cotton range from 29.5 – 223.7mg/kg (ILSI, 2016), well encapsulating all values observed for all entries. Therefore, the statistically significant differences in alpha tocopherol are not considered biologically relevant.

Anti-nutrients in cotton fuzzy seed (Table 7.9)

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for malvalic acid and sterculic acid.

No statistically significant differences were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated with trait-specific herbicides (Entry J) for dihydrosterculic acid. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides (Entry J and Entry K) for free gossypol and total gossypol. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GHB811 cotton treated with trait-specific herbicides (Entry K) for dihydrosterculic acid.

The means for all entries for free gossypol and total gossypol were within the range of means of the reference varieties and the tolerance intervals. The levels of dihydrosterculic acid for treated GHB811 (Entry K) fell outside of and below the range of means for the reference variety, but still within overall range of values for the reference varieties as well as the tolerance intervals. Literature values for dihydrosterculic acid content of fuzzy cotton seed were also explored. The ILSI crop composition database reports a range of values for dihydrosterculic acid content of cotton fuzzy seed of 0.105 – 1.666 % Total Fatty Acid (ILSI, 2016), which contains the value observed for treated GHB811 (0.169 % Total Fatty Acid, Table 7.9). Therefore, the statistically significant differences are not considered biologically relevant.

Of the 69 composition analytes, 54 had sufficient levels above LOQ for statistical analysis. Of the 54 analytes that were analyzed, statistically significant differences ($p < 0.05$) were observed for 11 analytes, six of which were statistically different between both the non-GM counterpart (Entry A) and GHB811 cotton not treated or treated with trait-specific herbicides

(Entry J and Entry K), and five of which were statistically different between the non-GM counterpart (Entry A) and GHB811 cotton treated with trait-specific herbicides (Entry K). However the means of all analytes were within the overall range of values of the reference varieties and the tolerance intervals. In most cases, values for the non-GM counterpart (Entry A), and both GHB811 entries (Entry J and K) fell within the range of means for reference varieties. In any case where they did not meet these criteria, they fell within the range of values provided in the ILSI crop composition database (ILSI, 2016). Therefore, the statistically significant differences are not considered biologically relevant.

Table 7.5. Comparison of proximates and fiber in fuzzy seed of GHB811 cotton with its non-GM counterpart[a]

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range (Entries B-G,N) (Min-Max)	Non-GM Reference Varieties Mean Range (Entries B-G,N) (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries B-G,N) [b] (Lower-Upper)	Comparison t-test A vs J [c] p-value	Comparison t-test A vs K [c] p-value
Moisture (% FW)	10.8 ± 1.9	11.1 ± 2.8	11.5 ± 2.7	8.26 - 23.7	9.34 - 16.08	2.51 - 20.2	0.534	0.177
Ash (% DW)	3.80 ± 0.29	3.80 ± 0.29	3.79 ± 0.42	3.07 - 5.16	3.58 - 4.41	2.66 - 4.99	0.983	0.919
Carbohydrates (% DW)	52.3 ± 2.8	53.3 ± 2.6	53.4 ± 2.6	46.3 - 61.0	49.0 - 56.7	46.5 - 63.0	0.104	0.084
Crude Fat (% DW)	20.5 ± 2.8	20.4 ± 2.5	20.5 ± 2.2	13.7 - 24.4	17.8 - 21.2	13.6 - 25.6	0.730	0.939
Crude Protein (% DW)	23.3 ± 2.6	22.5 ± 2.4	22.3 ± 2.6	15.8 - 28.7	20.4 - 26.0	14.9 - 28.8	0.029	0.008
Acid Detergent Fiber (% DW)	43.0 ± 5.6	42.9 ± 4.2	43.3 ± 3.7	36.6 - 54.1	42.1 - 45.5	33.1 - 54.2	0.944	0.721
Neutral Detergent Fiber (% DW)	45.6 ± 3.2	46.7 ± 3.1	46.9 ± 3.0	41.2 - 58.6	44.3 - 50.0	39.2 - 57.7	0.094	0.048
Total Dietary Fiber (% DW)	43.7 ± 8.8	47.4 ± 5.3	47.6 ± 6.6	25.2 - 77.0	42.4 - 49.4	30.3 - 64.9	0.067	0.058

[a] Composition samples were derived from eight field trials conducted in the United States in 2014 and 2015.

[b] 99% Tolerance Interval: Range of reference lines based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 7.6. Comparison of amino acids in fuzzy seed of GHB811 cotton with its non-GM counterpart[a] (% DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range (Entries B-G,N) (Min-Max)	Non-GM Reference Varieties Mean Range (Entries B-G,N) (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries B-G,N) [b] (Lower-Upper)	Comparison t-test A vs J [c] p-value	Comparison t-test A vs K [c] p-value
Alanine	0.910 ± 0.088	0.904 ± 0.090	0.928 ± 0.088	0.634 - 1.07	0.806 - 0.990	0.607 - 1.14	0.729	0.265
Arginine	2.43 ± 0.39	2.40 ± 0.35	2.44 ± 0.35	1.45 - 3.18	2.07 - 2.83	1.33 - 3.29	0.597	0.969
Aspartic Acid	2.10 ± 0.30	2.12 ± 0.29	2.12 ± 0.31	1.32 - 2.58	1.83 - 2.26	1.22 - 2.78	0.684	0.650
Cystine	0.439 ± 0.077	0.421 ± 0.082	0.408 ± 0.067	0.271 - 0.574	0.392 - 0.496	0.224 - 0.607	0.257	0.047
Glutamic Acid	4.53 ± 0.63	4.52 ± 0.58	4.55 ± 0.64	2.97 - 5.70	3.96 - 4.93	2.73 - 6.06	0.918	0.886
Glycine	1.013 ± 0.103	0.988 ± 0.100	1.009 ± 0.106	0.667 - 1.26	0.886 - 1.083	0.650 - 1.27	0.256	0.864
Histidine	0.680 ± 0.083	0.666 ± 0.072	0.676 ± 0.074	0.455 - 0.969	0.593 - 0.746	0.417 - 0.887	0.376	0.824
Isoleucine	0.743 ± 0.082	0.737 ± 0.076	0.744 ± 0.075	0.518 - 0.866	0.657 - 0.780	0.490 - 0.935	0.663	0.951
Leucine	1.38 ± 0.15	1.37 ± 0.14	1.39 ± 0.13	0.942 - 1.64	1.219 - 1.49	0.910 - 1.74	0.616	0.700
Lysine	1.05 ± 0.10	1.05 ± 0.12	1.07 ± 0.12	0.746 - 1.30	0.931 - 1.14	0.693 - 1.35	0.949	0.461
Methionine	0.319 ± 0.047	0.308 ± 0.055	0.297 ± 0.047	0.188 - 0.384	0.280 - 0.327	0.191 - 0.412	0.303	0.047
Phenylalanine	1.29 ± 0.17	1.26 ± 0.16	1.29 ± 0.15	0.801 - 1.57	1.104 - 1.42	0.754 - 1.68	0.450	0.953
Proline	0.877 ± 0.101	0.866 ± 0.093	0.884 ± 0.089	0.595 - 1.08	0.772 - 0.989	0.569 - 1.12	0.519	0.665
Serine	1.045 ± 0.112	1.025 ± 0.109	1.035 ± 0.140	0.707 - 1.66	0.921 - 1.111	0.626 - 1.39	0.465	0.723
Threonine	0.777 ± 0.071	0.769 ± 0.067	0.776 ± 0.074	0.556 - 0.894	0.694 - 0.813	0.537 - 0.954	0.558	0.928
Tryptophan	0.241 ± 0.034	0.249 ± 0.036	0.246 ± 0.034	0.177 - 0.293	0.213 - 0.259	0.156 - 0.310	0.258	0.460
Tyrosine	0.499 ± 0.069	0.473 ± 0.083	0.487 ± 0.068	0.176 - 0.608	0.426 - 0.542	0.250 - 0.688	0.100	0.443
Valine	1.036 ± 0.121	1.029 ± 0.115	1.041 ± 0.111	0.720 - 1.24	0.912 - 1.123	0.671 - 1.32	0.741	0.808

[a] Composition samples were derived from eight field trials conducted in the United States in 2014 and 2015.

[b] 99% Tolerance Interval: Range of reference lines based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 7.7. Comparison of fatty acids in fuzzy seed of GHB811 cotton with its non-GM counterpart[a] (% Total Fatty Acids)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range (Entries B-G,N) (Min-Max)	Non-GM Reference Varieties Mean Range (Entries B-G,N) (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries B-G,N) [b] (Lower-Upper)	Comparison t-test A vs J [c] p-value	Comparison t-test A vs K [c] p-value
C12:0 Lauric Acid	<LOQ	<LOQ	<LOQ	<LOQ - 0.0377	NA	NA	NA	NA
C14:0 Myristic Acid	0.651 ± 0.150	0.665 ± 0.145	0.665 ± 0.138	0.470 - 1.17	0.553 - 0.902	0.217 - 1.11	0.310	0.291
C16:0 Palmitic Acid	23.2 ± 1.8	23.2 ± 1.8	23.4 ± 1.6	19.7 - 27.6	21.6 - 25.0	17.7 - 27.7	0.837	0.342
C16:1 Palmitoleic Acid	0.486 ± 0.049	0.524 ± 0.052	0.525 ± 0.043	0.392 - 0.641	0.452 - 0.532	0.325 - 0.618	<.001	<.001
C17:0 Heptadecanoic Acid	0.0820 ± 0.0096	0.0827 ± 0.0116	0.0808 ± 0.0094	0.0653 - 0.1180	0.0771 - 0.0977	0.0517 - 0.1206	0.752	0.569
C17:1 Heptadecenoic Acid	0.0436 ± 0.0121	0.0446 ± 0.0116	0.0458 ± 0.0095	0.0175 - 0.0821	0.0391 - 0.0672	0.0050 - 0.0937	0.682	0.376
C18:0 Stearic Acid	2.37 ± 0.16	2.28 ± 0.17	2.26 ± 0.15	2.00 - 2.97	2.25 - 2.68	1.75 - 3.07	<.001	<.001
C18:1 Oleic Acid	14.2 ± 1.0	14.2 ± 1.0	14.2 ± 1.0	13.4 - 20.8	13.8 - 18.1	9.2 - 21.3	0.912	0.956
C18:2 Linoleic Acid	57.3 ± 2.9	57.5 ± 3.0	57.3 ± 2.8	46.3 - 60.7	51.1 - 59.2	45.2 - 67.8	0.682	0.877
C18:3 Linolenic Acid	0.229 ± 0.121	0.228 ± 0.113	0.210 ± 0.109	0.0301 - 0.547	0.2248 - 0.281	0 - 0.562	0.984	0.481
C18:4 Stearidonic Acid	<LOQ - 0.0653	<LOQ - 0.0562	<LOQ - 0.0509	<LOQ - 0.0832	NA	NA	NA	NA
C20:0 Arachidic Acid	0.254 ± 0.032	0.247 ± 0.035	0.244 ± 0.033	0.196 - 0.376	0.214 - 0.307	0.117 - 0.388	0.112	0.036
C20:1 Eicosenoic Acid	0.0666 ± 0.0154	0.0709 ± 0.0166	0.0663 ± 0.0136	0.0396 - 0.0980	0.0631 - 0.0797	0.0289 - 0.1129	0.268	0.931
C20:2 Eicosadienoic Acid	<LOQ	<LOQ - 0.0382	<LOQ - 0.0451	<LOQ	NA	NA	NA	NA
C20:5 Eicosapentaenoic Acid	<LOQ	<LOQ	<LOQ	<LOQ - 0.0427	NA	NA	NA	NA
C22:0 Behenic Acid	0.148 ± 0.021	0.149 ± 0.026	0.148 ± 0.023	0.110 - 0.212	0.126 - 0.186	0.074 - 0.224	0.829	0.903
C22:5 N3 Docosapentaenoic Acid	<LOQ - 0.0767	<LOQ - 0.128	<LOQ - 0.0712	<LOQ - 0.104	NA	NA	NA	NA
C22:5 N6 Docosapentaenoic Acid	<LOQ - 0.22	<LOQ - 0.259	<LOQ - 0.185	<LOQ - 0.277	NA	NA	NA	NA
C24:0 Lignoceric Acid	0.117 ± 0.054	0.099 ± 0.059	0.109 ± 0.071	0.0295 - 0.285	0.0900 - 0.129	0 - 0.285	0.238	0.603

[a] Composition samples were derived from eight field trials conducted in the United States in 2014 and 2015.

[b] 99% Tolerance Interval: Range of reference lines based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

NA=Not Applicable because more than 1/3 of the values are <LOQ. Minimum and maximum are reported instead of mean and standard deviation.

Table 7.8. Comparison of minerals and alpha tocopherol in fuzzy seed of GHB811 cotton with its non-GM counterpart[a] (mg/kg DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range (Entries B-G,N) (Min-Max)	Non-GM Reference Varieties Mean Range (Entries B-G,N) (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries B-G,N) [b] (Lower-Upper)	Comparison t-test A vs J [c] p-value	Comparison t-test A vs K [c] p-value
Calcium	983 ± 288	1022 ± 296	1039 ± 270	702 - 1960	1044 - 1846	456 - 1930	0.435	0.263
Copper	6.19 ± 2.35	6.21 ± 2.55	6.15 ± 2.27	1.99 - 11.5	4.52 - 9.27	0 - 13.3	0.937	0.835
Iron	37.2 ± 6.7	36.1 ± 6.1	37.1 ± 6.8	22.1 - 60.1	31.9 - 52.6	13.2 - 60.0	0.415	0.937
Magnesium	4013 ± 489	4002 ± 486	3917 ± 355	2848 - 5328	3517 - 4324	2380 - 5438	0.896	0.226
Manganese	12.9 ± 2.5	12.5 ± 2.0	12.4 ± 2.2	9.94 - 25.2	12.27 - 22.9	5.35 - 23.4	0.111	0.077
Phosphorus	6613 ± 1298	6749 ± 960	6449 ± 1277	3888 - 9333	5841 - 8896	3137 - 10251	0.466	0.381
Potassium	12287 ± 1158	12508 ± 1385	12193 ± 943	10263 - 15727	11677 - 12618	9269 - 15027	0.342	0.684
Sodium	30.3 ± 15.5	37.3 ± 29.8	32.5 ± 24.9	10.0 - 148	22.0 - 63.0	0 - 74.9	0.116	0.626
Zinc	33.7 ± 5.4	33.7 ± 5.8	33.2 ± 5.8	21.5 - 49.6	31.9 - 44.2	17.2 - 50.3	0.957	0.553
Alpha Tocopherol	121.6 ± 19.1	109.8 ± 18.1	109.1 ± 15.0	49.8 - 141	87.3 - 108.4	37.0 - 157	0.002	0.001

[a] Composition samples were derived from eight field trials conducted in the United States in 2014 and 2015.

[b] 99% Tolerance Interval: Range of reference lines based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 7.9. Comparison of anti-nutrients in fuzzy seed of GHB811 cotton with its non-GM counterpart[a]

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range (Entries B-G,N) (Min-Max)	Non-GM Reference Varieties Mean Range (Entries B-G,N) (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries B-G,N) [b] (Lower-Upper)	Comparison t-test A vs J [c] p-value	Comparison t-test A vs K [c] p-value
Free Gossypol (% DW)	0.591 ± 0.145	0.534 ± 0.122	0.526 ± 0.118	0.273 - 0.941	0.412 - 0.828	0.106 - 1.15	0.007	0.002
Total Gossypol (% DW)	0.804 ± 0.167	0.700 ± 0.160	0.735 ± 0.145	0.346 - 1.34	0.537 - 1.146	0.153 - 1.53	0.002	0.033
Cyclopropenoic Fatty Acids (% Total Fatty Acids)								
Dihydrosterculic Acid	0.198 ± 0.052	0.188 ± 0.062	0.169 ± 0.048	0.126 - 0.407	0.2245 - 0.292	0.0972 - 0.438	0.405	0.021
Malvalic Acid	0.380 ± 0.176	0.384 ± 0.147	0.355 ± 0.172	0.014 - 0.983	0.275 - 0.567	0 - 1.062	0.928	0.533
Sterculic Acid	0.146 ± 0.066	0.136 ± 0.056	0.127 ± 0.062	0.014 - 0.366	0.136 - 0.233	0 - 0.423	0.527	0.236

[a] Composition samples were derived from eight field trials conducted in the United States in 2014 and 2015.

[b] 99% Tolerance Interval: Range of reference lines based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

NA=Not Applicable because more than 1/3 of the values are <LOQ. Minimum and maximum are reported instead of mean and standard deviation.

7.5. Conclusions from compositional analyses

Comparison of key nutrient and anti-nutrient levels of GHB811 cotton fuzzy seed (not treated and treated with trait-specific herbicides) to the non-GM counterpart revealed statistically significant differences for 11 of the 54 analytes examined. However, the means of 9 of the 11 analytes were within the range of means of the reference varieties and the tolerance intervals, and the remaining 2 were within the ranges provided by the ILSI crop composition database. Therefore, the statistically significant differences are not considered biologically relevant. Based on the comparative assessment, nutrient and anti-nutrient levels in GHB811 cotton fuzzy seed are comparable to that of the non-GM counterpart and reference varieties.

8. Agronomic and phenotypic evaluation

8.1. History of field activities

GHB811 cotton has been field tested in adapted growing regions of the United States, Argentina and Chile. The field trials and associated permit and notification numbers in the U.S., Argentina and Chile are summarized in Table 8.1, Table 8.2 and Table 8.3, respectively. Field trials were conducted for the development of regulatory data, product development and characterization, and production of seed for the latter activities.

Table 8.1. Summary of field activities under USDA notifications for GHB811 cotton

Agency	Year	Permit Type	Permit Number
USDA APHIS BRS	2016	Notification: Interstate movement and environmental release	16-069-101n
USDA APHIS BRS	2015	Notification: Interstate movement and environmental release	15-247-104n
USDA APHIS BRS	2015	Notification: Interstate movement and environmental release	15-111-101n
USDA APHIS BRS	2015	Notification: Interstate movement and environmental release	15-065-104n
USDA APHIS BRS	2015	Notification: Interstate movement and environmental release	15-058-108n
USDA APHIS BRS	2014	Notification: Interstate movement and environmental release	14-241-101n
USDA APHIS BRS	2014	Notification: Interstate movement and environmental release	14-104-103n
USDA APHIS BRS	2014	Notification: Interstate movement and environmental release	14-069-106n
USDA APHIS BRS	2014	Notification: Interstate movement and environmental release	14-068-101n
USDA APHIS BRS	2013	Notification: Interstate movement and environmental release	13-255-102n
USDA APHIS BRS	2013	Notification: Interstate movement and environmental release	13-054-102n
USDA APHIS BRS	2012	Notification: Interstate movement and environmental release	12-247-102n

Table 8.2. Summary of field activities under CONABIA notifications for GHB811 cotton

Agency	Year	Permit Type	Permit Number
CONABIA Argentina	2016	Experimental	10.587-16
CONABIA Argentina	2016	Experimental	14.492-16
CONABIA Argentina	2015	Experimental	22.573-15
CONABIA Argentina	2014	Experimental	26.370-14
CONABIA Argentina	2013	Experimental	525.966-13
CONABIA Argentina	2012	Experimental	244.908-12

Table 8.3. Summary of field activities under SAG notifications for GHB811 cotton

Agency	Year	Permit Type	Permit Number
SAG-Chile	2016	Environmental release	SAG Res. 5655/2016

8.2. Field study of agronomic and phenotypic characteristics

To evaluate the agronomic performance of GHB811 cotton under field conditions representative of commercial cultivation, a multi-site field evaluation was undertaken in the U.S. during the 2014 and 2015 growing seasons. The agronomic assessment included 15 locations (seven sites conducted in 2014 and eight sites conducted in 2015) representative of diverse cotton growing regions of the United States (Table 8.4).

Table 8.4. Field trial locations for agronomic and phenotypic evaluation

Year	Site Code	Nearest Town or City	State	County or Parish
2014	02	East Bernard	Texas	Wharton
	03	Kerman	California	Fresno
	07	Chula	Georgia	Tift
	08	West Memphis	Arkansas	Crittenden
	09	Cheneyville	Louisiana	Rapides
	10	Greenville	Mississippi	Washington
	11	Elko	South Carolina	Barnwell
2015	13	Chula	Georgia	Tift
	14	West Memphis	Arkansas	Crittenden
	15	Wall	Texas	Tom Green
	16	Greenville	Mississippi	Washington
	17	Hertford	North Carolina	Perquimans
	18	Groom	Texas	Carson
	20	Waller	Texas	Waller
	21	Edmonson	Texas	Hale

The 15 field trial site locations reported are representative of the range of receiving environments where cotton is commercially grown. Wide geographical distribution of the sites in regions representative of commercial cotton production in the U.S. is indicated in Figure 8.1. All field sites were managed by methods typical of commercial cotton production.

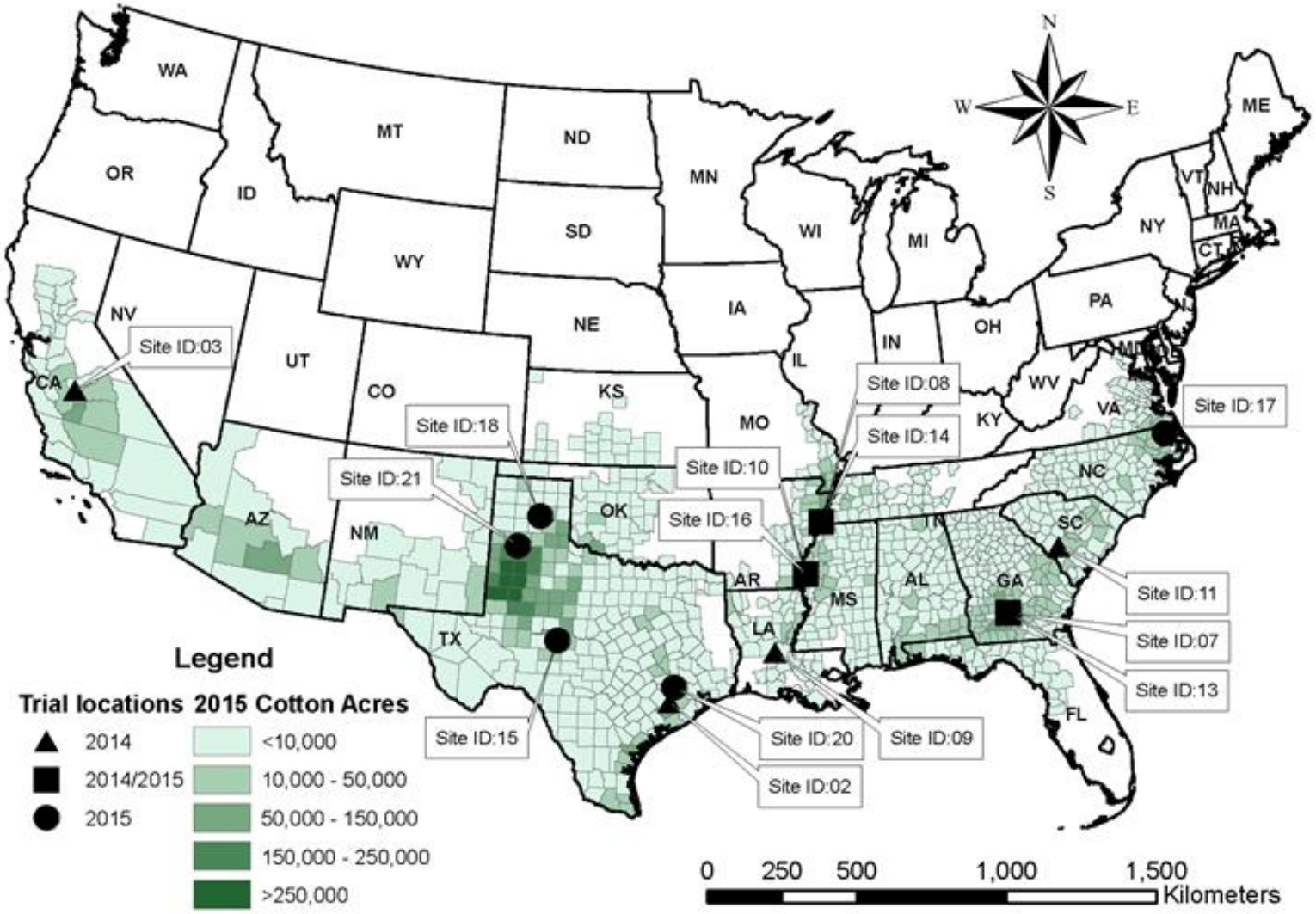


Figure 8.1. Field trial locations for agronomic and phenotypic evaluation overlaid on 2015 commercial cotton acreage

Entries relevant for the agronomic assessment of GHB811 included at each field trial site are presented in Table 8.5. Each entry was replicated four times in a randomized complete block design. Seven total non-GM reference varieties were used, and each field trial contained three reference varieties adapted to each respective location.

All plots within a field site were subjected to the same growing conditions and management (*i.e.* cultivation, irrigation, fertilizer, maintenance pesticide treatments). Each plot within each field trial was identically sized and separated from other plots by non-GM cotton buffers or fallow alleyways. Plots were a minimum of 27.9 m^2.

The GHB811 cotton plots treated with the trait specific herbicide received one spray application of each trait-specific herbicide. One application of IFT was made at a rate of 100.3 to 115.2 grams active ingredient per hectare (g ai/ha) before or shortly after emergence (BBCH 00 to 13). One application of GLY was made at a rate of 1067 to 1222 g ai/ha at the six to nine leaf growth stage (BBCH 16 to 19).

Table 8.5. Description of entries in agronomic and phenotypic evaluation trials

Entry ID	Material Name	Herbicide Treatment	Site Placements
A	Coker 312	Not Treated	All
J	GHB811	Not Treated	All
K	GHB811	Treated with IFT and GLY	All
B	FM958	Not Treated	02, 03, 15, 18, 20, 21
C	FM989	Not Treated	02, 03, 15, 18, 20, 21
D	ST457	Not Treated	07, 08, 09, 10, 11, 13, 14, 16, 17
E	DP399	Not Treated	07, 08, 09, 10, 11, 13, 14, 16, 17
F	ST468	Not Treated	07, 08, 09, 10, 11, 13, 14, 16, 17
G	Acala Maxxa	Not Treated	02, 03
N	FM966	Not Treated	15, 18, 20, 21

The following agronomic parameters were measured throughout the growing season at each field trial site. Data are reported for each individual plot in each field trial.

Continuous Parameters:

- Early Stand Counts: Counts of plants in one row representative of each plot (or a minimum of six meters of row), expressed as plants per m^2.
    - The first stand count was done when each plot was at cotyledon to three leaf stage (BBCH 10 to 13) at 13 to 22 days after planting.
    - The second stand count was done when each plot was at the three to eight leaf stage (BBCH 13 to 18) at 27 to 36 days after planting.
- Percent Ground Cover: Visual estimation of the percent ground cover in three representative 1 m^2 quadrats within each plot. Data is reported as an average of the three quadrats.
- Days and Heat Units to First Flower: The number of days elapsed from planting to the date when 10% of plants in each plot had at least one open flower. Heat Units to First Flower are the accumulated heat units (in °C, from a base temperature of 15.6°C) from planting to first flower.
- Days and Heat Units to First Open Bolls: The number of days elapsed from planting to the date when 50% or more plants in each plot had at least one cracked boll. Heat Units to First Open Bolls are the accumulated heat units (in °C, from a base temperature of 15.6°C) from planting to first open bolls.
- Percent Open Bolls: A same-day visual evaluation of the percentage of bolls open within each plot rated when the non-GM comparator (Coker 312) was at 40% to 60% open bolls (BBCH 84 to 86).
- Final Stand Count: Counts of plants in one row representative of each plot (or row length of a minimum of six meters), expressed as plants per m^2. Final stand counts were performed at BBCH 84 to 99.
- Boll Properties: Boll properties were determined from a 25 boll sample from each plot. Bolls were sampled from non-end rows within each plot. The 25 boll seed cotton samples were ginned to produce fuzzy seed and lint. The bolls, fuzzy seed and lint were independently weighed. The following parameters were analyzed:
    - Boll Weight: The average single boll seed cotton mass from 25 bolls in grams.
    - Percent Lint: The lint mass from 25 bolls divided by boll weight of 25 bolls expressed as a percentage.
    - Hundred Seed Weight (Seed Index): mass of 100 fuzzy seeds in grams.
    - Seeds per Boll: Average weight of seeds per boll divided by average weight of each seed. The source data was derived from the mass of seed from 25 bolls and the Hundred Seed Weight .
- Seed Cotton Yield: The total weight of the seed cotton (seed and lint) harvested from two rows in each plot. The data was converted to kilograms per hectare (kg/ha).
- Lint Yield: Lint yield was calculated from plot weights of seed cotton and Percent Lint. Data is reported on a kg/ha basis.

- Lint (Fiber) Properties: Lint for analysis was obtained from the same 25 boll sample that provided source data for Boll Properties. Lint samples were analyzed by Bayer CropScience Breeding Station Fiber Analysis Lab, Leland, MS. Standard High Volume Instrument (HVI) tests for length, strength, elongation, micronaire and uniformity index was conducted for all samples submitted. HVI lint parameters are:
- Micronaire (mic): a measure of air permeability in compressed cotton fibers. This parameter indicates cell wall thickness (higher values) and lint fineness (lower values).
- Length: average length (in inches) of upper 50% of fiber lengths.
- Uniformity Index: ratio between Length and the mean length (in inches) of fibers. This parameter is also known as Length Uniformity.
- Strength: tensile strength of lint reported in grams (of force) per tex. A tex is the weight of 1,000 meters of fiber in grams.
- Elongation: the percent of maximum length extension applied during the strength test.

- Plant Mapping: Five representative plants per plot were selected for plant mapping BBCH 85 to 99 and within one month of harvest. Each node on the plants' main stems were categorized as "empty," "vegetative branch," "fruiting branch boll," or "missing boll" (*i.e.* bud scar), or "last node" (*i.e.* terminal bud). Numbers of vegetative bolls were counted on vegetative branches, and secondary fruiting positions (fruiting branch boll or missing boll) on lateral fruiting branches were noted. Fruiting branch bolls were also characterized as "harvestable," "normal (not open)," or "abnormal" (*e.g.* diseased or damaged). Height was also measured. These notations captured the following information:
  - Plant Height, Number of Nodes, and Height to Node Ratio:
    - Height from the cotyledonary node to the terminal (in cm). The cotyledonary node is distinguished by bud scars that are of opposite positioning on the stem (other branches follow an alternate pattern of positioning).
    - Number of Nodes: The number of nodes from the node above the cotyledon to the terminal bud.
    - Height to Node Ratio: Height (in cm) divided by number of nodes.
  - Branch Assessments:
    - First Fruiting Branch: Lowest node (counted up from cotyledonary node) containing a fruiting branch.
    - Number of Vegetative Branches: Count of vegetative branches. Vegetative branches are distinguishable from fruiting branches by morphological differences including longer internode length and an appearance of succulence.
  - Boll (Fruit) Counts:
    - Number of Fruiting Branch Bolls: Count of bolls borne on fruiting branches.
    - Number of Vegetative Bolls: Count of bolls borne on vegetative branches.
    - Number of Bolls: Sum counts of fruiting branch bolls and vegetative branch bolls.

- Fruiting Site Descriptions
  - Number of Potential Fruiting Sites: Sum of fruiting branch bolls and missing bolls (bud scars).
  - Percent Fruit Retention: Number of Fruiting Branch Bolls divided by Number of Potential Fruiting Sites, expressed as a percent.
  - Percent Harvestable Fruiting Branch Bolls: Number of harvestable (i.e. normal and open) fruiting branch bolls divided by Number of Fruiting Branch Bolls.

Categorical Parameters:

- Stressor Ratings: Independent assessments of insect, disease and abiotic stress based on a 1-9 scale. In cases where pest, disease or abiotic stressors were present, the most prevalent stressors were noted at the time of rating. Scale designations were as follows:

  1: Little to no stressor present

  3: Stressor present but symptoms are light or patchy and effect on yield and plant growth are likely negligible

  5: Stressor symptoms apparent and more consistent through the plot; effects on yield and plant growth somewhat uncertain but certainly possible

  7: Stressor symptoms are obvious; likely to affect yield/quality

  9: Stressor symptoms are severe; crop damage and yield loss are certain and significant

  Point ratings 2, 4, 6, and 8 were used for borderline plots.

  Four stressor ratings were made during the growing season at each site:

  - 1st Stressor Rating: Two leaf-stage to floral initiation (BBCH 12 to 52)
  - 2nd Stressor Rating: Floral bud enlargement to peak bloom (BBCH 54 to 65)
  - 3rd Stressor Rating: Flowering (BBCH 61 to 69)
  - 4th Stressor Rating: Boll maturation (BBCH 81 to 89)

- Boll Type: At maturity and within three weeks of harvest, a rating which indicates the tightness of the lint in bolls on the cotton plants. Boll Type relates to ease of harvest (lower ratings), and is analogous to potential seed loss especially in windy areas.Each plot was rated 1 to 9 based on the following scale:

  1: Loose in boll: >80% loose lint
  2: >70% loose lint
  3: >60% loose lint
  4: >50% loose lint
  5: Intermediate: >40% loose lint;
  6: >30% loose lint
  7: >20 % loose lint
  8: >10 % loose lint
  9: Stormproof: no loose lint in any bolls

- Plant Lodging: The amount of lodging at BBCH 81 to 99 prior to harvest. A rating of 1 to 9 was assigned to each plot based on the following scale:

  1: <10% of plants show lodging or mildly lodged
  2: 11 – 20% of plants show lodging
  3: 21 – 30% of plants show lodging
  4: 31 – 40% of plants show lodging
  5: 41 – 50% of plants show lodging
  6: 51 – 60% of plants show lodging
  7: 61 – 70% of plants show lodging
  8: 71 – 80% of plants show lodging
  9: >80% of plants show lodging

Statistical Analysis

Agronomic observations for the non-GM counterpart (Coker 312) (Entry A) were compared to GHB811 cotton not treated with IFT and GLY (Entry J), and also compared to GHB811 cotton treated with IFT and GLY (Entry K).

All data management and statistical analyses were conducted using SAS version 9.3.

Descriptive statistics for the continuous agronomic parameters are summarized in Table 8.6 for the combined site analysis. Comparative assessments between the different entries (A vs J and A vs K) were performed using a mixed model analysis of variance:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta_{k(j)} + \varepsilon_{ijk}$$

where Y_{ijk} is the individual value measurement, μ the overall mean, α_i the fixed effect associated with entry, β_j the random effect associated with site, $\alpha\beta_{ij}$ the random effect for the interaction of entry by site, $\delta_{k(j)}$ the random effect associated with block nested within site and ε_{ijk} the random error.Based on the mixed model, entry differences (A vs J and A vs K) were estimated and presented with 95% confidence intervals, along with the *p*-values (*t*-test) for the entry differences. Statistical significance was evaluated at $p<0.05$ level. Studentized residuals based on the mixed model were visually checked for model assumptions, including normality. Overall no serious departure from model assumptions was observed for majority of the parameters. A small proportion of the parameters show a moderate departure from normality, in which situation the use of the mixed model is still valid based on the robustness of analysis of variance to moderate departures from normality of the error distribution (Schabenberger and Pierce*,* 2002; Jacqmin-Gadda *et al.,* 2007). In addition, tolerance intervals for continuous parameters, specified to contain 99% of the population with 95% confidence, were calculated for the reference varieties (Entries B, C, D, E, F, G, and N) over all sites in Table 8.6.

Descriptive statistics for the categorical parameters are summarized in Table 8.7. For the categorical data the Cochran-Mantel-Haenszel test (CMH-test) (Breslow and Day, 1980) was used to compare the different entries (A vs J and A vs K). The CMH test is a method to analyze categorical data with adjustment for sites. Mean ± SD were listed in Table 8.7 to help with the interpretation since the rating scores have meaningful order (ordinal categorical data), however the means were not used to conduct CMH test. Instead the CMH test was

based on counts and proportions, and the related outputs are provided in Table 8.8. Statistical significance was evaluated at $p<0.05$ level.

The combined site summary of the statistical analysis for the 31 continuous agronomic parameters is presented in Table 8.6. Statistically significant differences were detected for the continuous parameters Final Stand Count, Seed Cotton Yield, Lint Yield, and Height to Node Ratio between the non-GM counterpart (Coker 312) and GHB811 cotton not treated with trait-specific herbicides. Statistically significant differences were also detected for Boll Weight between the non-GM counterpart and both GHB811 cotton entries (treated and not treated). All mean values of the continuous agronomic parameters of GHB811 cotton (treated or not treated) were within the range of means of the reference varieties with the exception of Boll Weight. Boll Weight for both GHB811 cotton entries were within the overall range of values for reference varieties and tolerance intervals, but fell below the range of means for the reference varieties. To further explore biological relevance of this difference, literature values for Boll Weight were explored. The CottonGen database provides Boll Weight values for cotton (*G. hirsutum*) ranging from 0.1 to 63 g/boll (Yu *et al.,* 2014), demonstrating the wide variability of Boll Weight within the *G. hirsutum* species. Values for Coker 312 are not specifically provided in the database, however comparable varieties in the Coker pedigree have values ranging from 3.7 to 7.5 g/boll (Yu *et al.,* 2014). Thus, statistically significant differences were considered not biologically relevant (Table 8.6).

The combined site summary of statistical results for the categorical parameters of Boll Type, Plant Lodging, four insect stressor ratings, four disease stressor ratings, and four abiotic stressor ratings are presented in Table 8.7 and Table 8.8. No statistically significant differences, as defined by CMH test *p*-values <0.05, were detected for thirteen of the fourteen categorical parameters. Statistically significant differences were observed for the third disease stressor rating between the non-GM counterpart and both GHB811 cotton entries (treated and not treated). All mean values for GHB811 cotton (treated or not treated) in the third disease stressor rating fell within the range of the reference varieties and thus statistically significant differences were considered not biologically relevant (Table 8.7). The prevalent abiotic and biotic stressors at each evaluation period and site are provided in Table 8.9. Treated and not treated GHB811 plots, non-GM Coker 312 comparator, and the reference varieties, qualitatively, interacted with the abiotic and biotic environment in a comparable manner.

Based on the agronomic assessment, GHB811 cotton demonstrated no biologically relevant differences from the non-GM counterpart and showed equivalent agronomic performance to non-GM reference varieties.

Table 8.6. Summary of continuous agronomic parameters

Parameter	Non-GM Conventional Counterpart (Entry A)	GHB811 Not Treated (Entry J)	GHB811 Treated (Entry K)	Non-GM Reference Varieties Range[a]	Non-GM Reference Varieties Mean Range (Entries B-G,N) [a]	Tolerance Interval Non-GM Reference Varieties[b]	Comparison *t*-test A vs J [c]	Comparison *t*-test A vs K [c]
	Mean ± SD	Mean ± SD	Mean ± SD	(Min-Max)	(Min-Max)	(Lower-Upper)	*p*-value	*p*-value
Stand Count 1 (Plants/ m²)	11.03 ± 2.37	10.81 ± 2.38	10.71 ± 2.17	3.70 - 14.97	9.08 - 12.34	4.28 - 16.36	0.505	0.346
Stand Count 2 (Plants/ m²)	11.26 ± 2.01	10.83 ± 2.07	10.72 ± 2.01	2.59 - 14.83	9.11 - 12.26	4.35 - 16.47	0.274	0.175
Percent Ground Cover	57.98 ± 16.66	54.41 ± 14.66	56.55 ± 15.65	23.33 - 86.67	47.08 - 60.56	4.08 - 102.35	0.198	0.602
Days to 10% flower	55.8 ± 5.8	56.1 ± 5.7	56.2 ± 5.7	48 - 71	54.7 - 64.5	41.7 - 72.8	0.432	0.406
Heat Units to 10% flower (°C)	639.3 ± 76.2	643.8 ± 75.5	645.1 ± 77.3	530.7 - 786.1	613.5 - 772.1	450.0 - 863.6	0.513	0.330
Days to First Open Bolls	108.7 ± 12.5	109.1 ± 12.8	109.3 ± 12.6	90 - 141	107.7 - 114.8	70.9 - 150.8	0.488	0.371
Heat Units to First Open Bolls (°C)	1191.2 ± 63.4	1196.2 ± 69.4	1198.1 ± 70.7	1083.0 - 1341.3	1168.3 - 1251.8	1027.5 - 1379.7	0.458	0.288
Percent Open Bolls	56.5 ± 7.5	50.6 ± 12.8	52.6 ± 13.0	10 - 80	41.3 - 57.8	13.3 - 83.9	0.060	0.199
Final Stand Count (Plants/ m²)	11.07 ± 3.04	10.11 ± 2.90	10.61 ± 2.62	5.77 - 18.13	10.03 - 12.31	3.53 - 18.34	0.035	0.300
Hundred Seed Weight (g)	11.6 ± 1.2	11.6 ± 1.4	11.4 ± 1.3	8.3 - 15.1	9.5 - 12.5	6.9 - 14.6	0.758	0.277
Boll Weight (g)	5.3 ± 0.8	5.0 ± 0.7	5.1 ± 0.8	3.5 - 7.8	5.2 - 6.1	3.3 - 7.9	0.008	0.011
Number of Seeds per Boll	27.0 ± 3.7	26.4 ± 3.6	26.7 ± 3.9	21.1 - 42.4	23.5 - 31.9	20.3 - 39.0	0.251	0.668
Total Seed Cotton Yield (kg/ha)	3053 ± 1188	2789 ± 1205	2989 ± 1235	398 - 5356	1816 - 3510	0 - 6624	0.018	0.578
Percent Lint	39.0 ± 3.1	38.3 ± 4.1	38.4 ± 3.2	32 - 53	40.4 - 45.0	33.3 - 49.7	0.069	0.143
Lint Yield (kg/ha)	1198 ± 491	1070 ± 501	1152 ± 505	172 - 2353	803 - 1436	0 - 2781	0.010	0.346
Average Plant Height (cm)	84.0 ± 20.5	86.4 ± 22.3	83.8 ± 21.0	34.8 - 123.2	55.1 - 92.8	24.0 - 141.8	0.099	0.991
First Fruiting Branch	5.38 ± 1.34	5.33 ± 1.31	5.21 ± 1.22	1.0 - 8.8	4.65 - 6.29	1.22 - 10.35	0.642	0.208
Number of Bolls	9.5 ± 3.2	10.2 ± 3.9	10.0 ± 3.9	4.0 - 21.2	7.7 - 11.5	0 - 21.5	0.124	0.324
Number of Fruiting Branch Bolls	8.3 ± 2.3	8.5 ± 2.8	8.3 ± 2.4	4.0 - 15.4	6.2 - 9.5	1.4 - 16.1	0.417	0.931
Number of Potential Fruiting Sites	19.1 ± 4.9	18.9 ± 5.1	18.4 ± 3.8	8.8 - 33.0	12.6 - 20.4	4.4 - 32.2	0.810	0.339

Parameter	Non-GM Conventional Counterpart (Entry A)	GHB811 Not Treated (Entry J)	GHB811 Treated (Entry K)	Non-GM Reference Varieties Range[a]	Non-GM Reference Varieties Mean Range (Entries B-G,N) [a]	Tolerance Interval Non-GM Reference Varieties[b]	Comparison *t*-test A vs J [c]	Comparison *t*-test A vs K [c]
	Mean ± SD	Mean ± SD	Mean ± SD	(Min-Max)	(Min-Max)	(Lower-Upper)	*p*-value	*p*-value
Number of Vegetative Bolls	1.23 ± 1.51	1.64 ± 1.84	1.64 ± 2.20	0.0 - 10.8	1.19 - 2.48	0 - 7.52	0.122	0.138
Number of Vegetative Branches	1.69 ± 1.32	1.84 ± 1.26	1.65 ± 1.14	0.0 - 7.2	1.30 - 3.18	0 - 6.65	0.340	0.645
Percent Fruit Retention	45.2 ± 13.4	46.7 ± 15.2	46.2 ± 13.3	23.0 - 88.6	45.2 - 54.6	10.7 - 87.8	0.328	0.782
Percent Harvestable Fruiting Branch Bolls	82.3 ± 14.8	78.3 ± 18.1	83.1 ± 18.1	0.0 - 100.0	67.4 - 85.4	19.1 - 135.9	0.100	0.809
Average Node Count	17.3 ± 3.0	17.2 ± 3.1	17.1 ± 2.6	7.8 - 26.8	15.0 - 18.4	8.8 - 26.4	0.733	0.488
Height to Node Ratio	4.86 ± 1.00	5.02 ± 1.02	4.90 ± 1.10	2.31 - 7.54	3.65 - 5.21	1.61 - 7.90	0.018	0.516
Lint Length	1.23 ± 0.05	1.24 ± 0.05	1.24 ± 0.05	1.01 - 1.29	1.13 - 1.18	1.01 - 1.30	0.075	0.055
Lint Micronaire	4.90 ± 0.49	4.80 ± 0.58	4.82 ± 0.53	3.5 - 6.3	4.61 - 5.16	3.56 - 6.21	0.135	0.214
Lint Elongation (%)	7.43 ± 0.93	7.30 ± 0.98	7.41 ± 1.09	4.5 - 12.3	5.45 - 10.22	3.34 - 13.88	0.372	0.922
Lint Strength (g/tex)	32.8 ± 1.7	32.4 ± 1.5	32.6 ± 1.4	25.9 - 38.6	31.7 - 34.7	26.5 - 39.3	0.230	0.492
Lint Uniformity Index	84.7 ± 1.2	84.3 ± 1.2	84.6 ± 1.4	78.7 - 87.2	83.2 - 84.8	80.3 - 88.0	0.090	0.672

[a] Range of results from seven reference varieties lines (Entries B, C, D, E, F, G, and N).

[b] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.

[c] *t*-Test *p*-value: Pairwise comparison to the non-GM conventional counterpart (Entry A).

Table 8.7. Summary of categorical agronomic parameters

Timing of Rating	Parameter	Non-GM Conventional Counterpart (Entry A) Mean ± SD	GHB811 Not Treated (Entry J) Mean ± SD	GHB811 Treated (Entry K) Mean ± SD	Non-GM Reference Varieties Range[a] (Min-Max)	A vs J CMH-Test *p*-value[b]	A vs K CMH-Test *p*-value[b]
Rating 1 BBCH 12-52	Abiotic Stressor (1-9)	1.6 ± 1.2	1.6 ± 1.2	1.5 ± 1.2	1 - 5	1.000	0.317
	Disease Stressor (1-9)	1.3 ± 1.0	1.3 ± 1.0	1.3 ± 1.0	1 - 5	0.980	0.980
	Insect Stressor (1-9)	1.9 ± 1.2	1.9 ± 1.2	2.0 ± 1.2	1 - 5	1.000	0.285
Rating 2 BBCH 54-65	Abiotic Stressor (1-9)	1.4 ± 1.3	1.5 ± 1.3	1.3 ± 0.6	1 - 8	0.811	0.165
	Disease Stressor (1-9)	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1 - 2	0.317	0.317
	Insect Stressor (1-9)	2.2 ± 1.1	2.2 ± 1.1	2.2 ± 1.1	1 - 4	0.247	0.074
Rating 3 BBCH 61-69	Abiotic Stressor (1-9)	1.3 ± 1.2	1.3 ± 1.1	1.0 ± 0.0	1 - 8	1.000	0.072
	Disease Stressor (1-9)	1.5 ± 1.1	1.4 ± 1.1	1.4 ± 1.1	1 - 5	0.022	0.022
	Insect Stressor (1-9)	2.2 ± 1.4	2.2 ± 1.4	2.2 ± 1.4	1 - 5	0.364	1.000
Rating 4 BBCH 81-89	Abiotic Stressor (1-9)	1.5 ± 1.4	1.5 ± 1.4	1.4 ± 1.2	1 - 6	0.910	0.268
	Disease Stressor (1-9)	1.9 ± 1.7	2.0 ± 1.8	2.0 ± 1.8	1 - 7	0.495	0.747
	Insect Stressor (1-9)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1 - 2	0.317	0.317
Maturity and within 3 weeks of harvest	Boll Type (1-9)	5.9 ± 1.6	6.1 ± 1.5	6.0 ± 1.6	1 - 9	0.128	0.417
BBCH 81-89	Plant Lodging (1-9)	1.2 ± 0.5	1.1 ± 0.4	1.2 ± 0.6	1 - 5	0.463	0.498

[a] Range of results from seven reference varieties (Entries B, C, D, E, F, G, and N).

[b] Cochran-Mantel-Haenszel Test *p*-value: comparison to the non-GM conventional counterpart (Entry A).

Table 8.8. Analysis of categorical agronomic parameters

					p-value	
		Entry			CMH-test	
Parameter	Rating	A N (%)	J N (%)	K N (%)	A vs J	A vs K
Abiotic Stressor Rating 1 (1-9)	1	46 (78.0%)	47 (78.3%)	48 (80.0%)	1.000	0.317
	3	9 (15.3%)	9 (15.0%)	8 (13.3%)		
	5	4 (6.8%)	4 (6.7%)	4 (6.7%)		
Disease Stressor Rating 1 (1-9) #	1	55 (93.2%)	56 (93.3%)	56 (93.3%)	0.980	0.980
	5	4 (6.8%)	4 (6.7%)	4 (6.7%)		
Insect Stressor Rating 1 (1-9)	1	33 (55.9%)	34 (56.7%)	33 (55.0%)	1.000	0.285
	2	4 (6.8%)	4 (6.7%)	3 (5.0%)		
	3	18 (30.5%)	18 (30.0%)	20 (33.3%)		
	5	4 (6.8%)	4 (6.7%)	4 (6.7%)		
Abiotic Stressor Rating 2 (1-9)	1	48 (81.4%)	49 (81.7%)	50 (83.3%)	0.811	0.165
	2	5 (8.5%)	4 (6.7%)	5 (8.3%)		
	3	4 (6.8%)	4 (6.7%)	5 (8.3%)		
	5	0	1 (1.7%)	0		
	7	1 (1.7%)	2 (3.3%)	0		
	8	1 (1.7%)	0	0		
Disease Stressor Rating 2 (1-9)	1	59 (100.0%)	59 (98.3%)	59 (98.3%)	0.317	0.317
	2	0	1 (1.7%)	1 (1.7%)		
Insect Stressor Rating 2 (1-9)	1	24 (40.7%)	24 (40.0%)	24 (40.0%)	0.247	0.074
	2	5 (8.5%)	5 (8.3%)	4 (6.7%)		
	3	26 (44.1%)	26 (43.3%)	26 (43.3%)		
	4	4 (6.8%)	5 (8.3%)	6 (10.0%)		
Abiotic Stressor Rating 3 (1-9)	1	56 (94.9%)	57 (95.0%)	60 (100.0%)	1.000	0.072
	3	1 (1.7%)	0	0		
	5	0	1 (1.7%)	0		
	6	0	1 (1.7%)	0		
	7	1 (1.7%)	1 (1.7%)	0		
	8	1 (1.7%)	0	0		
Disease Stressor Rating 3 (1-9)	1	48 (81.4%)	51 (85.0%)	51 (85.0%)	0.022	0.022
	2	1 (1.7%)	1 (1.7%)	1 (1.7%)		
	3	7 (11.9%)	4 (6.7%)	4 (6.7%)		
	5	3 (5.1%)	4 (6.7%)	4 (6.7%)		
Insect Stressor Rating 3 (1-9)	1	28 (47.5%)	30 (50.0%)	29 (48.3%)	0.364	1.000
	2	6 (10.2%)	6 (10.0%)	6 (10.0%)		
	3	13 (22.0%)	12 (20.0%)	13 (21.7%)		
	4	8 (13.6%)	8 (13.3%)	8 (13.3%)		
	5	4 (6.8%)	4 (6.7%)	4 (6.7%)		

Entry A: Non-GM conventional counterpart, not treated with trait-specific herbicides
Entry J: GHB811, not treated with trait-specific herbicides
Entry K: GHB811, treated with trait-specific herbicides
CMH-test: Cochran-Mantel-Haenszel test
#) Adjusting for sites was not possible due to singular covariance matrices

Table 8.8. Analysis of Categorical Agronomic Parameters (continued)

					p-value	
		Entry			CMH-test	
Parameter	Rating	A N (%)	J N (%)	K N (%)	A vs J	A vs K
Abiotic Stressor Rating 4 (1-9)	1	50 (84.7%)	50 (83.3%)	54 (90.0%)	0.910	0.268
	2	0	1 (1.7%)	0		
	3	4 (6.8%)	6 (10.0%)	3 (5.0%)		
	4	1 (1.7%)	0	0		
	5	1 (1.7%)	0	1 (1.7%)		
	6	1 (1.7%)	0	0		
	7	2 (3.4%)	3 (5.0%)	2 (3.3%)		
Disease Stressor Rating 4 (1-9)	1	39 (70.9%)	38 (67.9%)	39 (69.6%)	0.495	0.747
	2	4 (7.3%)	4 (7.1%)	3 (5.4%)		
	3	5 (9.1%)	6 (10.7%)	6 (10.7%)		
	5	4 (7.3%)	4 (7.1%)	4 (7.1%)		
	7	3 (5.5%)	4 (7.1%)	4 (7.1%)		
Insect Stressor Rating 4 (1-9)	1	52 (88.1%)	52 (86.7%)	52 (86.7%)	0.317	0.317
	2	7 (11.9%)	8 (13.3%)	8 (13.3%)		
Boll Rating (1-9)	3	4 (6.8%)	5 (8.3%)	3 (5.0%)	0.128	0.417
	4	8 (13.6%)	5 (8.3%)	6 (10.0%)		
	5	14 (23.7%)	7 (11.7%)	18 (30.0%)		
	6	12 (20.3%)	18 (30.0%)	12 (20.0%)		
	7	6 (10.2%)	10 (16.7%)	5 (8.3%)		
	8	15 (25.4%)	15 (25.0%)	13 (21.7%)		
	9	0	0	3 (5.0%)		
Lodged Plants (1-9)	1	50 (84.7%)	53 (88.3%)	50 (83.3%)	0.463	0.498
	2	7 (11.9%)	6 (10.0%)	6 (10.0%)		
	3	2 (3.4%)	1 (1.7%)	4 (6.7%)		

Entry A: Non-GM conventional counterpart, not treated with trait-specific herbicides
Entry J: GHB811, not treated with trait-specific herbicides
Entry K: GHB811, treated with trait-specific herbicides
CMH-test: Cochran-Mantel-Haenszel test

Table 8.9. Most prevalent abiotic and biotic stressors observed in field trials

Site	Growth stage[1]	Most prevalent stressors[2]	
		Abiotic	Biotic
Site 2 – Wharton County, TX 2014	**BBCH 12 to 52**	No stress observed	No stress observed
	BBCH 54 to 65	No stress observed	No stress observed
	BBCH 61 to 69	No stress observed	Alternaria leaf spot
	BBCH 81 to 89	No stress observed	Alternaria leaf spot
Site 3 – Fresno County, CA 2014	**BBCH 12 to 52**	No stress observed	No stress observed
	BBCH 54 to 65	No stress observed	No stress observed
	BBCH 61 to 69	No stress observed	No stress observed
	BBCH 81 to 89	No stress observed	No stress observed
Site 7 – Tift County, GA 2014	**BBCH 12 to 52**	No stress observed	No stress observed
	BBCH 54 to 65	No stress observed	Armyworms
	BBCH 61 to 69	No stress observed	Bollworms
	BBCH 81 to 89	No stress observed	No stress observed
Site 8 – Crittenden County, AR 2014	**BBCH 12 to 52**	Excess moisture	Damping off, Thrips
	BBCH 54 to 65	Drought	Leaf spot, plant bugs
	BBCH 61 to 69	Drought	Leaf spot, bollworms
	BBCH 81 to 89	Drought	Bollworm, boll rot – cause undefined
Site 9 – Rapides County, LA 2014	**BBCH 12 to 52**	Drought	Fusarium, lygus
	BBCH 54 to 65	Drought	Fusarium, lygus
	BBCH 61 to 69	Drought	Lygus, boll rot – cause undefined
	BBCH 81 to 89	Wind	Bollworm, boll rot – cause undefined
Site 10 – Washington County, MS 2014	**BBCH 12 to 52**	Heat	Rhizoctonia, thrips
	BBCH 54 to 65	Excess moisture	Foliar leaf spots, tarnished plant bugs
	BBCH 61 to 69	Heat	Leaf spots, plant bugs
	BBCH 81 to 89	Excess moisture	Stinkbugs, boll rot – cause undefined
Site 11 – Barnwell County, SC 2014	**BBCH 12 to 52**	Heat	Beet armyworms, seedling diseases (Rhizoctonia, Pythium)
	BBCH 54 to 65	No stress observed	Beet armyworm damage but pest not present and plants recovering
	BBCH 61 to 69	No stress observed	Bollworms, stinkbugs
	BBCH 81 to 89	No stress observed	Boll rot – cause undefined

Site	Growth stage[1]	Most prevalent stressors[2]	
		Abiotic	Biotic
Site 13 – Tift County, GA 2015	**BBCH 12 to 52**	No stress observed	Thrips
	BBCH 54 to 65	Heat	Beet armyworms
	BBCH 61 to 69	No stress observed	Bollworms
	BBCH 81 to 89	Potassium deficiency observed among rows of plots for non-GM Coker 312 (Entry A), reference variety ST468 (Entry F), GHB811 not treated (Entry J), GHB811 treated (Entry K).	No stress observed
Site 14 – Crittenden County, AR 2015	**BBCH 12 to 52**	Cool and wet	Damping off, thrips
	BBCH 54 to 65	Drought	Leaf spot, lygus
	BBCH 61 to 69	Heat	Verticillium wilt, lygus
	BBCH 81 to 89	Drought	Leaf spot, lygus
Site 15 – Tom Green County, TX 2015	**BBCH 12 to 52**	No stress observed	No stress observed
	BBCH 54 to 65	No stress observed	No stress observed
	BBCH 61 to 69	No stress observed	Verticillium wilt unevenly distributed across plots and entries. Non-GM Coker 312 (Entry A) showed stress in all plots. All other entries, with the exception of reference variety FM966 (Entry N), showed verticillium wilt stress in one plot. No stress was observed for FM966.
	BBCH 81 to 89	No stress observed	Verticillium wilt detected in one plot of reference variety FM966 (Entry N)
Site 16 – Washington County, MS 2015	**BBCH 12 to 52**	Heat	Damping off, aphids
	BBCH 54 to 65	Heat	Cotton leaf spot, tarnished plant bug
	BBCH 61 to 69	No stress observed	Whiteflies, aphids present in all treatments. Some variability whether one or both pests present in plots of some treatments.
	BBCH 81 to 89	Nutrient deficiency	Leaf spot, stinkbugs
Site 17 – Perquimans County, NC 2015	**BBCH 12 to 52**	Heat, drought	Damping off, lygus, thrips
	BBCH 54 to 65	Heat, drought	Leaf spot, stinkbug, lygus
	BBCH 61 to 69	Heat, drought	Leaf spot, stinkbug, lygus
	BBCH 81 to 89	Heat, drought, nutrient deficiency	Leaf spot, stinkbug, bollworm

Site	Growth stage[1]	Most prevalent stressors[2]	
		Abiotic	Biotic
Site 18 – Carson County, TX 2015	**BBCH 12 to 52**	Heat, wind, excess moisture	Fusarium, verticilium, leaf blight, thrips, leafhopper, armyworm
	BBCH 54 to 65	Heat, hail, wind	Fusarium, leaf spot, phytophthora, armyworm, leaf hopper, cabbage looper
	BBCH 61 to 69	Heat, hail, wind	Bacterial leaf blight, fusarium, verticillium, bollworm complex, aphid, looper
	BBCH 81 to 89	Heat, hail, wind	Bacterial leaf blight, fusarium, verticillium, bollworm complex, aphid, looper
Site 20 – Waller County, TX 2015	**BBCH 12 to 52**	Excess moisture	Thrips
	BBCH 54 to 65	Moisture stress	Leaf spot, general insect pest stress
	BBCH 61 to 69	Moisture stress	Stemphylium, stinkbugs
	BBCH 81 to 89	Nutrient deficiency	Stemphylium, aphids
Site 21 – Hale County, TX 2015	**BBCH 12 to 52**	Excess moisture	Aschycota leaf blight, thrips (ladybugs also noted in each plot)
	BBCH 54 to 65	Wind, hail, excess moisture	Aschycota leaf blight, verticillium wilt, fleahoppers (Orius, beneficicial insect, also noted in each plot)
	BBCH 61 to 69	No stress observed	Verticillium, cotton aphids
	BBCH 81 to 89	No stress observed	No stress observed

[1]BBCH 12 to 52 = Two-leaf stage to floral initiation; BBCH 54 to 65 = Floral bud enlargement to peak bloom; BBCH 61 to 69 = Flowering; BBCH 81 to 89 = Boll maturation

[2]Unless otherwise described in the table, all stressors were observed evenly across field trial entries and plots.

8.3. Seed germination and dormancy evaluation

To compare the germination potential of GHB811 cotton to that of the non-genetically modified (non-GM) counterpart (Coker 312) cotton, the warm-cold germination test was conducted in a growth chamber. The GHB811 seed and non-GM Coker 312 seed used for the germination test were produced under identical field conditions.

For warm germination, GHB811 cotton had an overall germination percentage of 96.25% while Coker 312 had an overall germination percentage of 95.50% (Table 8.10). In the cold germination test, GHB811 had an overall germination percentage of 94.00% while Coker 312 had an overall germination percentage of 94.50% (Table 8.11).

Evaluation of the germination potential of GHB811 cotton compared to the non-GM counterpart, Coker 312 cotton, demonstrated that there was no significant difference in the germination potential of the two genotypes under the two germination conditions.

Table 8.10. Comparison of germination categories between GHB811 and Coker312 in the warm germination test

Genotype	Category	Count	Percent	p-value [a]
Coker 312	Abnormal/Un-germinated	18	4.50	
Coker 312	Normal	382	95.50	
GHB811	Abnormal/Un-germinated	15	3.75	
GHB811	Normal	385	96.25	
				0.594

[a] Chi-square Test p-value for comparison of germination categories between GHB811 and Coker312 in the Warm Germination Test

Table 8.11. Comparison of germination categories between GHB811 and Coker312 in the cold germination test

Genotype	Category	Count	Percent	p-value [a]
Coker 312	Abnormal/Un-germinated	11	5.50	
Coker 312	Normal	189	94.50	
GHB811	Abnormal/Un-germinated	12	6.00	
GHB811	Normal	188	94.00	
				0.830

[a] Chi-square Test p-value for comparison of germination categories between GHB811 and Coker312 in the Cold Germination Test

9. Environmental safety and impact on agronomic practices

9.1. Environmental safety

Persistence, weediness and invasiveness

Cotton is a domesticated crop that requires human intervention to survive in non-cotton production areas. It has been grown for centuries throughout the world without any reports of being a serious weed pest (OGTR, 2013). Also, cotton has no relatives that are considered as problematic weeds. Modern cotton cultivars and varieties do not possess any of the attributes normally associated with weediness such as seed dormancy, persistence in soil, rapid vegetative growth, short life cycle, or seed dispersal mechanisms (OECD, 2008; OGTR, 2013).

In addition, the comparative assessment (section 8, Agronomic and phenotypic evaluation) has confirmed the substantial equivalence of GHB811 to its non-GM counterpart for all phenotypic and agronomic parameters except the predicted traits, herbicide tolerance to glyphosate and to HPPD inhibitors, such as isoxaflutole, confirming that it is very unlikely that GHB811 plants would be more persistent or would present different weed-related characteristics than the conventional counterpart. Should volunteer GHB811 cotton occur in years subsequent to cultivation there are multiple options for control including chemical and mechanical methods which vary based on the crop in which the volunteers occur (Morgan *et al.*, 2011a; Morgan *et al.*, 2011b).

In conclusion there are no differences between GHB811 and its non-GM counterpart that could contribute to increased weediness potential of GHB811.

Gene flow and its consequence

In plants, genetic material may escape from an area by seed or by pollen. Movement of seed from the area is mainly executed by human activity during harvest or transport. Cotton was originally a woody perennial tree, which has been domesticated and converted to an annual crop; it is harvested and planted annually and is not considered to have weedy characteristics. Seeds are the only survival structures. Wild species of cotton generally have a fairly high percentage of "hard seed", *i.e.* seed that survives one or more generations before germination. This is a positive survivability mechanism in wild cotton, and plant breeders have bred it out of modern cultivars. Cultivated cotton is not considered to have seed which can persist in the environment for long periods of time: cottonseed is not dormant and looses viability quickly under moist conditions. If incorporated before the soil temperature reaches 15°C, it is likely to rot in the soil. Following germination, the seedling is relatively “tender” and may not be able to push its way through the soil and emerge if the soil layer above the seed is too hard (Munro, 1987; Oosterhuis and Jernstedt, 1999).

Movement of genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to those allotetraploids with AADD genomes. In the United States, this would only include *G. hirsutum*, *G. barbadense* and *G. tomentosum*. Movement to *G. hirsutum* and *G. barbadense* is possible if suitable insect pollinators are present and if there is a short distance from plant to plant. Physical barriers, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement.

Cotton pollen is very large (between 120 and 200 µm), heavy and sticky, and thus not wind-borne. The pollen can be transferred by various wild bees (*Mellisodes*), bumble bees (*Bombus* spp.), as well as honey bees (*Apis mellifera*), or even Coleoptera (OGTR, 2013). The rate of cross-pollination varies with the insect pollinator population and declines rapidly as distance increases from the pollen source.

Extensive use of insecticides for control of insect pests will essentially limit the extent of cross-pollination. However as the advent of Bt cotton has reduced insecticide applications (Sankula, 2006), pollinator activity in fields may have increased in recent years (van Deynze, *et al*., 2005). Nevertheless recent studies (Heuberger *et al*., 2010) showed most cross-pollinations involve plants situated in close vicinity (rate below 1% at 10 m).

Therefore, the likelihood of gene flow between GHB811 cotton and other cotton varieties, although possible, is minimal due to its physiological characteristics and various environmental and biological factors.

Potential selective advantage to wild relatives

GHB811 cotton produces the 4-hydroxyphenylpyruvate dioxygenase protein (HPPD W336) and the 5-enolpyruvylshikimate 3-phosphate synthase protein (2mEPSPS), which confer tolerance to HPPD inhibitors (*e.g*., isoxaflutole) and glyphosate herbicides, respectively. Any GHB811 cotton plant that would germinate would only have a selective advantage in those cases where the herbicides glyphosate and/or HPPD inhibitiors, such as isoxaflutole are used.

Cotton (*Gossypium hirsutum*) is largely considered as a self-pollinating plant. Cross-pollination can occur, albeit at relatively low incidence rates, mainly through the activity of pollinating insects. Pollen and flower characteristics, relatively short pollen viability times, and lack of wild or weedy cotton species in geographical proximity to commercial cotton production greatly limit chances of outcrossing among any *Gossypium* species.

Therefore, the likelyhood of gene flow between GHB811 cotton and other cotton varieties, although possible, is minimal due to its physiological characteristics and various environmental and biological factors and there is no selective advantage for cotton varieties with GHB811 in the natural environment. If gene flow would occur, resulting hybrids could be controlled with commercially available herbicides (*e.g.,* glufosinate ammonium, other herbicides controlling cotton).

Potential for horizontal gene transfer

Scientific evidence to date supports that horizontal gene transfer (HGT) is an extremely rare event in the environment under natural conditions (Nielsen *et al*., 1997; Keese, 2008).

The factors affecting the potential for HGT between genetically modified (GM) plants and microorganisms in the environment or in the gastrointestinal system have been extensively studied. Successful gene transfer via transformation requires many factors including available free DNA, the development of competent bacteria, gene sequence homology between plant and bacterial DNA, stable integration of DNA from plant cells into the bacterial genome, and a strong selective pressure (Keese, 2008) which are highly unlikely to be encountered under field conditions. Transformation frequencies (the frequency of foreign

DNA incorporation into the microbial genome) likely to be encountered in the field are exceedingly low, representing environmental significance only on an evolutionary time scale.

Altered disease/pest susceptibility

Biotic (disease and insect) stressors were evaluated in the U.S. during the 2014 and 2015 growing seasons (Section 8. Agronomic and phenotypic assessment).

1st Stressor Rating: Two leaf-stage to floral initiation (BBCH 12 to 52)

2nd Stressor Rating: Floral bud enlargement to peak bloom (BBCH 54 to 65)

3rd Stressor Rating: Flowering (BBCH 61 to 69)

4th Stressor Rating: Boll maturation (BBCH 81 to 89)

Descriptive statistics for insect and disease stressor ratings are summarized in Table 8.7. For the categorical data the Cochran-Mantel-Haenszel test (CMH-test) was used to compare the different entries (GHB811 treated vs. non-GM Coker312 and GHB811 not treated vs non-GM Coker312). Statistical significance was evaluated at $p<0.05$ level. No statistical differences were observed for seven of the eight in any of the biotic environmental interaction ratings in comparisons between the non-GM counterpart and both GHB811 cotton entries. Statistically significant differences were observed for the third disease stressor rating between the non-GM counterpart and both GHB811 cotton entries (treated and not treated). All mean values for GHB811 cotton (treated or not treated) in the third disease stressor rating fell within the range of the reference varieties and thus statistically significant differences were considered not biologically relevant (Table 8.4).

Therefore GHB811 cotton did not show altered susceptibility to plant disease or insects relative to its non-GM counterpart and conventional commercial varieties.

Potential interactions with abiotic environment

Abiotic stressors were evaluated in the U.S. during the 2014 and 2015 growing seasons(Section 8. Agronomic and phenotypic assessment). Four stressor ratings were made during the growing season:

1st Stressor Rating: Two leaf-stage to floral initiation (BBCH 12 to 52)

2nd Stressor Rating: Floral bud enlargement to peak bloom (BBCH 54 to 65)

3rd Stressor Rating: Flowering (BBCH 61 to 69)

4th Stressor Rating: Boll maturation (BBCH 81 to 89)

Descriptive statistics for abiotic stressor ratings are summarized in Table 8.7. For the categorical data the Cochran-Mantel-Haenszel test (CMH-test) was used to compare the different entries (GHB811 treated vs. non-GM Coker312 and GHB811 not treated vs. non-GM Coker312). Statistical significance was evaluated at $p<0.05$ level. No statistical differences were observed in any of the abiotic environmental interaction ratings in comparisons between the non-GM counterpart and both GHB811 cotton entries (treated and not treated).

Therefore, GHB811 cotton showed equivalent potential interactions with abiotic environment to non-GM counterpart.

Survival and dormancy

To compare the germination potential of GHB811 cotton to that of the non-genetically modified (non-GM) counterpart (Coker 312) cotton, a warm-cold germination test was conducted (Section 8. Agronomic and phenotypic assessment).

For warm germination, the seeds were incubated at 30 ± 5 °C for eight days without light. GHB811 cotton had an overall germination percentage of 96.25% while Coker 312 had an overall germination percentage of 95.50% (Table 8.8). In the cold germination test, the seeds were incubated at 10 ± 5 °C for seven days and then transferred to 30 ± 5 °C for eight days without light. GHB811 had an overall germination percentage of 94.00% while Coker 312 had an overall germination percentage of 94.50% (Table 8.9).

Evaluation of the germination potential of GHB811 cotton compared to the non-GM counterpart, Coker 312 cotton, demonstrated that there was no statistically significant difference in the germination potential of the two genotypes under the two germination conditions.

Potential effects on non-target organisms, including beneficial organisms and threatened and endangered species

Because of the specificity of the 2mEPSPS and HPPD W336 proteins, event GHB811 cotton is not expected to affect non-target organisms, including beneficial organisms, threatened or endangered species. No adverse effects on non-target organisms were observed during field trial testing in the U.S. under USDA notification. No unexpected interactions with pests were detected in replicated field trials (Section 8. Agronomic and phenotypic assessment) (Section 8).

The U.S. Fish & Wildlife Service (FWS) has accountability for endangered species under the Endangered Species Act (ESA), (16 USC 1531). Section 6 of the ESA requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact.

Event GHB811 cotton will not impact any currently listed species of concern. Species of concern that may inhabit areas close to commercial cotton operations would not be impacted by the use of event GHB811 cotton. Commercial agriculture routinely disturbs the ground in which crops are currently planted. As a result, perennial vegetative species would not grow in these areas.

Glyphosate is currently registered for use in glyphosate-tolerant cotton products. The environmental fate and ecological effects of glyphosate herbicides on non-target organisms and endangered species have been addressed by the EPA as part of its review process.

The U.S. EPA has not registered isoxaflutole (IFT) for use in cotton production. During the registration process for the cotton use pattern, EPA will address the environmental fate and ecological effects of IFT on non-target organisms and endangered species.

9.2. Impact on agronomic practices

Event GHB811 cotton is genetically modified to confer tolerance to glyphosate and HPPD-inhibiting herbicides, such as isoxaflutole (IFT). The agronomic comparisons between event GHB811 cotton and non-GM cotton showed no biologically relevant differences aside from the intended herbicide tolerance traits. Therefore, this section will focus on weed management in U.S. cotton production, as this is where cotton varieties containing event GHB811 would be expected to have an impact.

Importance of weed management in cotton

The potential of a cotton crop is determined in the 40 days following the planting (NCC, 2007). Therefore it is critical to implement Best Management Practices (BMPs) during this period to set the crop up for the best possible outcome, and central among these BMPs is effective weed management. According to "The First Forty Days", a multi-state cooperative extension collaboration coordinated and published by the National Cotton Council and the Cotton Foundation, ideally weeds are controlled beginning 3-weeks before planting to ensure efficient planting operations, and the crop maintained weed-free for the 40-day period following planting to prevent impact on yield (NCC, 2007). Weed control should be maintained out to 9 weeks after planting for harvest efficiency. Features of an effective weed management program described in this 2007 document include:

- Have a weed control plan for the entire farm before planting
- Start the season weed-free
- Scout for weeds, and manage in a timely manner
- Engage multiple (effective) sites of action
- Incorporate residual herbicides in the management plan
- Keep records of what weeds were problematic, and in which fields they occurred

Anticipated recommended use pattern of herbicides in cotton production with event GHB811 cotton

This section summarizes anticipated recommendations that Bayer CropScience LP will publicize in its communications regarding cropping systems utilizing event GHB811 cotton, stacks with GHB811 cotton, and the herbicides associated with these products. Bayer CropScience LP has not to date submitted a registration to U.S. EPA for the use pattern of HPPD-inhibitor herbicides, such as IFT, in cotton production.

The current commercial plans for event GHB811 cotton is to offer it in stacked trait varieties with three additional GM-events already deregulated by USDA: T304-40 (lepidopteran resistant and glufosinate tolerant), GHB119 (lepidopteran resistance and glufosinate tolerant), and COT102 (lepidopteran resistant). Therefore the commercial product will have herbicide tolerance to IFT, glyphosate, and glufosinate in addition to three independently-acting lepidopteran targeting proteins (Cry1Ab, Cry2Ae, and Vip3Aa19).

Bayer CropScience LP expects to specifically label IFT herbicide for use on HPPD-inhibitor tolerant cotton varieties developed with event GHB811 cotton. It is anticipated that IFT will

be labeled for pre-emergence (PRE) and early post-emergence (EPO) use patterns in cotton. The planned label will support tank mixes of IFT with glufosinate, glyphosate, and other herbicides labeled for use in cotton. The spectrum of weed control for IFT, based on the current corn label, is provided in Table 9.1.

The recommendation will be to use IFT in PRE or EPO use pattern. Applications made PRE or EPO will offer control of many agronomically important weeds (Table 9.1) and residual control of weeds after cotton emergence, with reactivation potential up to 6-weeks after application when 0.5 inches of precipitation occurs. For EPO applications of IFT, the recommendation will be to include glyphosate or glufosinate (particularly in the case of glyphosate resistant weeds) as a tank mix partner to control emerged weeds (up to 2-leaf weeds when using glufosinate).

Table 9.1. Weeds controlled or suppressed by IFT herbicide

Broadleaf Species	Control (C) or Suppress (S)	Grass Species	Control (C) or Suppress (S)
Amaranth, Palmer	C	Barnyardgrass	C
Buffalobur	C	Crabgrass, large	C
Burcucumber	S	Crabgrass, smooth	C
Buttercup, small flower	C	Cupgrass, woolly**	C
Chamomile spp.	C	Foxtail, bristly	C
Chickweed, common	C	Foxtail, giant	C
Copperleaf, hophornbeam	C	Foxtail, green	C
Dandelion (seedling)	C	Foxtail, robust purple	C
Deadnettle, purple	C	Foxtail, robust white	C
Galinsoga	C	Foxtail, yellow**	C
Henbit	S	Goosegrass	C
Jimsonweed	C	Johnsongrass, seedling	C
Kochia	C	Panicum, fall	C
Lambsquarters, common	C	Panicum, Texas	C
Mallow, Venice	C	Proso millet, wild**	C
Marestail	C	Sandbur, field**	S
Wild mustard	C	Shattercane**	S
Nightshade, black	C	Signalgrass, broadleaf**	C
Nightshade, eastern black	C		
Pennycress, field	C		
Pepperweed, Virginia	C		
Plantain, broadleaf	C		
Pigweed, prostrate	C		
Pigweed, redroot	C		
Pigweed, smooth	C		
Purslane, common	C		
Ragweed, giant*	S		
Russian Thistle	C		
Shepherds-purse	C		
Smartweed, Pennsylvania	C		
Spurge, toothed	C		
Velvetleaf	C		
Waterhemp, common	C		
Waterhemp, tall	C		

Source: BalancePro and Balance Flexx herbicide labels (EPA Reg No. 264-600, 264-1067, respectively)
*These weeds may require a post-emergence application of an additional appropriately labeled herbicide
**These weeds will be suppressed and / or be reduced in competition. Reduced competition weeds will be stunted ingrowth and / or be of reduced populations as compared to non-treated areas. Commercially acceptable control may require the application of an appropriate pre-emergence tank mixture or sequential post-emergence herbicide treatment.

Use of herbicides in cotton today

Based on the 2015 USDA National Agricultural Statistics Service (NASS) survey of agricultural chemical use, herbicides were applied on more planted acres of cotton than any other agricultural chemical surveyed, including fertilizers and other pesticides (NASS, 2016). Herbicides were applied on 92% of planted cotton acres in 2015, in comparison to nitrogen fertilizer at 78% of planted acres. This included 47% and 37% (combined 84%) of planted acres receiving at least one glyphosate application in the form of potassium salt or isopropylamine salt, respectively. On average these formulations of glyphosate were applied 1.9 to 2.1 times during the season for isopropylamine salt and potassium salt formulations, respectively (NASS, 2016).

Glyphosate (WSSA Group 9) is by far the most used herbicide in U.S. cotton production, a trend which began with the launch of glyphosate tolerant cotton varieties in 1998. However, glyphosate use in cotton is trending downward. Since the last NASS survey in 2007, the actual pounds of glyphosate applied to cotton in the U.S. has decreased from 16.6 million lbs in 2007 to 14.1 million lbs in 2015 (NASS, 2017). This trend can likely be attributed to: 1) a decrease in efficacy of glyphosate to several key weed species in cotton due to the development of glyphosate resistance in these species and, 2) increasing awareness among cotton farmers (and farmers in general) of the importance of weed resistance management and the pitfalls of overreliance on a single site of action in weed management programs.

Indeed, growers are increasing their use of herbicides with other sites of action, some in conjunction with genetically modified herbicide tolerant cotton varieties such as varieties with LibertyLink™ technology from Bayer CropScience. The LibertyLink trait confers post-emergence tolerance to glufosinate-ammonium (Liberty™) herbicide (WSSA Group 10). Glufosinate-ammonium is the only non-selective herbicide alternative to glyphosate formulations in cotton, and to date no weeds have developed resistance to glufosinate in U.S.row crop production.

As described previously, Bayer CropScience LP will seek labeled IFT use patterns PRE and EPO. There are several herbicide active ingredients and differing sites of action currently labeled for each of these use patterns in cotton. Table 9.2 provides sites of action along with WSSA group number corresponding to each of these possible IFT use patterns, based on recommendations by Texas AgriLife for Texas cotton producers (Morgan, *et al*., 2013). Based on this analysis, there are seven different recommended sites of action currently available to farmers for pre-plant (*i.e.,* burndown) and pre-emergence (*i.e.,* at planting) use patterns. There are six different sites of action recommended for EPO in cotton, including glyphosate and glufosinate uses on tolerant GE varieties. The recent labeling of WSSA group 4 synthetic auxins in tolerant GE cotton varieties brings in an additional post-emergence site of action (U.S. EPA, 2016; U.S. EPA, 2017). There is currently only one site of action recommended for the pre-plant incorporated use pattern in cotton, microtubule synthesis inhibition.

Table 9.2. Sites of action in cotton with comparable use patterns to the intended use pattern of IFT in event GHB811 cotton varieties (Morgan *et al*., 2013)

Timing	WSSA Group Number	Site of action	Example active ingredients
PrePlant Burndown	2	ALS Inhibitor	trifloxysulfuron, thifensulfuron-methyl, tribenuron, rimsulfuron
	4	Synthetic Auxins	2,4-D
	9	EPSP synthase inhibitor	Glyphosate
	10	Glutamine Synthetase inhibitor	Glufosinate
	14	PPG oxidase	oxyfluorfen, saflufenacil, flumioxazin
	15	Long chain fatty acid inhibitor	s-metolachlor
	22	Photosynthesis I diverter	Paraquat
PrePlant Incorporated	3	Microtubule assembly inhibitor	pendimethalin, trifluralin,
Preemergence (at planting)	2	ALS inhibitor	Pyrithiobac
	3	Microtubule assembly disruptor	Pendimthalin
	5	Photosystem II inhibitor	Prometryn
	7	Photosystem II inhibitor	fluometuron, diuron
	9	EPSP synthase inhibitor	Glyphosate
	13	Carotenoid Biosnthesis Inhibitor	Clomazone
	15	Long chain fatty acid inhibitor	s-metolachlor, acetochlor,
Early Postemergence	1	ACCase	fluazifop-p-buytl, fenoxaprop-p-ethyl, clethodim, quizalofop, sethoxydim
	2	ALS inhibitor	Pyrithiobac
	7	Photosystem II inhibitor	Flumeturon
	9*	EPSP synthase inhibitor	Glyphosate
	10**	Glutamine Synthetase inhibitor	Glufosinate
	15	Long chain fatty acid inhibitor	s-metolachlor, acetocholor

*Only when planting a GM-cotton variety with glyphosate tolerance

**Only when planting a GM-cotton variety with glufosinate tolerance, or using a hooded sprayer to shield non-glufosinate tolerant cotton.

Impact of GHB811 on agronomic practices and weed management

Event GHB811 cotton is genetically modified to confer herbicide tolerance to glyphosate and HPPD-inhibitor (WSSA Group 27) herbicides. Because of the relative saturation of the glyphosate tolerance trait in cotton varieties sold in the U.S. today, the glyphosate tolerance aspect of event GHB811 cotton is not considered in this impact assessment, as glyphosate will continue to be applied in U.S. cotton production with or without the introduction of this event.

HPPD-inhibitor tolerant cotton varieties have not been commercialized in the U.S. or other cotton cultivation countries to date. However, HPPD-inhibitors are already used in rotational crops to cotton such as corn, sorghum and small grains; therefore it will not be the first time HPPD-inhibitors are used in overall production systems that may include cotton cultivation. Further, two GM soybean events have been deregulated in the U.S. with HPPD-inhibitor tolerance. The use of HPPD-inhibitors has not yet been registered on soybean by the U.S. EPA, however the proposed label is currently under review by U.S. EPA, with expected approval in 2017. Soybean is also a common rotational crop with cotton. Once commercial, HPPD-inhibitor tolerant soybeans will be another use of this site of action in production systems that include cotton cultivation.

As discussed in previous sections, Bayer CropScience LP expects to label IFT herbicide for use on HPPD-inhibitor tolerant cotton varieties developed with event GHB811 with PRE and EPO use patterns. There are several herbicides active ingredients with differing sites of action from IFT currently labeled for each of these use patterns in cotton (Table 9.2). Therefore, the commercialization of cotton varieties containing event GHB811 will not change the overall use patterns of herbicides in cotton weed management programs.

The flexibility to apply IFT in PRE and EPO use patterns in cotton weed management systems will add a site of action that has never been available in cotton weed management systems before. This will alleviate resistance selection pressure from existing labeled chemistries, prolonging the efficacy of available herbicides. As with all herbicides, IFT is not immune to resistance. Therefore it will be necessary to use multiple effective sites of action in each application window, in order to decrease selection pressure for IFT resistance in weed populations. It has been demonstrated that the use of multiple effective sites of action within a given year greatly decreases the likelihood of resistance relative to rotating sites of action season to season. Using *Amaranthus tuberuclatus* as a model, Evans *et al*., (2015) demonstrated that a weed management system that averaged 2.5 effective sites of action per season was 83% less likely to develop glyphosate resistant *A. tuberculatus* 4-6 years later relative to a system averaging only 1.5 effective sites of action per season.

In addition to controlling over 30 broadleaf and over 15 grasses, IFT herbicide controls several important glyphosate resistant weeds in cotton (Heap, 2017) (Table 9.3). To date, resistance to IFT has only been observed in one weed species, *A. tuberculatus*, as a result of its use in continuous corn seed production in Iowa (Heap 2017). The properties of IFT make it particularly well suited to be included in a weed resistance management plan. IFT will have flexible application timing. This allows it to be combined as a second effective site of action at critical application windows. IFT has residual activity, with reactivation potential for up to 6 weeks when 0.5 inches of rainfall occurs. This allows PRE and EPO application of IFT to control late emerging weeds up to 2 inches tall into the early growing season, during the critical 40 days of cotton development following planting. This use pattern makes IFT and event GHB811 cotton unique relative to other herbicide active ingredients associated with GM cotton, presently or expected to be available soon, which control emerged weeds with little residual activity.

Overall, the impact of GHB811 cotton to U.S. agriculture will be positive, contributing a new effective site of action to cotton weed management for early season weed control and weed resistance management.

Table 9.3. Glyphosate resistant weeds in the U.S. (Heap, 2017) and extent of control by IFT

Species	Controlled (C), Supressed (S), or Not Controlled (NC**) by IFT
Amaranth, Palmer	C
Amaranth, spiny	NC
Waterhemp, tall	C
Ragweed, common	C
Ragweed, giant	S
Fleabane, hairy	NC
Marestail	C
Junglerice	NC
Goosegrass	C
Sunflower, common	NC
Kochia*	C
Ryegrass, Italian	NC
Ryegrass, rigid	NC
Ragweed, parthenium	NC
Bluegrass, annual	S
Russian-thistle*	NC
Johnsongrass	C

*Resistant species present in the U.S., however not detected in a cotton growing states
**Can be controlled postemergence with glufosinate.

Weed resistance management

Prior to commercialization, Bayer CropScience LP will develop and publicize a resistance management plan for IFT herbicide use in HPPD-inhibitor tolerant cotton. Current U.S. EPA policy for herbicide registrations associated with a GM herbicide tolerance trait includes a registration condition whereby the registrant must implement, maintain and report an herbicide (weed) resistance management plan. These conditions of registration have not yet been established for the IFT use pattern on cotton varieties developed with event GHB811.

Bayer CropScience LP, based on principles of good product stewardship, proactively publicizes resistance management strategies for crop protection products. The name of this program focused on weed resistance management is Respect the Rotation™. Respect the Rotation is a comprehensive educational and stewardship program from Bayer CropScience LP that promotes greater understanding of the importance of integrated weed management. Respect the Rotation works to assist growers in the adoption of the following best management practices:

- Rotate crops. Crop rotation diversifies and enhances weed management.
- Rotate herbicide-tolerant traits. Alternate herbicide-tolerant (HT) traits and/or use HT trait stacks for more efficient rotation.
- Use multiple herbicide sites of action (SOA). Use tankmix partners and multiple effective SOAs throughout each season and each year to reduce the selection pressure of a single SOA.
- Know your weeds, know your fields. Closely monitor problematic areas with difficult-to-control weeds or dense weed populations.
- Start with clean fields. Effective tillage or the use of a burndown herbicide program can control emerged weeds prior to planting.

- Stay clean – use residual herbicides. Regardless of tillage system, pre-emergence or early post-emergence soil-applied residual herbicides should be used when possible.
- Apply herbicides correctly. Ensure proper application, including correct timing, full use rates and appropriate spray volumes as well as the appropriate nozzle types.
- Control weed escapes. Consider spot herbicide applications, row wicking, cultivation or hand removal of weeds to stop weed seed production and improved weed engagement.
- Zero tolerance – reduce the weed seed bank. Do not allow surviving weeds to set seed, which will help decrease weed populations from year to year and prevent major weed shifts.
- Clean equipment. Prevent the spread of herbicide-resistant weed and their seeds.

Independent of what the U.S. EPA requires for herbicide resistance management as a condition of registration for the use pattern of IFT in cotton, Bayer CropScience LP will develop a resistance management plan which fits within the framework of Respect the Rotation. The principles of Respect the Rotation are aligned with that of Norsworthy *et al*. (2012), which is the basis for the U.S. EPA requirements for herbicide resistance management.

10. Statement of grounds unfavorable

Bayer CropScience LP knows of no study data and/or observations associated with event GHB811 cotton that will result in adverse environmental consequences for its introduction. The only biologically relevant phenotypic difference between event GHB811 cotton and conventional cotton is the expression of the 2mEPSPS and HPPD W336 proteins which provide tolerance to the application of glyphosate herbicide and isoxaflutole herbicide, respectively. Planting double-herbicide-tolerant cotton varieties, containing transformation event GHB811 cotton, will provide growers with new options for weed control using IFT tolerant cotton and other agricultural production systems. IFT herbicide offers an alternative weed control option for the cotton grower via a new herbicide mode of action for cotton that is efficacious against many of the herbicide resistant weeds currently found n cotton fields.

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